SYNTHESIS OF MALIC ACID BY REDIRECTION OF CELLULAR METABOLISM IN *ZYMOMONAS MOBILIS*

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by

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CERTIFICATE

This is to certify that the thesis titled "**Synthesis of malic acid by redirection of cellular metabolism in *Zymomonas mobilis***" being submitted by **Mr. Rohit Khandelwal** to the Indian Institute of Technology Delhi for the award of the degree of **Doctor of Philosophy** is a record of bona fide research work carried out by him under our supervision and guidance in conformity with the rules and regulations of Indian Institute of Technology Delhi.

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

Date

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ABSTRACT

Malic acid has a wide variety of applications in different industries and its demand has been increasing over the years. At present, malic acid is mainly produced by chemical methods which lead to various environmental sustainability concerns. Since it is naturally synthesized in the cell via TCA pathway, microorganisms offer an eco-friendly and cost-effective alternative for malic acid production. Additional advantages of microbial production include synthesis of pure L-form of malic acid. Several studies have been conducted earlier in different organisms. This study was an attempt to produce malic acid by genome engineering in *Zymomonas mobilis*.

The pyruvate decarboxylase enzyme, which constitutes significantly high amount of total protein in *Z. mobilis* and thus responsible for high production of ethanol, was selected for deletion to divert the flux towards malic acid. The replication and stability of pBBR1 and RK2 replicon was established in *Z. mobilis*. Recombineering was used and the *pdc* gene was replaced in the genome by a kan^R cassette by homologous recombination in the presence of a pSIM plasmid containing pBBR1 replicon and lambda *red* genes. The deletion of *pdc* gene was confirmed by PCR using region specific and gene specific primers and Southern blotting and hybridization. As a result of *pdc* gene deletion, malic acid production increased compared to that in wild type strain and more than 50% of theoretical yield was obtained. But the deletion resulted into disturbed redox balance due to which growth was hampered. The Δ*pdc* mutant cells were also found to be shrunken.

As an alternative strategy, gene encoding malic enzyme from *Escherichia coli* (*Ecmae*) was expressed in *Z. mobilis* under different promoters. To select the appropriate promoters, genome-wide analysis of promoters was conducted and -10 and -35 box consensus sequences of *Z. mobilis* promoters were predicted. The P*chap*, P*pap* and P*pdc* promoters from *Z. mobilis* were selected. The strengths of these promoters were determined and compared with P*tac*

promoter in *E. coli* by cloning gfp_{uv} gene downstream to them. The expression of gfp_{uv} was studied with respect to growth at different pH and temperatures. Based on the results, P*chap* and P*pdc* promoters from *Z. mobilis*, and P*tac* promoter from *E. coli* were used to express *Ecmae* gene in *Z. mobilis* to increase malic acid production. The mae^{+} recombinants were obtained by recombineering-based genomic integration of P*chap-mae*, P*tac-mae* and P*pdc-mae* sequences. Maximum malic acid yield was obtained in P*pdc-mae* recombinant, followed by P*tac-mae* and P*chap-mae* recombinants. In P*pdc-mae* recombinant, the yield of malic acid obtained in shake flask was ~31% of theoretical, while ~37% of theoretical yield was obtained in a batch fermenter, which were much higher than that in the wild type strain. This is the first report demonstrating the use of lambda *red* genes based recombineering for deletion as well as integration of genes in *Z. mobilis*. The methodology developed and the mutants of *Z. mobilis* constructed in the present study can be used for several other metabolic engineering applications.

सार

मैलिक अम्ल के विभिन्न उद्योगों में अनेक प्रकार के अनुप्रयोग हैं और इसकी मांग वर्षों से बढ़ रही है। वर्तमान में, मैलिक अम्ल मुख्य रूप से रासायनिक विधियों द्वारा निर्मित होता है जो विभिन्न पर्यावरणीय स्थिरता संबंधी चिंताओं को जन्म देता है। चूंकि यह स्वाभाविक रूप से टीसीए मार्ग के माध्यम से कोशिका में संश्लेषित होता है, सूक्ष्मजीव मैलिक अम्ल उत्पादन के लिए एक पर्यावरण के अनुकूल और लागत-प्रभावी विकल्प प्रदान करते हैं। सूक्ष्मजीवों द्वारा उत्पादन के अतिरिक्त लाभों में मैलिक अम्ल के शुद्ध एल-रूप का संश्लेषण शामिल है। इससे पहले विभिन्न जीवों में कई अध्ययन किए जा चुके हैं। यह अध्ययन *ज़ाइमोमोनास मोबिलिस* में जीनोम इंजीनियरिंग द्वारा मैलिक अम्ल का उत्पादन करने का एक प्रयास था।

पाइरूवेट डिकार्बोक्सिलेज एंज़ाइम, जो *ज़ा. मोबिलिस* में कुल प्रोटीन की उच्च मात्रा का गठन करता है और इस प्रकार इथेनॉल के उच्च उत्पादन के लिए जिम्मेदार है, को हटाने के लिए चुना गया था ताकि प्रवाह को मैलिक अम्ल की ओर मोड़ा जा सके। *ज़ा. मोबिलिस* में pBBR1 और RK2 प्रतिकृति और स्थिरता स्थापित की गई। पुनर्संयोजन का उपयोग किया गया और *pdc* जीन को pBBR1 प्रतिकृति और लैम्ब्डा रेड जीन युक्त pSIM प्लास्मिड की उपस्थिति में समजातीय पुनर्संयोजन द्वारा एक kanR कैसेट द्वारा जीनोम में विस्थापित कर दिया गया। पीसीआर द्वारा क्षेत्र विशिष्ट और जीन विशिष्ट प्राइमरों और सदर्न ब्लोटिंग और संकरण का उपयोग करके *pdc* जीन के विलोपन की पुष्टि की गई। *pdc* जीन विलोपन के परिणामस्वरूप, प्रकृतिकृत प्रकार के रूप की तुलना में मैलिक अम्ल उत्पादन में वृद्धि हुई और 50% से अधिक सैद्धांतिक उपज प्राप्त हुई। लेकिन विलोपन के परिणामस्वरूप रेडॉक्स संतुलन गड़बड़ा गया जिसके कारण विकास बाधित हुआ। *pdc* उत्परिवर्ती कोशिकाएँ भी सिकुड़ी हुई पाई गईं।

एक वैकल्पिक रणनीति के रूप में, *एस्चेरिचिया कोलाई* से मेलिक एंज़ाइम बनाने वाले जीन (*Ecmae*) को विभिन्न प्रमोटरों के तहत *ज़ा. मोबिलिस* में व्यक्त किया गया। उपयुक्त प्रमोटरों का चयन करने के लिए, प्रमोटरों का जीनोम-वाइड विश्लेषण किया गया और *ज़ा. मोबिलिस* प्रमोटरों के -10 और -35 बॉक्स सर्वसम्मति अनुक्रमों की अनुमान लगाया गया। *ज़ा. मोबिलिस* से P*chap*, P*pap* और P*pdc* प्रमोटरों का चयन किया गया। इन प्रमोटरों का सामर्थ्य निर्धारित किया गया और *ई. कोलाई* में P*tac* प्रमोटर के साथ gfp_{uv} जीन के तहत क्लोनिंग करके उनकी तुलना की गई। विभिन्न pH और तापमान पर वृद्धि के साथ gfp_{uv} की अभिव्यक्ति का अध्ययन किया गया। परिणामों के आधार पर, *ज़ा. मोबिलिस* के Pchap और Ppdc प्रमोटरों, और *ई. कोलाई* के P*tac* प्रमोटर का उपयोग *ज़ा. मोबिलिस* में *Ecmae* जीन को व्यक्त करके मैलिक अम्ल उत्पादन बढ़ाने के लिए किया गया। mae^+ पुनः संयोजक P*chap-mae*, P*tac-mae* और P*pdc-mae* अनुक्रमों के पुनर्संयोजन-आधारित जीनोमिक एकीकरण द्वारा प्राप्त किए गए। P*pdc-mae* पुनः संयोजक में अधिकतम मैलिक अम्ल उपज प्राप्त की गई, इसके बाद P*tac-mae* और P*chap-mae* पुनः संयोजक थे। P*pdc-mae* पुनः संयोजक में, शेक फ्लास्क में प्राप्त मैलिक अम्ल की उपज ~31% सैद्धांतिक थी, जबकि एक बैच किण्वक में ~37% सैद्धांतिक उपज प्राप्त की गई, जो कि प्रकृतिकृत प्रकार के रूप की तुलना में बहुत अधिक थी। यह पहली रिपोर्ट है जिसमें *ज़ा. मोबिलिस* में विलोपन के साथ-साथ जीन के एकीकरण के लिए लैम्ब्डा रेड जीन आधारित पुनर्संयोजन के उपयोग को प्रदर्शित किया गया है। विकसित की गई कार्यप्रणाली और वर्तमान अध्ययन में निर्मित *ज़ा. मोबिलिस* के उत्परिवर्ती का उपयोग कई अन्य चयापचय अभियांत्रिकी अनुप्रयोगों के लिए किया जा सकता है।

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ABBREVIATIONS AND SYMBOLS

Abbreviation/ Symbol	Expansion
°C	Degree centigrade
Δ	Delta/deletion
α	Alpha
β	Beta
λ	Lambda
µF	Microfarad(s)
µg	Microgram(s)
µL	Microliter(s)
µM	Micromolar
σ	Sigma
φ	Phi
Ω	ohm(s)
APS	Ammonium per sulphate
ATP	Adenosine triphosphate
AU	Arbitrary units
BLAST	Basic local alignment search tool
bp	base pair
cDNA	Complementary deoxyribonucleic acid
Cm	Chloramphenicol
CSPD	Disodium 3-(4-methoxyspiro {l,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl
DIG	Digoxigenin

DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
DTT	Dithiothreitol
Ecmae	Gene encoding malic enzyme in *E. coli*
EDTA	Ethylenediamine tetraacetic acid
EtBr	Ethidium bromide
g/L	Gram per liter
GFP_{UV}	Green fluorescent protein
h	Hour(s)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
IPTG	Isopropyl β-D-thiogalactopyranoside
Kan	Kanamycin
kb	kilo base pair
kDa	kilo Dalton
kV	kilo volt(s)
LA	Luria Agar
LB	Luria Broth
LC/Q-TOF	Liquid chromatography/quadrupole-time-of-flight
M	Molar
mae	Gene encoding malic enzyme
MCT	Microcentrifuge tube
min	Minute(s)
mL	Milliliter(s)
mm	Millimeter

mM	Millimolar
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MTCC	Microbial type culture collection
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NCBI	National Center for Biotechnology Information
ng	Nanogram(s)
OD_{600}	Optical density at 600 nm wavelength
PAP2	Phosphatidic acid phosphatases
PBS	Phosphate buffer saline
P*chap*	Promoter transcribing gene encoding chaperonin
PCR	Polymerase chain reaction
pdc	Pyruvate decarboxylase encoding gene
P*pap*	Promoter transcribing gene encoding phosphatase PAP2 family protein
P*pdc*	Promoter transcribing gene encoding pyruvate decarboxylase enzyme
P*tac*	*E. coli* promoter hybrid between the *trp* and *lac* UV5 promoters
recA	*recombinase A*
RID	Refractive index detector
RM	Rich medium
RNA	Ribonucleic acid
RNase A	Ribonuclease A enzyme
rpm	Revolutions per minute

RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	Second(s)
SSC	Saline sodium citrate
st-DNA	Salmon testes-deoxyribonucleic acid
TAE	Tris-glacial acetic acid-EDTA
TEMED	Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) amino methane
UV	Ultraviolet
UVD	UV-vis detector
V/m	volts per meter
vvm	Volume per volume per minute
WT	Wild type
Zmmae	Gene encoding malic enzyme in *Z. mobilis*