

**STUDIES ON MICROBIAL L-ASPARAGINASES AND AMIDASES
AND THEIR APPLICATIONS IN FOOD INDUSTRY**

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

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AND THEIR APPLICATIONS IN FOOD INDUSTRY**

by

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DEPARTMENT OF CHEMISTRY**

Submitted

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

to the



INDIAN INSTITUTE OF TECHNOLOGY DELHI

JULY 2023

Dedicated to my Ammi

CERTIFICATE

This is to certify that the thesis entitled “**Studies on microbial L-asparaginases and amidases and their applications in food industry**” being submitted by **MR. SHAHENVAZ ALAM** 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 him.

Mr. Shahenvaz Alam 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

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Acknowledgments

First and foremost, I would like to praise **Allah The Almighty**, the most Gracious, the most Merciful, the Ever Magnificent, for giving me strength, courage, endurance, and blessings to complete my work.

I am extremely grateful to my supervisor, **Prof. Sunil Kumar Khare**, for his valuable advice, continuous guidance, and patience during my PhD tenure. I am fortunate to be guided by a very encouraging and considerate Professor like him. My research journey has seen thick and thin moments. He has always advised me to keep moving and do good research.

I am thankful to the Head of the Department, **Prof. Siddharth Pandey**, and former HODs of the Department, **Prof. Ravi Shankar**, **Prof. A. K. Ganguli**, **Prof. A. J. Elias**, and **Prof. N. D. Kurur**, for providing adequate facilities and tutelage during my study.

I am incredibly thankful to my SRC members, **Prof. Shashank Deep** (SRC Chairperson), **Prof. Tanmay Dutta** (Internal Expert), and **Prof. Prashant Mishra** (External Expert, Department of Biochemical Engineering and Biotechnology, IIT Delhi) for helping me with valuable suggestions during my PhD work.

I am indebted to my research collaborators, **Prof. Bansi D. Malhotra** (Former Distinguished Professor, Delhi Technological University, New Delhi) and **Prof. John HT. Luong** (Distinguished Professor, University College Cork, Ireland), for guiding me into the new arena of biosensors and giving in-depth knowledge for the development of chemosensor of acrylamide. My sincere thanks to Nano Bioelectronic Laboratory (NBL), where I learned a lot of new things and made many good friends. My extended thanks to NBL friends **Dr. Shine Augustine**, **Dr. Niharika Gupta**, **Dr. Sharda**, **Dr. Shipra**, **Dr. Tarun Narayan**, **Mr. Udiptya**

Saha, and **Mr. Keshav Tody**. Special thanks to **Mr. Raghav Jain** for sharing and discussing scientific knowledge and valuable life lessons.

I would like to acknowledge the **Central research facility (CRF)** and **Nanoscale research facility (NRF)** at IIT Delhi for providing instrumentation facilities. 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 for their cooperation and assistance.

I would extend my gratitude to the **Indian Institute of Technology Delhi** for providing me Institute Fellowship and aiding financial support for my PhD.

The support from the **EMB Lab** (Enzyme and Microbial Biochemistry Laboratory) shaped me and helped me in my formative years in science. I am grateful to my lab seniors, **Dr. Anshu Gupta, Dr. Ruchi Gaur, Dr. Ram Karan, Dr. Arvind Sinha, Dr. Chetna Joshi, Dr. Sumit Kumar, Dr. Rajeshwari Sinha, Dr. R. Hemamalini, Dr. Ayesha Sadaf, Dr. Jasneet Grewal, Dr. Shubhrima Ghosh, Dr. Neerja Yadav, and Dr. Syeda Warisul Fatima**. Although they have left the lab and excelled in life, they are always available to extend help, support, and share belongingness with their juniors and lab mates.

I am thankful for my lovely juniors, **Mr. Nitin Srivastava, Ms. Nikky Goel, Ms. Pooja, Ms. Ankita, Ms. Huma Fatima, Ms. Sukriti Srivastava, Ms. Deeksha Gopaliya, Ms. Saniya Zaidi, Ms. Simran Kundral, Mr. Bibhuti B. Das, and Mr. Sachin** for keeping the lab environment cheerful.

I am thankful for M.Sc. and M. Tech students in my lab, **Mr. Vikas Gautam, Ms. Neeraj Yadav, Ms. Isha Jain, Ms. Anjali Maheshwari, Ms. Tanya Nagpal, Mr. Sayan Barua, Ms. Henadri, Ms. Shweta, and Ms. Alka**.

I am grateful to my Lab Post-Docs, **Dr. Shivani Chaturvedi, Dr. Rameshwar Tiwari, Dr. Razi Ahmed, Dr. Arti Pal, Dr. Sunaina Singh** and especially **Dr. Amrik Bhattacharya**, for supporting and providing valuable insights into the experiments and discussions.

I am most grateful to **Dr. Kumar Pranaw**, former lab Post-Doc, who helped me in my early scientific years and inculcated my scientific temper. His persistent guidance throughout my PhD is greatly appreciated.

I want to thank my extended family (my friends), **Mr. Nitin Srivastava** and **Mr. Pradeep Shah**, for always being with me. We have been friends for a decade now, and I always find them cheering for me and celebrating my happiness. They are my partners in crime.

I owe many thanks to my friends, **Dr. Dinesh Raj Pant, Nitesh, Supriya, Dr. Sonam Kumari, Imran, Parveen, Arun, Anil, Bhoomika**, and **Swati Raina**. Their friendships are invaluable to me.

I am most grateful to my parents, my mother, **Ms. Ishrat Jahan** (Ammi) and my father, **Shamsad Alam**, unfortunately, who passed away before my PhD journey. I dedicate my thesis to my mother. She has been my source of inspiration, always loved me, and supported me in every possible way. She believed in me and wanted the best for me. Without her, this PhD journey would not be possible.

I sincerely thank my elder brother, **Mr. Sarfaraz Alam Ansari**, for his constant love, care, encouragement and support. I thank my Sister-in-Law, **Ms. Rehana Khatoon**, for her care and affection. I am incredibly grateful to my nephew (**Shehryar Alam**). His little presence in the house always cheered me and filled me with joy.

I would like to thank my significant half, **Dr. Syeda Warisul Fatima**, for being the most beautiful chapter of my life. I am forever grateful for her love, tremendous support, and motivation.

I want to thank my vast extended family, parent-in-laws, **Dr. Syed Aijazzudin** (Papa) and **Ms. Zarrin Aijaz** (Mumma), brother-in-law, **Mr. Syed Nayeluddin** and my sisters-in-law, **Ms. Abeer Fatima** and **Ms. Manaam Fatima**. I am grateful for such a caring, loving, and supportive family. Special mention goes to **Donny**, for all his pawing warmth and affection.

At last, I would like to thank all my family members, friends, and colleagues for their good wishes, encouragement, and support during my PhD.

SHAHENVAZ ALAM

Abstract

The thesis encompasses the detailed study on the detection of acrylamide in various Indian food products. The study focused on acrylamide, various mitigation strategies viz. enzymatic and non-enzymatic, employed for acrylamide reduction in food systems, while the later studies more focussed on acrylamide degradation using amidase enzymes. The work features the development of the chemosensor for rapid detection of acrylamide in food samples.

To address the challenges of quantification of acrylamide in Indian food products (fried and baked), the extraction and determination of acrylamide was established. The detection reported high level of acrylamide in heat-processed food products through HPLC. Further, the non-enzymatic strategies involving natural antioxidant compounds resulted in alleviation of acrylamide content in food systems. The natural compounds, commonly found in Indian spices and herbs were utilized for inhibiting Maillard reaction, and eventually acrylamide formation. *In vitro* testing of cinnamyl alcohol on acrylamide formation revealed that there was 83.3% reduction in the formation. Furthermore, the antioxidant compounds present in guava leaves extracts were also employed in frying oils for mitigation of acrylamide formation in Indian staple food (poori). The deterioration of oils due to heating resulted in the generation of lipid oxidation products that are responsible for acrylamide formation. The antioxidant compounds, present in guava extracts, showed affinity towards lipid oxidation products and inhibited the formation of acrylamide up to 27%. These results of natural antioxidants were compared with synthetic antioxidant, BHT (butylated hydroxytoluene).

Moreover, the work intended to explore the enzymatic strategies using microbial L-asparaginases and their immobilized preparation as pre-treatment method for acrylamide reduction in food systems. L-asparaginases have gained attention in food industry as it prevents

the formation of acrylamide in starch-rich fried foods. The use of L-asparaginase has been regarded as a promising strategy among the various methods, established to reduce the content of acrylamide in baked, fried, and roasted foods.

Therefore, current research efforts have been directed to microbial production of L-asparaginases, nano immobilization and mitigation strategies to combat the levels of acrylamide. The nano immobilization offers unique properties to the enzymes. The L-asparaginase from two microbial sources, *Bacillus aryabhatai* and *E. coli* were employed for immobilization on functionalized magnetic nanoparticles. The nano immobilization increased their thermal stability up to four-fold at high temperature (70 °C). Moreover, the applications of these immobilized preparations were tested for acrylamide reduction in food system. The L-asparaginase from *Bacillus aryabhatai* demonstrated >90% reduction of acrylamide in starch-asparagine food model system, whereas, *E. coli* L-asparaginase resulted in >95% reduction in fried potato chips. These findings highlight the prospects of cost-effective, thermostable, and immobilized L-asparaginase as promising candidates for food processing applications.

Subsequently, the potential of amidase enzymes was also explored. Amidases are underappreciated yet versatile enzymes involved in amide metabolism and degradation of amide toxicants. They belong to the nitrilase superfamily, an important biocatalyst hydrolyzing a wide range of amides (such as short-chain/mid-chain aliphatic amides, α -aminoamides, acrylamides, and α -hydroxy amides, etc.).

During the course of research, a newly amidase-producing bacterial strain was isolated using soil enrichment technique, and their enzymes were studied in detail. The genomic insights

highlighted by whole genome sequencing, along with biochemical, structural, and kinetic characterization, were elucidated to decipher the catalytic mechanism of amidases. Besides, the amidase was purified by anion exchange chromatography. A 19-fold increase in amidases purification was achieved.

As the function of amidases was not clearly understood and thus, required further investigations. This led to envisaging molecular analysis, biochemical characteristics, and functional studies to gain insights into their diverse biochemical capabilities for biocatalysis. Further to it, amidase gene sequences were successfully amplified from *Burkholderia* sp. EMB 26. The ORF encoded a protein of 210 amino acids cloned in the pET-22b (+) vector for overexpression in *Escherichia coli* BL21 (DE3). The comprehensive study was further conducted on the biochemical and structural-functional characterization of purified and recombinant amidases that hold tremendous applications and may be useful in acrylamide degradation.

As an innovative part of the thesis, the chemosensor was developed by exploiting the DTT and gold nanoparticles. The study features a new and elegant approach to the detection of acrylamide in food systems. 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 chemosensors. Rapid and simple electroanalysis of acrylamide was feasible by a gold electrode modified with gold nanoparticles (AuNPs) and dithiothreitol (DTT) with enhanced detection sensitivity and selectivity. The limit of detection (LOD) and the limit of quantitation (LOQ) were estimated to be 3.11×10^{-9} M and 1×10^{-8} M, respectively, with wide linearity ranging from 1×10^{-8} M to 1×10^{-3} M. The estimated levels of acrylamide in both the cases, potato chips, and coffee samples by the sensor were in agreement with those of high-performance

liquid chromatography. These findings point towards effective, sensitive, and accurate quantification of acrylamide via chemosensor electroanalyses.

The following research goals were accomplished:

- The natural extracts of guava leaves contained antioxidants compounds that are employed for inhibition of Maillard reaction. For enzymatic process, the nano-immobilized L-asparaginases were investigated to alleviate the formation of acrylamide. During pre-treatment of food system (potato chips) with L-asparaginase immobilized preparations, a significant reduction (> 95%) of acrylamide formation was observed.
- An acrylamide-degrading bacteria *Burkholderia* sp. EMB 26, exhibiting amidase activity, was isolated, and its novel amidases were characterized. The heterologous expression of amidase was also attempted successfully.
- The major highlight of the study was the development of a chemosensor, which was sensitive to detect acrylamide at nano-level concentrations.
- Overall, the long-term goal was to devise simple, powerful electrochemical-based chemosensors as an adjuvant to the existing detecting tool for acrylamide and successfully quantify the level in food samples. Hence, the significance of detection levels of acrylamide in food systems was achieved.

सारांश

थीसिस में विभिन्न भारतीय खाद्य उत्पादों में एक्रिलामाइड की पहचान पर विस्तृत अध्ययन शामिल है। अध्ययन एक्रिलामाइड, विभिन्न शमन रणनीतियों पर केंद्रित है। एंजाइमेटिक और गैर-एंजाइमी, खाद्य प्रणालियों में एक्रिलामाइड कमी के लिए नियोजित, जबकि बाद के अध्ययनों में एमिडेज एंजाइम का उपयोग करके एक्रिलामाइड गिरावट पर अधिक ध्यान केंद्रित किया गया। कार्य खाद्य नमूनों में एक्रिलामाइड का तेजी से पता लगाने के लिए केमोसेंसर के विकास की विशेषता है।

भारतीय खाद्य उत्पादों (तला हुआ और बेक किया हुआ) में एक्रिलामाइड के परिमाणीकरण की चुनौतियों का समाधान करने के लिए, एक्रिलामाइड का निष्कर्षण और निर्धारण स्थापित किया गया था। पता लगाने से एचपीएलसी के माध्यम से गर्मी-संसाधित खाद्य उत्पादों में एक्रिलामाइड के उच्च स्तर की सूचना मिली। इसके अलावा, प्राकृतिक एंटीऑक्सीडेंट यौगिकों को शामिल करने वाली गैर-एंजाइमी रणनीतियों के परिणामस्वरूप खाद्य प्रणालियों में एक्रिलामाइड सामग्री का उन्मूलन हुआ। आमतौर पर भारतीय मसालों और जड़ी-बूटियों में पाए जाने वाले प्राकृतिक यौगिकों का उपयोग माइलार्ड प्रतिक्रिया को रोकने और अंततः एक्रिलामाइड बनाने के लिए किया जाता था। एक्रिलामाइड गठन पर सिनामाइल अल्कोहल के इन विट्रो परीक्षण से पता चला कि गठन में 83.3% की कमी आई थी। इसके अलावा, अमरूद के पत्तों के अर्क में मौजूद एंटीऑक्सीडेंट यौगिकों को भारतीय मुख्य भोजन (पूरी) में एक्रिलामाइड गठन को कम करने के लिए तलने के तेल में भी इस्तेमाल किया गया था। हीटिंग के कारण तेलों की गिरावट के परिणामस्वरूप लिपिड ऑक्सीकरण उत्पादों की उत्पत्ति हुई जो एक्रिलामाइड गठन के लिए जिम्मेदार हैं। अमरूद के अर्क में मौजूद एंटीऑक्सिडेंट यौगिकों ने लिपिड ऑक्सीकरण उत्पादों के प्रति आकर्षण दिखाया और 27% तक एक्रिलामाइड के गठन को रोक दिया। प्राकृतिक एंटीऑक्सीडेंट के इन परिणामों की तुलना सिंथेटिक एंटीऑक्सीडेंट, बीएचटी (ब्यूटिलेटेड हाइड्रोक्साइटोल्यूइन) से की गई थी।

इसके अलावा, खाद्य प्रणालियों में एक्रिलामाइड कमी के लिए पूर्व-उपचार विधि के रूप में माइक्रोबियल एल-एस्पैरागिनेस और उनकी स्थिर तैयारी का उपयोग करके एंजाइमेटिक रणनीतियों का पता लगाने के लिए काम किया गया। एल-एस्पैरागिनेज ने खाद्य उद्योग में ध्यान आकर्षित किया है क्योंकि यह स्टार्च युक्त तले हुए खाद्य पदार्थों में एक्रिलामाइड के निर्माण को रोकता है। पके हुए, तले हुए और भुने हुए खाद्य पदार्थों में एक्रिलामाइड की मात्रा को कम करने के लिए स्थापित विभिन्न तरीकों के बीच एल-एस्पैरागिनेज के उपयोग को एक आशाजनक रणनीति के रूप में माना गया है।

इसलिए, वर्तमान अनुसंधान प्रयासों को एकिलामाइड के स्तरों का मुकाबला करने के लिए एल-एस्पैरजाइनेस, नैनो स्थिरीकरण और शमन रणनीतियों के माइक्रोबियल उत्पादन के लिए निर्देशित किया गया है। नैनो स्थिरीकरण एंजाइमों को अद्वितीय गुण प्रदान करता है। दो माइक्रोबियल स्रोतों, बैसिलस आर्यभट्टई और ई. कोलाई से एल-एस्पैरगाइनेज को कार्यात्मक चुंबकीय नैनोकणों पर स्थिरीकरण के लिए नियोजित किया गया था। नैनो स्थिरीकरण ने उच्च तापमान (70 °C) पर उनकी तापीय स्थिरता को चार गुना तक बढ़ा दिया। इसके अलावा, खाद्य प्रणाली में एकिलामाइड कमी के लिए इन स्थिर तैयारियों के अनुप्रयोगों का परीक्षण किया गया। बैसिलस आर्यभट्टई के एल-एस्पैरगाइनेज ने स्टार्च-एस्पैरेजिन खाद्य मॉडल प्रणाली में एकिलामाइड की >90% कमी प्रदर्शित की, जबकि ई. कोलाई एल-एस्पैरगाइनेज के परिणामस्वरूप तले हुए आलू के चिप्स में >95% की कमी हुई। ये निष्कर्ष खाद्य प्रसंस्करण अनुप्रयोगों के लिए होनहार उम्मीदवारों के रूप में लागत प्रभावी, थर्मोस्टेबल और इमोबिलाइज्ड एल-एस्पैरागिनेज की संभावनाओं को उजागर करते हैं।

इसके बाद, एमिडेज़ एंजाइम की क्षमता का भी पता लगाया गया। एमिडेस की कम सराहना की जाती है, फिर भी एमाइड चयापचय में शामिल बहुमुखी एंजाइम और एमाइड टॉक्सिकेंट्स का क्षरण होता है। वे नाइट्रिलेस सुपरफैमिली से संबंधित हैं, एमाइड्स की एक विस्तृत श्रृंखला (जैसे शॉर्ट-चेन / मिड-चेन एलिफैटिक एमाइड्स, α -एमिनोमाइड्स, एकिलामाइड्स, और α -हाइड्रॉक्सी एमाइड्स, आदि) के रूप में एक महत्वपूर्ण बायोकेटलिस्ट हाइड्रोलाइजिंग।

अनुसंधान के दौरान, मिट्टी संवर्धन तकनीक का उपयोग करके एक नए एमिडेस-उत्पादक जीवाणु तनाव को अलग किया गया था, और उनके एंजाइमों का विस्तार से अध्ययन किया गया था। जैव रासायनिक, संरचनात्मक और काइनेटिक लक्षण वर्णन के साथ-साथ पूरे जीनोम अनुक्रमण द्वारा उजागर की गई जीनोमिक अंतर्दृष्टि को एमिडेस के उत्प्रेरक तंत्र को समझने के लिए स्पष्ट किया गया था। इसके अलावा, आयन एक्सचेंज क्रोमैटोग्राफी द्वारा एमिडेज़ को शुद्ध किया गया था। एमिडेस शुद्धि में 19 गुना वृद्धि हासिल की गई। जैसा कि एमिडेस के कार्य को स्पष्ट रूप से नहीं समझा गया था और इस प्रकार, आगे की जांच की आवश्यकता थी। इसने जैव-उत्प्रेरण के लिए उनकी विविध जैव रासायनिक क्षमताओं में अंतर्दृष्टि प्राप्त करने के लिए आणविक विश्लेषण, जैव रासायनिक विशेषताओं और कार्यात्मक अध्ययनों की परिकल्पना की। इसके अलावा, बर्कहोल्डरिया एसपी से एमिडेज़ जीन अनुक्रमों को सफलतापूर्वक प्रवर्धित किया गया।

थीसिस के एक अभिनव भाग के रूप में, डीटीटी और सोने के नैनोकणों का शोषण करके केमोसेंसर विकसित किया गया था। अध्ययन खाद्य प्रणालियों में एकिलामाइड का पता लगाने के लिए एक नया और सुरुचिपूर्ण दृष्टिकोण पेश करता है। वर्तमान कार्य में नवीनता संश्लेषण की विधि, उत्पाद की डिजाइनिंग और केमोसेंसर में वांछित विशेषताओं को प्राप्त करने के लिए प्रक्रिया अनुकूलन से संबंधित है। बढ़ी हुई पहचान

संवेदनशीलता और चयनात्मकता के साथ सोने के नैनोकणों (AuNPs) और dithiothreitol (DTT) के साथ संशोधित एक सोने के इलेक्ट्रोड द्वारा एकिलामाइड का तीव्र और सरल इलेक्ट्रोएनालिसिस संभव था। पता लगाने की सीमा (LOD) और मात्रा की सीमा (LOQ) क्रमशः 3.11×10^{-9} M और 1×10^{-8} M होने का अनुमान लगाया गया था, जिसमें 1×10^{-8} M से लेकर 1×10^{-3} तक की व्यापक रैखिकता थी। सेंसर द्वारा दोनों मामलों में एकिलामाइड के अनुमानित स्तर, आलू के चिप्स और कॉफी के नमूने एच पी एल सी क्रोमैटोग्राफी के अनुरूप थे। ये निष्कर्ष केमोसेंसर इलेक्ट्रोएनालिसिस के माध्यम से एसाइलामाइड के प्रभावी, संवेदनशील और सटीक परिमाणीकरण की ओर इशारा करते हैं।

निम्नलिखित अनुसंधान लक्ष्यों को पूरा किया गया:

- अमरूद की पत्तियों के प्राकृतिक अर्क में एंटीऑक्सीडेंट यौगिक होते हैं जो माइलार्ड प्रतिक्रिया को रोकने के लिए उपयोग किए जाते हैं। एंजाइमैटिक प्रक्रिया के लिए, एकिलामाइड के गठन को कम करने के लिए नैनो-स्थिर एल-एस्पैरागिनैस की जांच की गई। एल-एस्पैरागिनैस स्थिर तैयारी के साथ खाद्य प्रणाली (आलू के चिप्स) के पूर्व-उपचार के दौरान, एकिलामाइड गठन की एक महत्वपूर्ण कमी (> 95%) देखी गई।
- एक एकिलामाइड-डिग्रेडिंग बैक्टीरिया बुर्कहोल्डरिया एसपी. ईएमबी 26, एमिडेस गतिविधि प्रदर्शित करता है, अलग किया गया था, और इसके उपन्यास एमिडेसेस की विशेषता थी। एमिडेज़ की विषम अभिव्यक्ति का भी सफलतापूर्वक प्रयास किया गया।
- अध्ययन का प्रमुख आकर्षण एक केमोसेंसर का विकास था, जो नैनो-स्तर की सांद्रता पर एकिलामाइड का पता लगाने के लिए संवेदनशील था।
- कुल मिलाकर, दीर्घकालिक लक्ष्य एकिलामाइड के लिए मौजूदा पता लगाने वाले उपकरण के सहायक के रूप में सरल, शक्तिशाली इलेक्ट्रोकेमिकल-आधारित केमोसेंसर तैयार करना और खाद्य नमूनों में स्तर को सफलतापूर्वक निर्धारित करना था। इसलिए, खाद्य प्रणालियों में एकिलामाइड के स्तर का पता लगाने के महत्व को प्राप्त किया गया।

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