METABOLIC ENGINEERING OF *ZYMOMONAS MOBILIS* FOR LACTIC ACID PRODUCTION AND XYLOSE UTILIZATION

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INDIAN INSTITUTE OF TECHNOLOGY DELHI

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by

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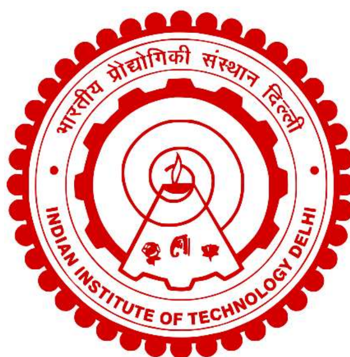

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Certificate

This is to certify that the thesis entitled **"Metabolic engineering of *Zymomonas mobilis* for lactic acid production and xylose utilization"** being submitted by **Mr. Arun Thapa** 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 him, 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 in any other University or Institute.

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Abstract

Zymomonas mobilis is an important microbial host that produces ethanol at high yields and titres, and has been used for industrial ethanol production. The synthesis of products other than ethanol at high titres in *Z. mobilis* requires redirection of flux away from ethanol; additionally, *Z. mobilis* is natively able to grow on hexose sugars but not pentose sugars. Here, we attempted to engineer *Z. mobilis* for the production of L-lactic acid and D-lactic acid; and utilization of xylose.

We identified highly active LDHs [L-LDH from *Bacillus subtilis* and D-LDH from *Lactobacillus delbrueckii subsp. Bulgaricus*] for LA production in *Z. mobilis*. We then targeted disruption of ADH by deletion of *adh1* and *adh2* genes that are known to be responsible for ethanologenic ADH activity. Expression of the identified *ldh* gene combined with deletion of *adh1* and *adh2* led to almost complete redirection of carbon flux from ethanol to LA. The resulting L-LA producing strain (Zm-BsLΔA1ΔA2) and D-LA producing strain (Zm-LdLΔA1ΔA2) formed LA at high yield (~0.4 g/g glucose) and had significantly reduced yields of ethanol (~0.03 g/g glucose).

However, these strains utilized low amounts of glucose and showed growth cessation that could not be overcome even after adaptively evolving the strains. We found that growth cessation was caused by a reduction in pH because of LA formation, and that pH-controlled fermentations could overcome it. Batch fermentations with pH control showed complete consumption of 40 g/l glucose with production of L-LA and D-LA at high yields (~0.85 g/g glucose) and titres (~32 g/l).

The best D-LA producing strain was evolved for growth at higher glucose concentrations under low oxygen availability to increase LA titres further. Batch fermentations of the evolved D-LA strain conducted in shake-flasks under limited oxygenation showed complete

utilization of 100 g/l glucose and production of ~83 g/l D-LA. The evolved strain was also able to grow on chemically-defined medium with starting glucose concentrations of 40 g/l glucose and produced ~33 g/l D-LA. Moreover, the strain could grow robustly in a bioreactor with low aeration requirements and showed efficient production of D-LA on rich and minimal media with titres and yields comparable to shake-flask experiments.

We also attempted molecular engineering strategies to alter transcription and translation efficiency of the *pdc* gene or completely disrupt it. However, there were no changes in ethanol production by use of these strategies.

For xylose utilization, we introduced four xylose pathway genes from *B. subtilis* via genome integration. The engineered strains were further evolved for growth on xylose using multiple ALE protocols. However, the evolved strains showed extremely weak growth on plates containing xylose and no growth in liquid medium containing xylose.

Overall, this work showed L-LA and D-LA production in an engineered *Z. mobilis* strain at yields and titres comparable to those of native LA producers. This demonstrates the use of metabolic engineering for redirecting flux away from ethanol in *Z. mobilis* and furthers its use for generating non-native products.

सार

ज़इमोमोनास मोबिलिस एक आकर्षक मेजबान सूक्ष्मजीव है, जो उच्च पैदावार और अनुमापांक पर इथेनॉल का उत्पादन करता है और इसका उपयोग औद्योगिक इथेनॉल उत्पादन के लिए किया गया है। जेड मोबिलिस में उच्च अनुमापांक पर इथेनॉल के अलावा अन्य उत्पादों के उत्पादन के लिए, प्रवाह को इथेनॉल से दूर पुनर्निर्देशन करने की आवश्यकता होती है; इसके अतिरिक्त, जेड मोबिलिस मूल रूप से हेक्सोज शर्करा पर विकसित होने में सक्षम है, लेकिन पेंटोज शर्करा पर नहीं। यहां, हमने एल-लैक्टिक एसिड (एल-एल ए) और डी-लैक्टिक एसिड (डी-एल ए) के उत्पादन और ज़ाइलोज़ के उपयोग के लिए जेड मोबिलिस को संशोधित करने का प्रयास किया।

हमने जेड मोबिलिस में एल ए उत्पादन के लिए अत्यधिक सक्रिय एलडीएच [बैसिलस सबटिलिस से एल-एलडीएच और लैक्टोबैसिलस डेलब्रुएकी सबस्प बुल्गारिकस से डी-एलडीएच] की पहचान की। इसके बाद हमने एडीएच१ और एडीएच२ जीनों को हटाकर एडीएच के विघटन को लक्षित किया, जो एथेनोजेनिक एडीएच गतिविधि के लिए जिम्मेदार माने जाते हैं। चयनित एलडीएच जीन की अभिव्यक्ति को एडीएच१ और एडीएच२ के विलोपन के साथ जोड़कर इथेनॉल से एलए तक कार्बन प्रवाह का लगभग पूर्ण पुनर्निर्देशन किया गया। परिणामी एल-एल ए उत्पादक उपभेद (Zm-BsLΔA1ΔA2) और डी-एल ए उत्पादक उपभेद (Zm-LdLΔA1ΔA2) ने उच्च उपज (लगभग ०.४ ग्राम/ग्राम ग्लूकोज़) पर एल ए का उत्पादन किया और इथेनॉल (लगभग ०.०३ ग्राम/ग्राम ग्लूकोज़) के उत्पादन में काफी कमी आई।

मगर, इन उपभेदों ने ग्लूकोज की कम मात्रा का उपयोग किया और ग्लूकोज की उपलब्धता के बावजूद भी अपने विकास को रोक दिआ, जो कि उपभेदों के अनुकूल रूप से विकसित होने के बाद भी पूरा नहीं हो सका। हमने पाया कि एलए के उत्पाद के कारण पीएच में कमी से उपभेदों का विकास बंद हो गया और इसे दूर करने के लिए, किण्वन के दौरान पीएच को नियंत्रण किया गया। पीएच नियंत्रण के साथ ४० ग्राम/ लीटर ग्लूकोज

की पूरी खपत दिखाई दी और बैच प्रयोगों में उच्च उपज (०.८५ ग्राम/ग्राम ग्लूकोज तक) और अनुमापांक (३२ ग्राम/लीटर तक) पर एल-एलए और डी-एलए का उत्पादन हुआ।

सबसे अच्छे डी-एलए उत्पादक उपभेद को, कम ऑक्सीजन की उपलब्धता के तहत, अधिक प्रारंभिक ग्लूकोज सांद्रता की वृद्धि में विकसित होने के अनुकूल बनाया गया। विकसित डी-एलए उत्पादक उपभेद ने, सीमित ऑक्सीजनेशन की स्थिति में, शेक-फ्लास्क बैच किण्वन में १०० ग्राम/लीटर ग्लूकोज की पूरी खपत दिखाई और लगभग ८३ ग्राम/लीटर के डी-एलए अनुमापांक का उत्पादन किया। विकसित उपभेद, ४० ग्राम/लीटर ग्लूकोज वाले न्यूनतम मीडिया पर भी विकास करने में सक्षम हुआ और लगभग ३३ ग्राम/लीटर डी-एलए का उत्पादन किया। इसके अलावा, उपभेद कम ऑक्सीजन की उपलब्धता में, बायोरिएक्टर में समृद्ध और न्यूनतम मीडिया में विकसित हुआ और डी-एलए का कुशल उत्पादन दिखाया जिसकी पैदावार और अनुमापांक शेक-फ्लास्क में हुए प्रयोगों से तुलनीय थी।

हमने एलए उत्पादक उपभेदों में पीडीसी जीन को कम विनियमित करने या बदलने के लिए आणविक इंजीनियरिंग रणनीतियों की कोशिशें की लेकिन पीडीसी गतिविधि को कम करने में असमर्थ रहे।

ज़ाइलोज़ उपयोग के लिए, हमने बी सबटिलिस से ज़ाइलोज़ पाथवे जीनो को जीनोम में डाला। फिर हमने विकसित उपभेद को ज़ाइलोज़ पर विकास के लिए अनुकूल बनाया। लेकिन उपभेदों ने ज़ाइलोज़ युक्त प्लेटों पर बेहद कमज़ोर वृद्धि दिखाई और ज़ाइलोज़ युक्त तरल माध्यम में कोई वृद्धि नहीं हुई।

कुल मिलाकर, इस काम ने एक इंजीनियर जेड मोबिलिस स्ट्रेन में एल–एलए और डी–एलए उत्पादन को उच्च पैदावार और अनुमापांक दिखाया, जो देशी एलए उत्पादकों के समान था। यह जेड मोबिलिस में इथेनॉल से दूर प्रवाह को पुनर्निर्देशित करने के लिए चयापचय इंजीनियरिंग के उपयोग को प्रदर्शित करता है और गैर–देशी उत्पादों को बनाने के लिए इसके उपयोग को आगे बढ़ाता है।

Table of Contents

Certificate i

Acknowledgements ii

Abstract iv

सार vi

Table of Contents viii

List of figures xi

List of tables xiv

Abbreviations xv

1 Introduction 1

2 Review of literature 4

2.1 A brief overview of *Z. mobilis* 4

2.2 Metabolism of *Z. mobilis* 4

2.3 Lactic acid and its application 7

2.4 *Z. mobilis* as a production host 9

2.5 Xylose utilization 11

3 Motivation and objectives 14

4 Materials and methods 15

4.1 Strains, medium, and growth conditions: 15

4.2 Construction of integration cassettes for L-LA producing strains: 16

4.3 Construction of integration cassettes for D-LA producing strains: 17

4.4 Construction of integration cassettes for strains to reduce PDC activity: 18

4.5 Construction of integration cassettes to introduce xylose uptake pathway: 19

4.6 DNA transformation: 21

4.7 Analytical methods: 21

4.8 Adaptive lab evolution: 22

4.9 Bioreactor operation: 23

4.10 Enzyme activity assays: 24

5 Results 25

5.1 Production of L-LA and D-LA by redirection of flux away from ethanol 25

5.1.1 Production of L-LA 25

5.1.1.1 Construction of genome-integrated strains expressing L-LDH and screening transformants for L-LA production 26

5.1.1.2 Improving L-LA production by deletion of *adh2* gene with concurrent L-LDH expression 28

5.1.1.3 Improving L-LA production by deleting *adh1 and adh2* in L-LA producing strain 29

5.1.2 Production of D-LA 35

5.1.2.1 Construction of genome-integrated strains expressing D-LDH and screening transformants for D-LA production 35

5.1.2.2 Improving D-LA production by D-LDH expression and step-wise deletion of *adh2* and *adh1* gene 36

5.2 Improvement of L-LA and D-LA Production 42

5.2.1 Process Strategies 42

5.2.1.1 Effect of pH control on growth of L-LA and D-LA producing strains 42

5.2.1.2 Adaptation of D-LA producing strain for growth at higher glucose concentration and low oxygen availability 47

5.2.1.3 Growth of adapted D-LA producing strain in minimal media 50

5.2.1.4 Growth of adapted D-LA producing strain in a bioreactor......51

5.2.2 Molecular engineering......55

5.2.2.1 Replacing the native *pdc* promoter with modified promoter-RBS......55

5.2.2.2 Replacing *pdc* gene with *ldh*-*pdc* (lacking native RBS) in an operon......57

5.2.2.3 Expression of NADP/NADPH co-utilizing mutant *Bs*LDH......59

5.3 Introduction of xylose utilization pathway......62

5.3.1 Construction of genome-integrated strains with xylose-utilizing pathway genes......62

5.3.2 Adaptation of strains with xylose-utilizing pathway......65

6 Discussion......70

6.1 Production of L-LA and D-LA by redirection of flux away from ethanol......70

6.2 Improvement of L-LA and D-LA Production:......73

6.3 Introduction of xylose utilization pathway......77

7 Research summary and future perspectives......79

8 References......82

9 Appendix......99

Biodata......108

List of figures

Figure No.	Title
2.1	Metabolic flux map of *Z. mobilis* (from Jacobson et al. 2019).
5.1	Schematic representation of metabolic engineering strategy for the production of LA in *Z. mobilis*.
5.2	Screening of different L-*ldh* genes by integrating at *Zm.ldh* locus of *Z. mobilis*.
5.3	Screening of different L-*ldh* genes by integrating at *adh2* gene locus of *Z. mobilis*.
5.4	Time course profile of L-LA producing strain with *adh2* knockout (Zm-BsLΔA2).
5.5	Verification of L-LA producing strain with *adh1* and *adh2* knockout (Zm-BsLΔA1ΔA2) using PCR-based screening.
5.6	Effect of *adh1* and *adh2* knockout on enzyme activities in L-LA producing strains.
5.7	Time course profile of L-LA producing strain with *adh1* and *adh2* knockout (Zm-BsLΔA1ΔA2).
5.8	Schematic representation of ALE process used for adaptation on LA.
5.9	Effect of adaptation with L-LA on L-LA producing strain (Zm-BsLΔA1ΔA2).
5.10	Screening of different D-*ldh* genes by integrating at *Zm.ldh* locus of *Z. mobilis*.
5.11	Screening of different D-*ldh* genes by integrating at *adh2* locus of *Z. mobilis*.
5.12	Time course profile of D-LA producing strain with *adh2* knockout (Zm-LdLΔA2).
5.13	Effect of *adh1* and *adh2* knockout on enzyme activities in D-LA producing strains.
5.14	Time course profile of D-LA producing strain with *adh1* and *adh2* knockout (Zm-LdLΔA1ΔA2).
5.15	Effect of adaptation with D-LA on D-LA producing strain (Zm-LdLΔA1ΔA2).
5.16	Time course profiles of L-LA (Zm-BsLΔA1ΔA2) and D-LA (Zm-LdLΔA1ΔA2) producing strains with pH measurement.
5.17	Time course profile of L-LA producing strain (Zm-BsLΔA1ΔA2) grown under pH-controlled conditions with 20 g/l of glucose.

5.18	Time course profile of L-LA producing strain (Zm-BsLΔA1ΔA2) grown under pH-controlled conditions with 40 g/l of glucose.
5.19	Time course profile of D-LA producing strain (Zm-LdLΔA1ΔA2) grown under pH-controlled conditions with 20 g/l of glucose.
5.20	Time course profile of D-LA producing strain (Zm-LdLΔA1ΔA2) grown under pH-controlled conditions with 40 g/l of glucose.
5.21	Time course profile of D-LA producing strain (Zm-LdLΔA1ΔA2) grown under pH-controlled conditions with 100 g/l of glucose.
5.22	Time course profile of D-LA producing strain (Zm-LdLΔA1ΔA2) grown under pH-controlled conditions with 150 g/l of glucose.
5.23	Schematic representation of ALE process used for adaptation on high glucose concentration.
5.24	Time course profile of D-LA producing strain (Zm-LdLΔA1ΔA2) after adaptation on high glucose concentrations.
5.25	Time course profile of adapted D-LA producing strain (Zm-LdLΔA1ΔA2) growing in minimal media with 40 g/l of glucose.
5.26	Time course profile of adapted D-LA producing strain (Zm-LdLΔA1ΔA2) grown in a bioreactor with 50 g/l of glucose.
5.27	Time course profile of adapted D-LA producing strain (Zm-LdLΔA1ΔA2) grown in a bioreactor with 100 g/l of glucose.
5.28	Time course profile of adapted D-LA producing strain (Zm-LdLΔA1ΔA2) grown in a bioreactor on minimal media with 40 g/l of glucose.
5.29	Plasmid maps of integration cassettes for the expression of *pdc* gene with modified promoter-RBS.
5.30	Product profiles of strains expressing *pdc* gene under weak promoter/RBS.
5.31	Plasmid maps showing integration cassettes for the expression of *Bs.ldh* and *pdc* gene lacking its native RBS.
5.32	Product profile of strain expressing *Bs.ldh* and *pdc* gene lacking its native RBS
5.33	Plasmid maps showing construction of integration cassette for the expression of NADH/NADPH co-utilizing mutant *Bs*LDH.
5.34	Product profile of strain expressing NADH/NADPH co-utilizing mutant *Bs*LDH.
5.35	Plasmids used for introducing xylose-utilization pathway genes in *Z. mobilis.*

5.36	Verification of strain with xylose-utilizing pathway integrated at single locus using PCR-based screening.
5.37	Verification of strain with xylose-utilizing pathway integrated at double locus using PCR-based screening.
5.38	Schematic representation of ALE process for adaptation on high xylose and low glucose.
5.39	Schematic representation of ALE process for adaptation on increasing xylose concentrations.
5.40	Growth curves of strains with genomically-integrated xylose pathway genes.
S1	Plasmid maps showing construction of pAB-T.
S2	Plasmid maps showing construction of pABTT-T.
S3	Plasmid maps showing construction of pABTT-T(L).
S4	Plasmid maps showing construction of pTT-C.
S5	Plasmid maps showing construction of pTT-C(M).
S6	Plasmid maps showing construction of pAB-T(L).

List of tables

Table No.	Title
1	Summary of L-LA (Zm-BsLΔA1ΔA2) and D-LA (Zm-LdLΔA1ΔA2) producing strains.
2	Summary of L-LA (Zm-BsLΔA1ΔA2) and D-LA (Zm-LdLΔA1ΔA2) producing strains with pH control (% MTY - Percentage of maximum theoretical yield).
3	Summary of D-LA production by adapted Zm-LdLΔA1ΔA2 strain in rich and minimal media under oxygen-limiting conditions (% MTY - Percentage of maximum theoretical yield).
4	Summary of D-LA production by adapted Zm-LdLΔA1ΔA2 strain in 5 L bioreactor under oxygen limiting conditions (% MTY - Percentage of maximum theoretical yield).
5	Summary of strains with genomically-integrated xylose pathway genes.
S1	List of primers
S2	List of plasmids
S3	List of strains
S4	K_m values (for pyruvate) of different LDHs used in this study.

Abbreviations

EMP	Embden-Meyerhof-Parnas
ED	Entner-Doudoroff
LA	Lactic acid
RM	Rich Medium
ALE	Adaptive lab evolution
PDC	Pyruvate decarboxylase
ADH	Alcohol dehydrogenase
LDH	Lactate dehydrogenase
G6PDH	Glucose-6-phosphate dehydrogenase
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
G6P	Glucose 6-phosphate
ATP	Adenosine triphosphate
RBS	Ribosome binding site
% MTY	Percentage of maximum theoretical yield

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