

**METABOLIC ENGINEERING OF *ZYMO MONAS*
MOBILIS FOR ACETOIN, D-(-)-2,3-BUTANEDIOL,
AND VALINE BRANCH INTERMEDIATE
PRODUCTION**

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INDIAN INSTITUTE OF TECHNOLOGY DELHI

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by

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**Department of Biochemical Engineering and
Biotechnology**

Submitted

in fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

to the



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Certificate

This is to certify that the thesis entitled “**Metabolic engineering of *Zymomonas mobilis* for acetoin, D-(-)-2,3-butanediol, and valine branch intermediate production**” being submitted by **Ms. Sakshi** to the Indian Institute of Technology Delhi, for the award of the degree of **Doctor of Philosophy**, is a record of the bonafide research work carried out by her, which has been prepared under my supervision in conformity with the rules and regulations of the Indian Institute of Technology, Delhi. The research reports and the results presented in this thesis have not been submitted for any degree or diploma at any other university or institute.

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Abstract

Zymomonas mobilis produces ethanol at high yields and titres due to its high ethanol tolerance. It utilises sugars via a highly active Entner–Doudoroff pathway, resulting in a rapid glucose consumption rate. *Z. mobilis* has an incomplete tricarboxylic acid cycle (TCA) and a deficient respiratory chain, which makes it dependent on glucose-to-ethanol fermentation for energy production and redox balance. It exhibits low biomass yield and extremely high ethanol yield, as a significant portion of the carbon flux (95-97%) is directed towards ethanol production. Due to these characteristics, *Z. mobilis* can be an excellent host for producing non-native compounds at high yields, provided the flux through the native ethanol pathway can be effectively eliminated.

In this work, *Z. mobilis* was engineered to produce acetoin and D-(-)-2,3-butanediol (2,3-BDO) by heterologously expressing the key pathway genes, acetolactate synthase (*alsS*)/acetohydroxyacid synthase (*ahasI*), acetolactate decarboxylase (*alsD*), and R, R-2,3-butanediol dehydrogenase (*bdhA*), through integration at two neutral gene loci. The engineered strain produced acetoin and 2,3-BDO at low yields, as most carbon flux (77-88%) was directed towards ethanol production. To improve yields, we targeted the ethanol pathway by deleting the *adh1* and *adh2* genes (based on previous work in our lab) while concurrently integrating the acetoin/2,3-BDO pathway genes (*ahasI*, *alsD*, and *bdhA*). The Zm_ΔA1ΔA2_HS1D strain exhibited a slight increase in acetoin yield compared to the wild-type strain and produced negligible ethanol (~0.8 g/l). However, the Zm_ΔA1ΔA2_HS1D strain could not consume more than 10-12 g/l of glucose. The decreased glucose consumption could be due to the low activity of the heterologous genes or the strain's inability to regenerate NAD(P)⁺ without ethanol production. The *nox* gene encoding NADH oxidase from *Lactococcus lactis* was expressed in the Zm_ΔA1ΔA2_HS1D strain to regenerate cofactor equivalents. The resulting Zm_ΔA1ΔA2_HS1DX strain was further improved by expressing a strong *alsS* gene from *Bacillus subtilis* to reroute more pyruvate flux towards 2,3-BDO and acetoin formation. The engineered strain, Zm_ΔA1ΔA2ΔM_HS1DXS (harbouring both *Ll.nox* and *Bs.alsS* genes), produced acetoin with a yield of 0.27 g/g of glucose, corresponding to 54.5% of the maximum theoretical yield (MTY). However, only 50-65% of the carbon flux was diverted towards acetoin, 2,3-BDO, and glycerol.

We deleted the *pdc* gene in the engineered acetoin/2,3-BDO production strain Zm_ΔA1ΔA2_HS1DX to improve yields. The Zm_ΔA1ΔA2ΔP_HS1DXS strain (with the

adh1, *adh2*, and *pdh* gene deletion) did not produce ethanol under any growth conditions. Under aerobic condition, the strain achieved an acetoin yield of 0.38 g/g of glucose, corresponding to 76% of the MTY. The Zm_ΔA1ΔA2ΔP_HS1DXS strain was cultivated in a bioreactor under different dissolved oxygen (DO) levels to optimize the growth conditions for acetoin/2,3-BDO production. Under 30-35% DO, the strain produced 39.09 g/l of acetoin and 7.86 g/l of 2,3-BDO from 128 g/l of glucose over 11 days. At a DO level below 10%, the strain produced 11.22 g/l of 2,3-BDO from 30 g/l of glucose in 5.6 days, achieving 75% of the MTY for 2,3-BDO. This study demonstrated the ability of a single recombinant strain to produce both acetoin and 2,3-BDO at high yields, with the production profile being dependent on the aeration conditions. The strain exhibited robust growth in bioreactors and consumed high glucose concentrations. The Zm_ΔA1ΔA2ΔP_HS1DXS strain was further engineered to increase acetoin yield by deleting the *bdhA* gene. The Zm_ΔA1ΔA2ΔP_KS1DXS strain (with the *bdhA* gene deletion) consumed up to 80 g/l of glucose in shake flask and accumulated acetoin with a yield of 0.44 g/g (88% of the MTY).

We sought to engineer *Z. mobilis* to produce valine or valine pathway intermediates by redirecting carbon flux from ethanol towards valine. The valine pathway genes (*Bs.alsD*, *Zm.ilvC*, *Zm.ilvD*, and *Bs.bcd*) were integrated at the *adh1* and *adh2* gene loci. The strong *B. subtilis* ALS was selected to catalyse the conversion of pyruvate to acetolactate, the first step towards valine production. For the subsequent two steps, native ketol-acid reductoisomerase (*ilvC*) and dihydroxy-acid dehydratase (*ilvD*) genes were overexpressed by placing them under a strong *Ppdc* promoter. *B. subtilis* NADH-specific L-leucine dehydrogenase (BCD) was selected to catalyse the last reductive step. The Zm_ΔA1ΔA2_S.Zm.CD.D strain did not produce valine, likely due to the inability of the native ILVC and ILVD enzymes to pull flux downstream towards valine synthesis. To address this limitation, the Zm_ΔA1ΔA2_S.Zm.CD.D strain was engineered to express the *E. coli ilvC* gene, resulting in the generation of a new strain, Zm_ΔA1ΔA2ΔAT_S.Zm.CD.D.EcC. However, valine production was not detected.

In this study, *Z. mobilis* was engineered to produce acetoin and 2,3-BDO at high yields by eliminating ethanol production through the introduction of an alternative pathway for redox regeneration. This work demonstrated that complete flux redirection in *Z. mobilis* is possible by deleting the *pdh* gene and introducing an alternative pathway.

सार

ज़इमोमोनास मोबिलिस एक इथेनॉल उत्पादक जीवाणु है जो अपनी उच्च इथेनॉल सहनशीलता और उपज के लिए जाना जाता है। यह अत्यधिक सक्रिय एंटनर-डौडोरोफ मार्ग के माध्यम से शर्करा का उपयोग करता है, जिसके परिणामस्वरूप ग्लूकोज की खपत दर तेजी से होती है। इसके अलावा, जेड मोबिलिस में अधूरा ट्राइकार्बोक्सिलिक एसिड चक्र और एक अपर्याप्त श्वसन श्रृंखला है, जो इसे ऊर्जा उत्पादन और रेडॉक्स संतुलन के लिए ग्लूकोज-से-इथेनॉल किण्वन पर निर्भर करती है। कार्बन प्रवाह का एक महत्वपूर्ण हिस्सा (९५ -९७ %) इथेनॉल उत्पादन की ओर निर्देशित होता है, जिससे जैवद्रव्यमान का निर्माण कम हो जाता है। ये विशेषताएँ जेड मोबिलिस को उच्च पैदावार पर गैर-देशी यौगिकों के उत्पादन के लिए एक उत्कृष्ट मेजबान प्रदान करती हैं, बशर्ते कि देशी इथेनॉल मार्ग के माध्यम से प्रवाह को प्रभावी ढंग से समाप्त किया जा सके।

हमने प्रमुख मार्ग जीन, एसिटोलैक्टेट सिंथेज़ (एएलएसएस)/एसिटोहाइड्रॉक्सीएसिड सिंथेज़ (एएचएस1), एसिटोलैक्टेट डिकार्बोक्सिलेज़ (एएलएसडी), और आर,आर-2,3-ब्यूटेनडियोल डिहाइड्रोजेनेज़ (बीडीएचए), दो तटस्थ जीनों ठिकाना में अभिव्यक्ति किया। इस उपभेद ने कम पैदावार पर एसीटोइन और 2,3-बीडीओ का उत्पादन किया, क्योंकि अधिकांश कार्बन प्रवाह (७७ - ७८%) इथेनॉल उत्पादन की ओर निर्देशित था। पैदावार में सुधार करने के लिए, हमने एसीटोइन/2,3-बीडीओ मार्ग जीन (एएचएस1, एएलएसडी, और बीडीएचए) को समवर्ती रूप से अभिव्यक्त करते हुए एडीएच1 और एडीएच2 दोनों जीनों को हटाकर इथेनॉल मार्ग को लक्षित किया। उपभेद ने नगण्य इथेनॉल उत्पादन (~ 0.८ ग्राम/लीटर तक) प्रदर्शित किया, जो दर्शाता है कि एडीएच1 और एडीएच2 जीन के विलोपन ने इथेनॉल उत्पादन को काफी कम कर दिया। हालाँकि, यह उपभेद १0-१२ ग्राम/लीटर से अधिक ग्लूकोज का उपभोग नहीं कर सका, और ग्लूकोज के उपयोग के मामले में जंगली-प्रकार के उपभेद की तुलना में एसिटोइन उपज में

केवल मामूली वृद्धि हुई थी। ग्लूकोज की खपत में कमी विषम जीन की कम गतिविधि और इथेनॉल उत्पादन के बिना एनएडी (पी) + को पुनर्जीवित करने में उपभेद की अक्षमता के संयुक्त प्रभाव के कारण थी। इन परिकल्पनाओं की जांच करने के लिए, एनएडी (पी)+ उत्पन्न करने के लिए लैक्टोकोकस लैक्टिस एनएडीएच ऑक्सीडेज (एनओएक्स) और बैसिलस सबटिलिस से एक मजबूत एलएसएस जीन को 2,3-बीडीओ और एसीटोइन उत्पादन की ओर अधिक पाइरूवेट प्रवाह को पुनर्निर्देशित करके उपभेद में और सुधार किया गया। पुनः संयोजक उपभेद ने 0.२७ ग्राम/ग्राम ग्लूकोज की उपज के साथ एसीटोइन का उत्पादन किया, जो अधिकतम सैद्धांतिक उपज (एमटीवाई) के ५४.५% के अनुरूप है। हालाँकि, कार्बन प्रवाह का केवल ५0-६५% एसीटोइन, 2,3-बीडीओ और ग्लिसरॉल के समग्र उत्पादन की ओर मोड़ा गया था, जो अन्य चयापचयों के संचय का संकेत देता है।

हमने पैदावार में सुधार के लिए एसीटोइन और 2,3-बीडीओ उत्पादन के लिए अभियंत किए गए उपभेद में पीडीसी जीन को हटा दिया। हटाए गए पीडीसी जीन वाले Zm_ΔA1ΔA2ΔP_HS1DXS उपभेद ने किसी भी विकास की स्थिति में इथेनॉल का उत्पादन नहीं किया। वातापेक्षी स्थिति के तहत, उपभेद ने 0.३८ ग्राम/ग्राम ग्लूकोज की एसीटोइन उपज हासिल की, जो एमटीवाई के ७६% के बराबर है। विकास स्थितियों को और अधिक अनुकूलित करने के लिए Zm_ΔA1ΔA2ΔP_HS1DXS उपभेद को एक रिएक्टर में विभिन्न घुलित ऑक्सीजन (डीओ) स्तरों के तहत विकसित किया गया था। ३०-३५% डीओ के तहत, उपभेद ने १२८ ग्राम/लीटर ग्लूकोज से ३९.0९ ग्राम/लीटर एसीटोइन और ७.८६ ग्राम/लीटर 2,3-बीडीओ का उत्पादन किया। १०% से कम डीओ स्तर पर, उपभेद ने ३० ग्राम/लीटर ग्लूकोज से ११.२२ ग्राम/लीटर 2,3-बीडीओ का उत्पादन किया, जिससे एमटीवाई के ७५% के करीब उपज प्राप्त हुई। इस अध्ययन ने उच्च पैदावार पर एसीटोइन और 2,3-बीडीओ दोनों का उत्पादन करने के लिए एक एकल संयोजक उपभेद की क्षमता का प्रदर्शन किया, जिसमें उत्पादन रूपरेखा विकास स्थितियों के आधार पर भिन्न होती है। इस उपभेद ने बायोरिएक्टर में मजबूत वृद्धि प्रदर्शित

की और उच्च ग्लूकोज सांद्रता का उपभोग करने की क्षमता प्रदर्शित की। Zm_ΔA1ΔA2ΔP_HS1DXS उपभेद को bdhA जीन को हटाकर एसीटोइन उपज बढ़ाने के लिए और अधिक इंजीनियर किया गया था Zm_ΔA1ΔA2ΔP_KS1DXS उपभेद (bdhA विलोपन के साथ) ने 0. ४४ g/g (MTY का ७८%) की उपज के साथ एसीटोइन का उत्पादन किया। शेक फ्लास्क में उपभेद ८0 ग्राम/लीटर तक ग्लूकोज की खपत करता है।

ज़ेड मोबिलिस को इथेनॉल से वेलिन की ओर कार्बन प्रवाह को पुनर्निर्देशित करके वेलिन या वेलिन मार्ग मध्यवर्ती का उत्पादन करने के लिए इंजीनियर किया गया था। वेलिन मार्ग जीन को एडीएच1 और एडीएच2 जीन के स्थान पर एकीकृत किया गया था। हमने पाइरूवेट को एसिटोलैक्टेट में बदलने के लिए उत्प्रेरित करने के लिए बी. सबटिलिस एएलएसएस जीन का चयन किया, जो वेलिन उत्पादन की दिशा में पहला कदम है। अगले दो चरणों के लिए, हमने देशी केटोल-एसिड रिडक्टोइसोमेरेज़ (आईएलवीसी) और डायहाइड्रॉक्सी-एसिड डीहाइड्रैटेज़ (आईएलवीडी) जीन को एक मजबूत पीपीडीसी आगे बढ़नेवाला के नियंत्रण में रखकर ऊपर अभिव्यक्त करना चुना। बी. सबटिलिस एनएडीएच-विशिष्ट एल-ल्यूसीन डीहाइड्रोजनेज (बीसीडी) को अंतिम रिडक्टिव चरण को उत्प्रेरित करने के लिए चुना गया था। पुनः संयोजक उपभेद ने वेलिन का उत्पादन नहीं किया, संभवतः देशी आईएलवीसी और आईएलवीडी किण्वन की वेलिन संश्लेषण की ओर प्रवाह को नीचे की ओर खींचने में असमर्थता के कारण। इस पर काबू पाने के लिए, एक और उपभेद का निर्माण किया गया जिसमें अतिरिक्त ई. कोली आईएलवीसी जीन शामिल था। फिर भी, वेलिन उत्पादन का अभी भी पता नहीं चला है।

इस अध्ययन में, रेडॉक्स पुनर्जनन के लिए वैकल्पिक मार्ग शुरू करके पूर्ण इथेनॉल उन्मूलन के साथ एक जेड मोबिलिस उपभेद बनाया गया था। इस कार्य से पता चला कि पीपीडीसी जीन जेड मोबिलिस में आवश्यक नहीं है और यदि रेडॉक्स समकक्ष पुनर्जनन के लिए एक वैकल्पिक मार्ग प्रदान किया जाता है तो इसे हटाया जा सकता है (इस अध्ययन में एनओएक्स)। जेड

मोबिलिस को इथेनॉल से लक्ष्य उत्पादों तक कार्बन प्रवाह को पूरी तरह से पुनर्निर्देशित करके उच्च पैदावार पर एसीटोइन और 2,3-बीडीओ का उत्पादन करने के लिए इंजीनियर किया गया था।

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Strain	Description
Zm_ΔA1H	<i>ZmΔadh1::Ppdc-Bs.bdhA-tetR</i>
Zm_ΔA1ΔA2_HS1D	<i>Zm_ΔA1H Δadh2::Ppdc-Ec.ahas1.Bs.alsD-cmR</i>
Zm_ΔA1ΔA2_HS1DX	<i>Zm_ΔA1H Δadh2::Ppdc-Ec.ahas1.Bs.alsD-Ll.nox-cmR</i>
Zm_ΔA1ΔA2ΔM_HS1DXS	<i>Zm_ΔA1H ΔA2_S1DX Δmrr::Ppdc-Bs.alsS-PspecR</i>
Zm_ΔA1ΔA2ΔP_HS1DXS	<i>Zm_ΔA1H ΔA2_S1DX Δpdc::Ppdc-Bs.alsS-PspecR</i>
Zm_ΔA1ΔA2ΔP_KS1DXS	<i>Zm_Δadh1::PkanR ΔA2_S1DX ΔP_S</i>
Zm_ΔA1-S	<i>Zm_Δadh1::Ppdc-Bs.alsS-tetR</i>
Zm_ΔA1ΔA2_S.Zm.CD.D	<i>Zm_ΔA1S Δadh2::Ppdc-Zm.ilvC.Zm.ilvD.Bs.bcd-cmR</i>
Zm_ΔA1ΔA2ΔP_S.Zm.CD.D.EcC	<i>Zm_ΔA1S ΔA2_S.Zm.CD.D with Ppdc-Ec.ilvC-PkanR at the pdc gene locus</i>

Abbreviations

BDO	Butanediol
EMP	Embden-Meyerhof-Parnas
ED	Entner-Doudoroff
RM	Rich Medium
YE	Yeast extract
LB	Luria Bertani
CDS	Coding DNA sequence
PDC	Pyruvate decarboxylase
PDH	Pyruvate dehydrogenase
ADH	Alcohol dehydrogenase
ADH1	Zinc-containing alcohol dehydrogenase
ADH2	Iron-containing alcohol dehydrogenase
G6PDH	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G3P	Glyceraldehyde 3-phosphate
TPI	Triose phosphate isomerase
DHA	Dihydroxyacetone

TCA	Tricarboxylic Acid Cycle
ATP	Adenosine Triphosphate
PPP	Pentose Phosphate Pathway
KO	Knockout
PEP	Phosphoenolpyruvate
NAD ⁺	Nicotinamide adenine dinucleotide
NADP ⁺	Nicotinamide adenine dinucleotide phosphate
LA	Lactic acid
OAA	Oxaloacetate
GFOR	Glucose-fructose oxidoreductase
OGDH	2-oxoglutarate dehydrogenase
MDH	Malate dehydrogenase
SEVA	Standard European Vector Architecture
RE	Restriction enzyme
LDH	Lactate dehydrogenase
Ppdc	Pyruvate decarboxylase gene's promoter
ALS	Acetolactate synthase
ALD	Acetolactate decarboxylase

BDH	Butanediol dehydrogenase
AHAS	Acetohydroxyacid synthase
NOX	NADH oxidase
ILVC	Ketol-acid reductoisomerase
ILVD	Dihydroxy-acid dehydratase
BCD	L-leucine dehydrogenase
ILVE	Transaminase
RPM	Revolutions per minute
TSS	Transformation and storage solution
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
RBS	Ribosome binding site
DO	Dissolved oxygen
PCR	Polymerase chain reaction
HPLC	High-performance liquid chromatography
RID	Refractive index detector
DAD	Diode array detector
OD	Optical Density

BGSC	Bacillus Genetic Stock Centre
GRAS	Generally Regarded As Safe
% MTY	Percentage of maximum theoretical yield

*Gene names are written in lowercase and italicized. Protein names are written in uppercase