BIOTRANSFORMATION OF AMIDES USING *Delftia acidovorans* WHOLE CELLS AND CROSS-LINKED ENZYME AGGREGATES (CLEA)

TUSHAR DASH

DEPARTMENT OF BIOCHEMICAL ENGINEERING AND BIOTECHNOLOGY

INDIAN INSTITUTE OF TECHNOLOGY DELHI

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by

TUSHAR DASH

Department of Biochemical Engineering and Biotechnology

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DEDICATED

TO

MY BELOVED

PARENTS

CERTIFICATE

This is to certify that the thesis entitled **"Biotransformation of amides using *Delftia acidovorans* whole cells and cross linked enzyme aggregates (CLEA)"** being submitted by **Mr. Tushar Dash** is worthy of consideration for the award of the degree of **Doctor of Philosophy.** The thesis has been prepared by him under my supervision and guidance in conformity with the rules and regulations of Indian Institute of Technology Delhi and is a record of the original bonafide research work. The results presented in this thesis have not been submitted in part or full to any other universities or institutes for the award of any other degree or diploma.

Dr. Prashant Mishra

Professor

Department of Biochemical Engineering & Biotechnology

Indian Institute of Technology Delhi

Hauz Khas New Delhi -110016

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Abstract

In today's world of growing population with limited natural resources to cater to, white biotechnological practices have become sine qua non for most of the countries of the world. Biotransformation is one of those practices which employ different kinds of living cells and their products to transform one compound to another compound with added value in terms of nutrition, texture, quality, toxicity etc. There has been always a search for a novel biocatalyst with a potential to catalyze a broad spectrum of substrates into useful products which can be recycled. Amidase is not an exception to this search since it has proved its efficacy in converting the carcinogenic acrylamide to non carcinogenic acrylic acid. In the present study amidase from *D. acidovorans* was used to biotransform different amides such as acrylamide, acetamide, propionamide, malonamide, nicotinamide to their corresponding carboxylic acids. Amidase from this strain was used in various forms such as enzyme isolate, whole cell biocatalyst (free and immobilized form), cross linked enzyme aggregate.

D. acidovorans whole cells were screened with different permeabilizing agents, out of which 15% toluene gave the maximum extent of permeabilization. The whole cell biocatalyst was found to be effective in biotransforming different amides upto 3 cycles. Acrylamide was the substrate which showed a maximum conversion of 65% at 40°C in 12 hours. Further the reusability of the whole cells was increased upto 5 cycles on immobilization in the alginate matrix. The immobilized whole cells were found to be thermally stable for 5 hours at 40°C and became unstable only after 10 hours.

Further to improve the biotransformation efficiency of *D. acidovorans* amidase, it's crosslinked enzyme aggregates (CLEA) were prepared by using glutaraldehyde as the crosslinker. Two kinds of CLEAs were prepared using organic solvents such as acetone and water soluble polymers such as PEI (Polyethyleneimine). Different parameters such as addition of additives such as BSA, time of cross linking, type of crosslinker used, temperature, pH which affects the performance of CLEA were optimized. CLEAs prepared by using acetone as precipitant, BSA as inert additive and crosslinked for 4 hours using 25 mM glutaraldehyde were found to be the most active biocatalyst. Acetamide showed 77% conversion by using CLEA of amidase from *D.acidovorans*. These CLEAs also had a high reusability factor upto 8 cycles. The CLEAs prepared by using water soluble polymers were found to be highly thermostable for a period more than 2 hours at 40°C to 50 °C.

Amidase was isolated using a less energy intensive process such as microwave at 300 watt for 10 mins. It was found that amidase isolated by the conventional sonication method had the same specific activity (0.2 U/mg) as that of the microwave isolated amidase. Hence in future this process can be scalable to an industrial level. The efficiency of the microwave isolated amidase was tested in terms of its catalysis of different amide substrates. Nicotinamide showed the highest enzyme activity i.e. 10 times as compared to the conventionally isolated amidase thereby proving the fact that on microwave irradiation enzyme ability to catalyze reaction increases. The amidase isolated by sonication method was partially purified with an yield of 30% and a purification fold of 12.

सार

आज के सीमित प्राकृतिक संसाधनों के साथ बढ़ती आबादी की दुनिया में, अधिकांश देशों के लिए श्वेत जैव प्रौद्योगिकी प्रथाएं अनिवार्य हो गई हैं। बायोट्रांसफॉर्मेशन उन प्रथाओं में से एक है, जो विभिन्न प्रकार की जीवित कोशिकाओं और उनके उत्पादों को एक यौगिक को दूसरे यौगिक में बदलने के लिए नियुक्त करते हैं, पोषण, बनावट, गुणवत्ता, विषाक्तता आदि के मामले में अतिरिक्त मूल्य के साथ। एक अनोखा बायोकेटलिस्ट के लिए हमेशा खोज की गई है जिसमें सब्सट्रेट के एक व्यापक स्पेक्ट्रम को उपयोगी उत्पादों में उत्प्रेरित करने की क्षमता तथा जिसे पुनर्नवीनीकरण किया जा सकता है। एमिडेज़ इस खोज का अपवाद नहीं है क्योंकि यह कार्सिनोजेनिक एक्रिलामाइड को गैर कार्सिनोजेनिक ऐक्रेलिक एसिड में परिवर्तित करने में अपनी प्रभावशीलता साबित कर चुका है। डी.एसिडोवोरन्स से एमिडेज़ के वर्तमान अध्ययन में एक्रिलामाइड, एसिटामाइड, प्रोपियोनामाइड, मैलोनामाइड, निकोटिनमाइड जैसे अलग-अलग अमाइड्स को उनके अनुरूपी अलग-अलग कार्बोक्जिलिक एसिड में बायोट्रांसफॉर्म करने के लिए इस्तेमाल किया गया था। इस स्ट्रेन से एमिडेज़ को विभिन्न रूपों में इस्तेमाल किया गया था जैसे कि एंजाइम आइसोलेट, पूरे सेल बायोकैटलिस्ट (मुक्त और स्थिर रूप), क्रॉस लिंक्ड एंजाइम एग्रीगेट।

डी. एसिडोवोरेंस की पूरी कोशिकाओं को अलग-अलग पारगम्य एजेंटों के साथ जांचा गया था, जिसमें से 15% टोल्यूनि ने परमैबिलाइजेशन की अधिकतम सीमा दी थी। पूरे सेल बायोकैटलिस्ट को 3 चक्रों तक अलग-अलग अमाइड में बायोट्रांसफॉर्मिंग में प्रभावी पाया गया था । एक्रिलामाइड सब्सट्रेट था जिसने 12 घंटे में 40°C पर 65% का अधिकतम रूपांतरण दिखाया। इसके अलावा एल्गिन मैट्रिक्स में स्थिरीकरण पर 5 चक्रों तक पूरे कोशिकाओं की पुन: प्रयोज्यता बढ़ाई गई थी। इम्मोबिलाइज्ड पूरी कोशिकाएं 40°C पर 5 घंटे के लिए थर्मल रूप से स्थिर पाई गईं और केवल 10 घंटों के बाद अस्थिर हो गईं थी।

डी. एसिडोवोरेंस एमिडेज़ की बायोट्रांसफॉर्मेशन दक्षता में सुधार करने के लिए, उसका क्रॉसलिंक एंजाइम एग्रीगेट (CLEA) को ग्लूटेराल्डिहाइड का उपयोग करके क्रॉसलिंकर के रूप में तैयार किया गया था। कार्बनिक सॉल्वैंट्स जैसे कि एसीटोन और पानी में घुलनशील पॉलिमर जैसे पीईआई (पॉलीएथिलीनिमाइन) का उपयोग करके दो प्रकार के क्रॉसलिंक एंजाइम एग्रीगेट (CLEA) तैयार किए गए थे। विभिन्न मापदंडों को अनुकूलित किया गया था जैसे कि बीएसए जैसे एडिटिव्स, क्रॉस लिंकिंग का समय, इस्तेमाल किए जाने वाले क्रॉसलिंकर का प्रकार, तापमान, पीएच जो क्रॉसलिंक एंजाइम एग्रीगेट (CLEA) के प्रदर्शन को प्रभावित करता है, । एसीटोन को अवक्षेपक के रूप में, बीएसए को अक्रिय

योज्य के रूप में और 25 mM ग्लूटाराल्डिहाइड का उपयोग करके 4 घंटे के लिए क्रॉस लिंकिंग करके तैयार किए गए CLEAs सबसे सक्रिय बायोकाटलिस्ट पाए गए थे। एसिटामाइड ने डी. एसिडोवोरेंस से एमिडेज़ के CLEA का उपयोग करके 77% रूपांतरण दिखाया। इन CLEAs में 8 चक्र तक एक उच्च पुन: प्रयोज्य कारक भी था। पानी में घुलनशील पॉलिमर का उपयोग करके तैयार किए गए CLEAs 40°C से 50°C तक 2 घंटे से अधिक की अवधि के लिए अत्यधिक थर्मोस्टेबल पाए गए।

10 मिनट के लिए 300 वाट पर माइक्रोवेव जैसे कम ऊर्जा गहन प्रक्रिया का उपयोग करके एमिडेज़ को अलग किया गया था। यह पाया गया कि पारंपरिक sonication विधि द्वारा पृथक एमिडेज़ में वही विशिष्ट उद्योगिता (0.2 U/mg) थी जो माइक्रोवेव से पृथक एमिडेज़ की थी। इसलिए भविष्य में यह प्रक्रिया एक औद्योगिक स्तर तक आरोह्य हो सकती है। अलग-अलग अमाइड सब्सट्रेट्स के इसके कैटेलिसिस के संदर्भ में माइक्रोवेव पृथक एमिडेज़ की दक्षता का परीक्षण किया गया था। निकोटिनामाइड ने पारंपरिक रूप से पृथक एमिडेज़ की तुलना में सबसे अधिक एंजाइम उद्योगिता यानी 10 गुना दिखाया, जिससे यह तथ्य साबित होता है कि प्रतिक्रिया को उत्प्रेरित करने के लिए माइक्रोवेव विकिरण की एंजाइम क्षमता पर असर है । Sonication विधि द्वारा पृथक एमिडेज़ को आंशिक रूप से 30% की उपज और 12 के शुद्धि गुना के साथ शुद्ध किया गया था।

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°C	Degree Celsius
CLEA	Cross linked enzyme aggregate
dew	Dry cell weight
DEAE	Diethyl amino ethane
DS	Dextran sulphate
HCl	Hydrochloric acid
H_2O	Water
kDa	Kilo Dalton
L	Litre
NaH_2PO_4	Sodium di-hydrogen phosphate
Na_2HPO_4	di-Sodium hydrogen phosphate
NH_3	Ammonia
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimeter
mM	Millimolar
MTCC	Microbial Type Culture Collection
MW	Molecular weight
PAGE	Polyacrylamide gel electrophoresis
PEI	Polyethylene imine
pH	Potential of hydrogen
rpm	Revolutions per minute
sp.	Species
SDS	Sodium dodecyl sulphate
$T_{1/2}$	Half life
U	Units
v/v	Volume by volume
α	Alpha
β	Beta
µg	Microgram
µl	Microlitre