STUDIES ON *EXIGUOBACTERIUM* SP. TBG-PICH-001 AND ITS PROTEASES: POLYEXTREMITIES AND MOLECULAR ASPECTS

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by

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DEPARTMENT OF CHEMISTRY

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in fulfillment of the requirements of the Degree of

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Dedicated to My Papa

CERTIFICATE

This is to certify that the thesis entitled "**Studies on *Exiguobacterium* sp. TBG-PICH-001 and its proteases: Polyextremities and molecular aspects**" being submitted by **Mr. Nitin** 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. Nitin has worked under my guidance and supervision and has fulfilled the requirements for the thesis submission, which, to my knowledge, has reached the requisite standard.

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

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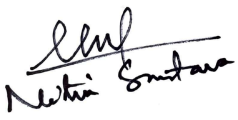

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Abstract

Alkaline proteases contribute to a significant share among industrial enzymes and are essential for various industries, viz. baking, beverage, dairy, and detergent. These are also useful for meat and leather processing, producing pharmaceutical drugs, developing photographs, synthesizing artificial sweeteners, processing textiles, and as additives in animal feeds. The versatile nature of these proteases makes them attractive candidates for wide industrial applications.

The major bottlenecks in implementing such proteases for their applicability in industrial sectors are often their low production yield and lack of stability. Bacteria are the major source of alkaline proteases for industrial purposes. The advancement in the genomic studies of bacteria allows suitable mining of their putative proteases. The heterologous expression of such proteases leads to high enzymatic titers inexpensively within a limited time. However, the stability of such proteases still remains a challenge. In this context, the halophiles producing proteases stable in high salts, alkaline pH, temperature, solvents, and surfactants are valuable candidates. These are reservoirs of robust proteases applicable for detergent industries and peptide synthesis. Moreover, they can be suitably modified through genetic manipulation techniques to tailor enzymes with enhanced catalytic efficiency and higher stability toward detergents, surfactants, and organic solvents.

The thesis encompasses the detailed genomic studies of a halotolerant *Exiguobacterium* sp. TBG-PICH-001 and characterization of its proteases. The soil sample from the Pichavaram estuary, situated on the south-east coast of Tamil Nadu, India, was used to isolate the *Exiguobacterium* sp. TBG-PICH-001. It was screened for protease production during its isolation on skim-milk agar media. The isolate exhibited protease activity as a clear zone of hydrolysis. The crude protease(s) showed pH optima at 9.0 and temperature

optima at 37 °C. These were found highly stable in the alkaline pH range (7-11) and temperatures up to 55 °C. The proteases possessed efficient surfactant and solvent stability. Its complete taxonomic position was established to comprehend the isolate's potential and correctly annotate the unique proteases. The genome of *Exiguobacterium* sp. TBG-PICH-001 was analysed and submitted to GenBank with an accession number-JADOYC000000000. The genomic sequences of the *Exiguobacterium* sp. TBG-PICH-001 genome was compared with all similar genomes in the NCBI database based on homology, orthology, and phylogenetic studies, resulting in its precise species delineation as '*Exiguobacterium indicum*'. All putative proteases were mined, enlisted, and screened for their heterologous expression, purification, and characterization.

Furthermore, the growth and adaptation mechanisms of the isolate were investigated in the presence of various organic solvents. It grew better in highly hydrophobic solvents (*log P* above 3.4), whereas the growth decreased in less hydrophobic and hydrophilic solvents. The electron micrographs revealed that the cell membrane was intact when the bacterial cells were grown in highly hydrophobic solvents like tetradecane, dodecane, and decane. In contrast, disorganized cell membranes and aggregated cells were observed in the presence of less hydrophobic solvents, such as octane, hexane, etc. The cell permeability and viability assays further confirmed the above effects. The molecular adaptations were studied through proteomic and transcriptomic analysis, which showed the upregulation of chaperonin groEL, alkaline dehydrogenase, BMP Family ABC Transporter substrate-binding protein, elongation factor Tu, and downregulation of flagellin. These results corresponded with the relative expression of these genes in the presence of hexane as a solvent. Additionally, the microbe produced alkaline protease(s), even while cultivated in various solvents. Such solvent-stable

bacteria and alkaline proteases could be useful in whole-cell biotransformation, detergent applications & peptide synthesis, respectively.

The heterologous expression of two protease genes, metalloprotease (*rsep,*1.23 Kb) and serine protease (*tpa*, 0.879 Kb) from the genome of the isolate in *Escherichia coli* BL21(DE3) was attempted. The recombinant protease yields were 6.3 and 6.7 IU/mg, 11 and 12-fold higher than the crude native protease(s). The *rsep* and *tpa* showed soluble expression at 46 and 32 kDa, respectively. These were purified to homogeneity through Ni-NTA affinity chromatography. The *rsep* and *tpa* used a wide range of protease substrates with a maximum affinity towards casein, K_m of 391 and 495.3 μM, respectively. Interestingly, the recombinant proteases were similarly stable in the alkaline pH range (7-10), surfactants, commercial detergents, and hydrophobic solvents, rendering them attractive candidates as detergent additives and for biocatalysis in non-aqueous bioprocesses.

The characterization, structure, and directed evolution studies were further investigated. The error-prone mutagenesis approach has been employed to develop a mutant metalloprotease with enhanced enzymatic potential. Four hundred PCR reactions were performed for mutant gene library preparation. Genes amplification was precisely observed in 18 modified and a single control at the predicted gene size of 1230 bp. The mutant and wild-type genes were cloned into the pET22b+ vector for overexpression. All the recombinants showed considerable expression in the soluble fraction. A screened mutant metalloprotease showed an approximately 8-fold and 4-fold increase in protease activity and catalytic efficiency, respectively. It was purified to homogeneity. It had a molecular size of 92 kDa. The structural elucidation studies through bioinformatic tools, sequencing data, and comparative biophysical studies of the wild and mutant metalloprotease revealed the presence of an increased amount of histidine residues in the mutant metalloprotease besides the

appearance of acidic residues like glutamic, aspartic acid, etc. These modifications in the protein structure of the mutant metalloprotease possibly led to a better interaction between the substrates and the mutant enzyme, which enhanced its affinity and catalytic efficiency.

Overall, the work in the thesis describes (i) genomic studies of a novel halotolerant *Exiguobacterium* sp. TBG-PICH-001. (ii) Its extracellular protease(s) which were interestingly stable in alkaline pH, a range of organic solvents, and surfactants. (iii) Recombinant proteases from its genome with higher yields. (iv) Mutagenesis studies for directed evolution of its recombinant protease to establish the structural alterations implicating significant amino acids for enhanced catalytic efficiency, substrate affinity, and alkali/solvent stability.

सार

अल्कलाइन प्रोटीएज एंजाइम जगत का एक महत्वपूर्ण एवं व्यापक अंग है। यह विविध प्रकार के उद्योगों में कार्यरत कर जाने की क्षमता रखता है। यह उद्योग है जैसे मांस या लेदर के कारखाने दवा उद्योग आप प्राकृतिक मिष्ठान टेक्सटाइल और पशुओं के खाद्य से संबंधित उद्योग प्रोटीज का बहुमुखी होना इन्हें उद्योग की दृष्टि से अधिक अधिक आकर्षक बनाता है। प्रोटीज का उपयोग बहुदा उपरोक्त उद्योगों में सफलतापूर्वक किया जाता रहा है परंतु इन्हें इनकी विशिष्ट क्षमता तक कार्यशील बनाने के लिए जैसे, कम मात्रा, कार्यशीलता एवं स्थिरता में कमी का सामना करना पड़ता है। इन पर विजय प्राप्त करने हेतु हमें हेलोफाइल्स नमक माइक्रोब्स का उपयोग करना अनिवार्य है। जिन ओवर एक्सप्रेशन प्रणाली का उपयोग करके हम इन प्रोटीज की मात्रा स्थिरता एवं कार्यशीलता को कार्यशीलता में वृद्धि ला सकते हैं इसी संदर्भ में इस थीसिस में हमने इक्सिगोबैक्टीरिया इंडीकॉम टीबी पी आई सी ह 0 0 1 नामक बैक्टीरिया का आइसोलेशन किया है जिसके उपरांत हमने प्रोटीज प्रोडक्शन को इसमें माप है प्रोटीज की प्राप्ति के बाद हमने प्रोटीज की स्थिरता को जच्चा और पाया कि यह इन्हें ऑर्गेनिक सॉल्वेंट्स में और डिटर्जेंट में अधिकांश रूप से स्थिर है इसके उपरांत हमने पाया कि यह प्रोटीन कम मात्रा में बना रहे हैं मात्र की वृद्धि के लिए हमने हेट्रोलॉगआउस एक्सप्रेशन कराया और उनकी इनकी इकाई को बढ़ाया एवं इनकी मात्रा को 11 और 12 गुना बढ़ा दिया। दो प्रोटिजेस पर हमने कार्य किया जिनमें मेटालर्फ्राटियर्स को बेहतर बनाने की कोशिश की एंजाइमेटिक क्षमता को हमने बढ़ाने के लिए एरर प्रोन पीसीआर का उपयोग किया इसके उपरांत हेट्रोलॉगआउस एक्सप्रेशन करके हमने इस प्रोटियस की कार्यशीलता को बढ़ा दिया को 8 गुना बढ़ा दिया को 4.2 गुना बढ़ाया। इन सब से हमने यह भी जांच की यह माइक्रो ऑर्गेनिक सॉल्वेंट्स में किस हद तक स्थिर है इसकी पूर्णता जांच करने के हेतु हमने माइक्रोस्कॉपी जीवन चित्रों का सहारा प्राप्त किया। यह चित्र इस बात का सूचक बन की हाइड्रोफोबिक सॉल्वेंट्स में यह बैक्टीरिया बेहतर ग्रोथ प्रोटियस प्रोडक्शन एवं स्थिरता का प्रचार एवं प्रसाद

प्रसार करता है इसके अन्यथा में यह बैक्टीरिया हाइड्रोफोबिक सॉल्वेंट्स की उपस्थिति में कम मात्रा में एंजाइम प्रोड्यूस करता है। हमने यह भी पाया मॉलेक्युलर संदर्भ में यह बैक्टीरिया किन जींस का उपयोग करता है जिससे कि वह उनके विषैला इफेक्ट से बच सके यह पाया गया कि कई सारे इलेक्ट्रॉन आएं पंप्स एवं प्लैगलिन प्रोटीन का असामान्य एक्सप्रेशन हो रहा है। इलेक्ट्रॉन पस के एक्सप्रेशन में वृद्धि एवं प्लैगलिन के एक्सप्रेशन में गिरावट देखी गई जब बैक्टीरिया को सॉल्वेंट्स की उपस्थिति में बड़ा किया जाता है। यह थीसिस हमें इस बात पर गौर करने के लिए विवश करती है कि हेलो फाइल बैक्टीरिया का उपयोग करके हम उपयोग करके भारी मात्रा में प्रोटीन जैसे एंजाइम का की एंजाइम की पैदावार कर सकते हैं और इनकी स्थिरता में भी वृद्धि ला सकते हैं। जेनेटिक इंजीनियरिंग का इस्तेमाल करके का उपयोग करके हम इन प्रोटीज का हेट्रोलॉगआउस एक्सप्रेशन करने के उपरांत उनकी पैदावार में वृद्धि, इनकी सॉल्वेंट के उपस्थिति में स्थिरता, एवं इनकी कैटालिटिक एफिशिएंसी में वृद्धि लाने में सफल हो सकते हैं।

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