

**STUDIES ON HALOPHILIC *MARINOBACTER* SP. EMB5
LIPASE: NOVEL CHARACTERISTICS, AGGREGATION
AND PROTEOMICS**

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LIPASE: NOVEL CHARACTERISTICS, AGGREGATION AND
PROTEOMICS**

by

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Submitted

in fulfillment of the requirements of the degree of DOCTOR OF PHILOSOPHY

to the



INDIAN INSTITUTE OF TECHNOLOGY DELHI
SEPTEMBER 2017

Dedicated to my family

CERTIFICATE

This is to certify that the thesis entitled “**Studies on halophilic *Marinobacter* sp. EMB5 lipase: novel characteristics, aggregation and proteomics**” being submitted by **Ms. R. HEMAMALINI** 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. Hemamalini 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:

Dr. S. K. Khare
Professor of Biochemistry
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R. Hemamalini

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Abstract

This thesis concerns the studies on a lipase from a halophilic bacterium, *Marinobacter* sp. EMB5. The major part of the work deals with lipase production and optimization, along with its purification and characterization. The aggregation tendency of the lipase, being a unique feature among halophilic enzymes has also been studied in detail. In addition to that, the halophilic adaptations of the strain using a proteomics approach and its comparison with the proteomic profile of a non-halophilic system also form an important part of the thesis.

Halophilic bacteria are a relatively important group of extremophilic microbes that thrive in presence of high concentration of salts. In recent years, there is an increasing interest in their lipase for use in industrial enzymology as they have shown remarkable solvent stability. The halophilic strain EMB5, isolated from the marine waters of Kozhikode, Kerala was selected as a source for lipase.

A preliminary characterization of the strain and its lipase was done. The strain was found to be moderately halophilic, and was identified as *Marinobacter* sp. using 16S rRNA gene sequencing. The lipase produced by the strain was found to be stable in a wide range of organic solvents.

To enhance the production level of this lipase, medium optimization was carried out by one-at-a-time approach. Optimization enhanced lipase production by 40.0 fold over that of un-optimized media. Starch as the carbon source (10.0 g/L), peptone, (8.0 g/L) and yeast extract (5 g/L) as nitrogen and vitamin sources, gum arabic (1 g/L) as the surfactant, olive oil (5 mL/L) as the inducer, MgSO₄ (0.039 g/L) and FeSO₄ (0.0001 g/L) as metal ions, NaCl-KCl (40 g/L and 10 g/L) as salts in culture media (pH 9.0) and inoculum of 4 % (v/v) were determined as optimum conditions for lipase production.

During early purification attempts it was realized that the *Marinobacter* sp EMB5 lipase had a tendency to aggregate. In order to understand the aggregation tendency of the EMB5 lipase, the effect of different additives and process conditions were studied on aggregation. Molecular weight of the aggregates was determined to be 180 kDa by using size-exclusion chromatography. While molecular weight of the disaggregated monomer was established to be 10 kDa using same method, suggesting octadecamer units in aggregates. The morphology revealed by electron microscopy showed irregular aggregates which were prone to disintegration in presence of high concentration of 2-propanol. This disaggregation using 2-propanol also resulted in a drastic decrease in the enzyme activity. However, disaggregated form showed better structural integrity compared to aggregated form of enzyme as suggested by secondary and tertiary structure analysis of the lipase protein. It emerged from these studies that the aggregates were products of random non-specific association between lipase molecules and were important for the hydrolytic activity of the lipase.

Marinobacter sp. EMB5 lipase was purified 28.2 fold with 5.6 % recovery by ultrafiltration and Sephacryl S-200 gel filtration chromatography. It was recovered in the form of aggregates. The relative molecular mass of enzyme was estimated to be ~82 kDa. It preferred medium chain fatty acid ester (C8) as substrate. The purified EMB5 lipase exhibited K_m : 1.43 μ M and V_{max} : 1.43 μ mol/mL/min towards *p*-nitrophenyl octanoate (pNPO) as substrate. The lipase was most active at 50 °C and pH 8.5. The EMB5 lipase was remarkably stable in hydrophobic organic solvents and majority of the detergents tested.

In an attempt to study the proteins responsible for halophilic adaptation in *Marinobacter* sp. EMB5, a two dimensional gel electrophoresis-based proteomics approach was adopted. The intracellular proteome was extracted from the cells prior exposed to

optimum and less-than-optimum NaCl concentrations. Overall, there was a 32 % reduction in protein expression levels under sub-optimal NaCl concentration. This indicates that a significant proportion of proteins expressed in the cell were involved in adaptation to a high salt environment.

Since organic solvents are known to mimic high salt conditions in the proximity of proteins, therefore a non-halophilic, organic solvent tolerant bacterial system was also chosen for studying the proteomic adaptation in presence of organic solvents. *Pseudomonas aeruginosa* PseA, an established organic solvent-tolerant strain was taken as the model system and the changes were studied at the level of membrane and extracellular proteome in the presence and absence of organic solvents. The studies revealed that the outer membrane porins, structural protein like flagellin and stress-induced proteins such as GroEL and superoxide dismutase were significantly affected in cells exposed to organic solvents.

The present study focuses on extremophiles, which are interesting systems of study. This work opens up new avenues of research into halophilic lipase aggregation and extremophile proteomics.

सार

यह थीसिस हालोफिलिक जीवाणु, मरिनोबैक्टर एसपी से एक लाइपेस पर अध्ययन से संबंधित है। EMB5। काम का प्रमुख हिस्सा अपने शुद्धि और लक्षण वर्णन के साथ, लाइपेस उत्पादन और अनुकूलन के साथ काम करता है। लाइपेस की एकत्रीकरण प्रवृत्ति, हेलोफिलिक एंजाइमों के बीच एक अनोखी विशेषता होने के कारण विस्तार से अध्ययन किया गया है। इसके अलावा, एक प्रोटीओमिक्स दृष्टिकोण का उपयोग करते हुए तनाव के हालोफोनिक रूपांतरों और गैर-हालोफिलिक प्रणाली के प्रोटीओमिक प्रोफाइल के साथ इसकी तुलना भी थीसिस का एक महत्वपूर्ण हिस्सा है।

हामोफिलिक बैक्टीरिया एक अपेक्षाकृत महत्वपूर्ण समूह है जो अकथोफिलिक रोगाणुओं का होता है जो लवण की उच्च एकाग्रता की उपस्थिति में कामयाब होता है। हाल के वर्षों में, औद्योगिक एंजाइमोलॉजी में उपयोग के लिए उनके लाइपेस में एक बढ़ती हुई रुचि है क्योंकि उन्होंने उल्लेखनीय विलायक स्थिरता दिखायी है। कोझिकोड के समुद्री जल से पृथक हालेफोइलिक तनाव ईएमबी 5, केरल को लाइपेस के लिए एक स्रोत के रूप में चुना गया था।

तनाव और इसकी लाइपेस का प्रारंभिक लक्षण वर्णन किया गया था। तनाव मध्यम हालफिलिक पाया गया था, और उसे मारिनोबैक्टर एसपी के रूप में पहचाना गया था। 16 एस आरआरएनए जीन अनुक्रमण का उपयोग करना तनाव से उत्पन्न लिपेज कार्बनिक सॉल्वेंट्स की एक विस्तृत श्रृंखला में स्थिर पाया गया था।

इस लाइपेस के उत्पादन स्तर को बढ़ाने के लिए, मध्यम अनुकूलन एक-एक-समय के दृष्टिकोण से किया गया था। ऑप्टिमाइजेशन ने अप्रयुक्त मीडिया के मुकाबले 40.0 गुना बढ़ाकर लाइपेस उत्पादन बढ़ाया। नाइट्रोजन और विटामिन स्रोत, गम अरबी (1 ग्रा / एल) के रूप में कार्बोन स्रोत (10.0 ग्राम / एल), पेप्टाण, (8.0 ग्राम / एल) और खमीर निकालने (5 ग्राम / एल) के रूप में स्टार्च, जैतून का तेल (5 एमएल / एल) इंडियस्टर के रूप में, एमजीएसओ 4 (0.039 ग्राम / एल) और फेएसओ 4 (0.0001 ग्राम / एल) के रूप में धातु आयनों, NaCl-KCl (40 ग्राम / एल और 10 ग्राम / एल) के रूप में संस्कृति मीडिया (पीएच 9.0) और 4% (वी / वी) के inoculum lipase उत्पादन के लिए इष्टतम शर्तों के रूप में निर्धारित किया गया।

शुरुआती शुद्धि प्रयासों के दौरान यह महसूस किया गया कि मारिनोबैक्टर एसईएम एम्बी 5 लाइपेस में कुल मिलाकर एक प्रवृत्ति थी। ईएमबी 5 लाइपेस की एकत्रीकरण प्रवृत्ति को समझने के लिए, एकत्रीकरण पर विभिन्न योजक और प्रक्रिया की स्थिति का अध्ययन किया गया। समुच्चय के आणविक भार को आकार-बहिष्करण क्रोमैटोग्राफी का उपयोग करके 180 केडीए होना निर्धारित किया गया था। जबकि पृथक मोनोमर का आणविक भार 10 डिग्री केडीए समान विधि का उपयोग करके स्थापित किया गया था, जबकि समुच्चय में ओकटैडेकर इकाइयों का सुझाव दिया गया था। इलेक्ट्रॉन माइक्रोस्कोपी द्वारा पता चला आकृति विज्ञान अनियमित समुच्चय दिखाता है जो 2 प्रोपेनॉल की उच्च एकाग्रता की

उपस्थिति में विघटन के लिए प्रवण थे। 2-प्रोपेनॉल का उपयोग करने वाली यह असंतुलन भी एंजाइम गतिविधि में भारी कमी आई है। हालांकि, असंतृप्त रूप में एंजाइम के समग्र रूप की तुलना में बेहतर संरचनात्मक अखंडता दिखाया गया है जैसा कि लाइपेस प्रोटीन के माध्यमिक और तृतीयक संरचना विश्लेषण द्वारा सुझाई गई है। यह इन अध्ययनों से उभरा है कि समुच्चय लाइपेस अणुओं के बीच यादृच्छिक गैर-विशिष्ट सहयोग के उत्पाद थे और लाइपेस की हाइड्रोलाइटिक गतिविधि के लिए महत्वपूर्ण थे।

मारिनोबैक्टर एसपी ईएमबी 5 लाइपेस को 28.2 गुना शुद्ध और अल्ट्राफिल्टरेशन और सेफैक्रील एस -200 जेल निस्पंदन क्रोमैटोग्राफी द्वारा 5.6% की वसूली के साथ शुद्ध किया गया था। यह समुच्चय के रूप में वसूल किया गया था एंजाइम के रिश्तेदार आणविक द्रव्यमान का अनुमान ~ 82 केडीए था इसे मध्यम श्रृंखला फैटी एसिड एस्टर (सी 8) को सब्सट्रेट के रूप में पसंद किया गया था। शुद्ध ईएमबी 5 लाइपेस का प्रदर्शन किलोग्राम: 1.43 माइक्रोग्राम और वीएमएक्स: सब्सट्रेट के रूप में पी-नाइट्रोफेनील ऑक्टोनेट (पीएनपीओ) की तरफ $1.43 \mu\text{mol} / \text{एमएल} / \text{मिन}$ । लाइपेस 50 डिग्री सेल्सियस और पीएच 8.5 में सबसे अधिक सक्रिय था। ईएमबी 5 लाइपेस हाइड्रोफोबिक कार्बनिक सॉल्वेंट्स में निश्चित रूप से स्थिर था और परीक्षण के अधिकांश डिटर्जेंट थे।

मैरिनोबैक्टर एसपी में हालोफिलिक अनुकूलन के लिए जिम्मेदार प्रोटीन का अध्ययन करने की कोशिश में ईएमबी 5, एक दो आयामी जेल वैद्युतकणसंचलन-आधारित

प्रोटीओमिक्स दृष्टिकोण अपनाया गया था। मैरीनोबैक्टर एसपी के संपर्क में आने वाली कोशिकाओं से इंटरसेल्युलर प्रोटीम निकाला गया था। ईएमबी 5 लाइपेस को 28.2 गुना शुद्ध और अल्ट्राफिल्टरेशन और सेफैक्रील एस -200 जेल निस्पंदन क्रोमैटोग्राफी द्वारा 5.6% की वसूली के साथ शुद्ध किया गया था। यह समुच्चय के रूप में वसूल किया गया था। एंजाइम के रिश्तेदार आणविक द्रव्यमान का अनुमान ~ 82 केडीए था। इसे मध्यम श्रृंखला फैटी एसिड एस्टर (सी 8) को सब्सट्रेट के रूप में पसंद किया गया था। शुद्ध ईएमबी 5 लाइपेस का प्रदर्शन किलोग्राम: 1.43 माइक्रोग्राम और वीएमएक्स: सब्सट्रेट के रूप में पी-नाइट्रोफेनील ऑक्टोनेट (पीएनपीओ) की तरफ $1.43 \mu\text{mol} / \text{एमएल} / \text{मिन}$ । लाइपेस 50 डिग्री सेल्सियस और पीएच 8.5 में सबसे अधिक सक्रिय था। ईएमबी 5 लाइपेस हाइड्रोफोबिक कार्बनिक सॉल्वेंट्स में निश्चित रूप से स्थिर था और परीक्षण के अधिकांश डिटर्जेंट थे।

मैरीनोबैक्टर एसपी में हालोफिलिक अनुकूलन के लिए जिम्मेदार प्रोटीन का अध्ययन करने की कोशिश में ईएमबी 5, एक दो आयामी जेल वैद्युतकणसंचलन-आधारित प्रोटीओमिक्स दृष्टिकोण अपनाया गया था। इंटरसेल्युलर प्रोटीम कोशिकाओं से निकाला गया था जो पहले इष्टतम और कम-इष्टतम NaCl सांद्रता के सामने था। कुल मिलाकर, उप-इष्टतम NaCl एकाग्रता के तहत प्रोटीन अभिव्यक्ति के स्तर में 32% की कमी आई थी। इससे पता चलता है कि सेल में व्यक्त प्रोटीन का एक महत्वपूर्ण अनुपात उच्च नमक वातावरण के अनुकूलन में शामिल था।

चूंकि कार्बनिक सॉल्वेंट्स प्रोटीन के निकट उच्च नमक की स्थिति की नकल करने के लिए जाने जाते हैं, इसलिए कार्बनिक सॉल्वेंट्स की उपस्थिति में प्रोटॉमिक अनुकूलन का अध्ययन करने के लिए एक गैर-हेलोफिलिक, कार्बनिक विलायक सहिष्णु बैक्टीरियल सिस्टम भी चुना गया था। *स्यूडोमोनस एरुगिनोसा* पीएसए, एक स्थापित कार्बनिक विलायक-सहिष्णु तनाव मॉडल प्रणाली के रूप में लिया गया था और परिवर्तनों में उपस्थिति और कार्बनिक सॉल्वेंट्स की उपस्थिति में झिल्ली और बाह्य प्रोटीम के स्तर पर अध्ययन किया गया था। अध्ययनों से पता चला है कि बाह्य झिल्ली porins, flagellin जैसे स्ट्रक्चरल प्रोटीन और ग्रोएल और सुपरऑक्साइड dismutase जैसे तनाव से प्रेरित प्रोटीन कार्बनिक सॉल्वेंट्स के संपर्क में कोशिकाओं में काफी प्रभावित थे।

वर्तमान अध्ययन extremophiles पर केंद्रित है, जो अध्ययन के दिलचस्प सिस्टम हैं। यह काम हैलोफिलिक लाइपेस समूह और एक्सट्रोफाइल प्रोटिओमिक्स में अनुसंधान के नए रास्ते खोलता है।

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