

**SYNTHESIS OF MALIC ACID BY  
REDIRECTION OF CELLULAR METABOLISM  
IN *ZYMO MONAS MOBILIS***

**ROHIT KHANDELWAL**



**DEPARTMENT OF BIOCHEMICAL  
ENGINEERING AND BIOTECHNOLOGY  
INDIAN INSTITUTE OF TECHNOLOGY DELHI**

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by

**ROHIT KHANDELWAL**

DEPARTMENT OF BIOCHEMICAL ENGINEERING AND  
BIOTECHNOLOGY

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In fulfilment of the requirements of the degree of Doctor of  
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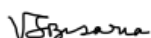


## CERTIFICATE

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



Prof. Virendra Swarup Bisaria  
Department of Biochemical Engineering  
and Biotechnology  
Indian Institute of Technology Delhi  
New Delhi- 110016

Prof. Preeti Srivastava  
Department of Biochemical Engineering  
and Biotechnology  
Indian Institute of Technology Delhi  
New Delhi- 110016



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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<sup>R</sup>* 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  $\Delta pdc$  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 *Pchap*, *Ppap* and *Ppdc* promoters from *Z. mobilis* were selected. The strengths of these promoters were determined and compared with *Ptac*

promoter in *E. coli* by cloning *gfp<sub>uv</sub>* gene downstream to them. The expression of *gfp<sub>uv</sub>* was studied with respect to growth at different pH and temperatures. Based on the results, *Pchap* and *Ppdc* promoters from *Z. mobilis*, and *Ptac* promoter from *E. coli* were used to express *Ecmae* gene in *Z. mobilis* to increase malic acid production. The *mae*<sup>+</sup> recombinants were obtained by recombineering-based genomic integration of *Pchap-mae*, *Ptac-mae* and *Ppdc-mae* sequences. Maximum malic acid yield was obtained in *Ppdc-mae* recombinant, followed by *Ptac-mae* and *Pchap-mae* recombinants. In *Ppdc-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 *जाइमोमोनास मोबिलिस* में जीनोम इंजीनियरिंग द्वारा मैलिक अम्ल का उत्पादन करने का एक प्रयास था।

पाइरूवेट डिकार्बोक्सिलेज एंजाइम, जो *जा. 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। *जा. मोबिलिस* में pBBR1 और RK2 प्रतिकृति और स्थिरता स्थापित की गई। पुनर्संयोजन का उपयोग किया गया और *pdc* जीन को pBBR1 प्रतिकृति और लैम्बडा 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 मैलिक एंजाइम 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 *Pchap*, *Ppap* और *Ppdc* प्रमोटरों का चयन किया गया। इन प्रमोटरों का सामर्थ्य निर्धारित किया गया और *ई. कोलाई* में *Ptac* प्रमोटर के साथ *gfp<sub>uv</sub>* जीन के तहत क्लोनिंग करके उनकी तुलना की गई। विभिन्न pH और तापमान पर वृद्धि के साथ *gfp<sub>uv</sub>* की अभिव्यक्ति का अध्ययन किया गया। परिणामों के आधार पर, *ज़ा. मोबिलिस* के *Pchap* और *Ppdc* प्रमोटरों, और *ई. कोलाई* के *Ptac* प्रमोटर का उपयोग *ज़ा. मोबिलिस* में *Ecmae* जीन को व्यक्त करके मैलिक अम्ल उत्पादन बढ़ाने के लिए किया गया। *mae<sup>+</sup>* पुनः संयोजक *Pchap-mae*, *Ptac-mae* और *Ppdc-mae* अनुक्रमों के पुनर्संयोजन-आधारित जीनोमिक एकीकरण द्वारा प्राप्त किए गए। *Ppdc-mae* पुनः संयोजक में अधिकतम मैलिक अम्ल उपज प्राप्त की गई, इसके बाद *Ptac-mae* और *Pchap-mae* पुनः संयोजक थे। *Ppdc-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 लैम्बडा 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।

# CONTENTS

Certificate	i
Acknowledgments	iii
Abstract	vii
Contents	xi
List of Figures	xv
List of Tables	xxiii
Abbreviations and Symbols	xxv
<b>Chapter 1 Introduction and Objectives</b>	<b>3</b>
<b>Chapter 2 Literature Review</b>	
2.1 Physicochemical properties and uses of malic acid	9
2.2 Metabolic pathways involved in microbial production of malic acid	12
2.3 Microbial production of malic acid	17
2.4 Genome engineering and malic acid production by engineered microorganisms	22
2.5 Production of malic acid from coproducts of biofuel production process and lignocellulosic biomass	27
2.6 Production of malic acid via polymalic acid	31
2.7 Physiological characteristics of <i>Zymomonas mobilis</i>	33
2.8 Molecular machinery and methods studied for metabolites' production in <i>Z. mobilis</i>	34
2.9 Selection of <i>Z. mobilis</i> strain and genome engineering tool for the present study	38
<b>Chapter 3 Materials and Methods</b>	
3.1 Strains, plasmids and primers	45

3.2 Media composition and growth conditions	48
3.3 Molecular biology techniques	48
3.4 Plasmid stability studies	61
3.5 Cloning of <i>Z. mobilis</i> promoters in plasmid pEPR1	61
3.6 Fluorimetry analysis	63
3.7 Selection of malic enzyme and its activity analysis	63
3.8 Promoter selection for expression of <i>Ecmae</i> , the malic enzyme gene	65
3.9 Proteomics methods	65
3.10 Southern blotting and hybridization	70
3.11 Evaluation of growth and metabolic product analysis	77
3.12 Microscopic analysis of recombinants	78
3.13 Bioinformatic analysis	78
3.14 Malic acid production by recombinants in fermenter	80
<b>Chapter 4 Results and Discussion</b>	
4.1 Optimization of parameters for electroporation of recombinating plasmids in <i>Z. mobilis</i>	83
4.2 Stability of plasmids pSIM7 and pSIM9	84
4.3 Deletion of <i>pyruvate decarboxylase</i> gene by recombineering	85
4.4 Confirmation of $\Delta pdc$ mutant of <i>Z. mobilis</i>	87
4.5 Cellular morphology and growth of $\Delta pdc$ mutant	90
4.6 Metabolic analysis of $\Delta pdc$ mutant in shake flask fermentation	92
4.7 Investigation of promoters in <i>Z. mobilis</i>	93
4.8 Functionality of <i>Pchap</i> , <i>Ppap</i> and <i>Ppdc</i> in <i>E. coli</i>	100
4.9 Promoter activity at different temperatures and pH	105
4.10 Selection of malic enzyme for expression in <i>Z. mobilis</i>	107

4.11 <i>In silico</i> prediction of binding of ligands to malic enzyme	107
4.12 Activity of malic enzyme from <i>E. coli</i>	110
4.13 Preparation of constructs for <i>Ecmae</i> expression in <i>Z. mobilis</i>	112
4.14 Integration of Promoter- <i>Ecmae</i> fragments at the target site in genome	116
4.15 Verification of <i>mae</i> <sup>+</sup> recombinants	118
4.16 Cellular morphology and growth of <i>mae</i> <sup>+</sup> mutants	122
4.17 Metabolic analysis of <i>mae</i> <sup>+</sup> recombinants in shake flask fermentation	126
4.18 Metabolic analysis of <i>Ppdc-mae</i> recombinant in a fermenter	132
<b>Chapter 5 Conclusions and Future prospects</b>	
5.1 Conclusions	139
5.2 Future prospects	140
References	141
Appendix	159
Curriculum Vitae	173



## LIST OF FIGURES

Figure No.	Title	Page No.
2.1	The value chain approach used in the down-selection process depicting indirect roles of malic acid (Adapted from Werpy & Petersen, 2004).	11
2.2	Metabolic pathways involved in the production of malate (shown in dark blue box) from glucose. NAD and NADP refer to NAD <sup>+</sup> and NADP <sup>+</sup> , respectively.	14
2.3	Principle of genome engineering by homologous recombination. To delete a gene, an antibiotic-resistance marker (Ab <sup>R</sup> ) is PCR-amplified from its source plasmid such that it possesses flanks matching the gene to be disrupted. For insertion of a gene, the gene is amplified with flanks matching the target site in the genome.	23
2.4	Central metabolic pathways for sugar metabolism. Red dotted arrows represent the enzymes missing in this organism. (Adapted from Seo <i>et al</i> , 2005).	40
3.1	PCR profile showing temperature and duration for different steps. 1x, cycle runs 1 time; 30x, cycle runs 30 times.	53
3.2	Temperature profile for RT-PCR.	60
3.3	Schematic representation of constructing different plasmids by cloning of <i>Pchap</i> , <i>Ptac</i> , <i>Ppdc</i> and <i>Ppap</i> promoters upstream of <i>gfp<sub>uv</sub></i> gene in pEPR1, a promoter-probe vector, which resulted in construction of pDJ1, pDJ2, pDJ3 and pPSM1 vectors, respectively.	62

3.4	Graphical representation showing different steps of southern blotting and hybridization.	74
4.1	Segment of lambda bacteriophage genome present in pSIM plasmids for recombineering. The Red genes <i>exo</i> , <i>bet</i> and <i>gam</i> are expressed under the control of lambda thermo-sensitive repressor (ts). $t_L$ , transcription terminators; $p_L$ , promoter; $o_L$ , operator; <i>cat</i> , <i>chloramphenicol acetyltransferase</i> gene which provides resistance to chloramphenicol.	83
4.2	(a) Plasmids pSIM7 and pSIM9 on 1% agarose gel after transformation in <i>Z. mobilis</i> ZM4. Lane 1, pSIM7; lane 2, pSIM9. (b) Graph showing stability of pSIM7 and pSIM9 in <i>Z. mobilis</i> up to 40 generations.	84
4.3	The complete sequence of <i>pdc</i> gene showing highlighted parts of <i>pdcRec.F</i> (light gray) and <i>pdcRec.R</i> (dark gray) primers, which were required for recombineering-based <i>pdc</i> deletion.	86
4.4	(a) Schematic representation of PCR amplification using <i>pdc</i> gene-specific primers <i>pdc.F</i> and <i>pdc.R</i> , and screening primers <i>kanInternal.F</i> and <i>pdcScreen.R</i> . (b). Red-marked fragment represents the inserted kanamycin-resistance cassette. M, 1 kb DNA ladder; lane 1, wild type; lane 2, $\Delta pdc$ mutant of <i>Z. mobilis</i> .	88
4.5	Southern blotting and hybridization using <i>pdc</i> gene-based probe. (a) Restriction digestion of genomic DNA of wild type and $\Delta pdc$ recombinant <i>Z. mobilis</i> ZM4. Lanes (1) and (2) represent $\Delta pdc$ and wild type digested with HindIII, (3) and (4) represent $\Delta pdc$ and wild type digested with XmnI, (5) and (6) represent $\Delta pdc$	90

- and wild type digested with FspI, and (7) and (8) represent  $\Delta pdc$  and wild type digested with PstI. (b) X-ray film of southern blot showing respective bands in lanes (2), (4) and (8) which is wild type.
- 4.6 Phase contrast microscopic images showing cellular morphology of (a) wild type, and (b)  $\Delta pdc$  mutant in aerobic and anaerobic conditions. The oil immersion objective with 100 $\times$  was used for imaging. 91
- 4.7 Growth of and metabolites' production by (a) wild type, and (b)  $\Delta pdc$  mutant in aerobic and anaerobic conditions in shake flask fermentation. 93
- 4.8 (a) The sequence logo depicting graphical representation of -10 and -35 elements of conserved sequences in promoter region, (b) distribution of promoters in *Z. mobilis* ZM4 in terms of spacer length, and (c) GC content distribution of promoter spacer regions. 95
- 4.9 (a) Comparison of hexamers of *Pchap*, *Ppap* and *Ppdc* with the consensus sequences, and (b) transcript levels of *chap*, *pap* and *pdc* in *Z. mobilis* in exponential phase and stationary phase. A constitutive gene *recombinase A (recA)* was used to compare the differential level of transcripts. 98
- 4.10 (a) Colonies and (b) cell pellets showing expression of GFP<sub>UV</sub> in *E. coli* containing (1) promoter-probe vector pEPR1, (2) pDJ1 (*Pchap* promoter), (3) pDJ3 (*Ppdc* promoter), (4) pPSM1 (*Ppap* promoter), and (5) pDJ2 (*Ptac* promoter). 101

4.11	Bar graph depicting transcript levels of <i>gfp<sub>uv</sub></i> in <i>E. coli</i> transformants harboring pEPR1, pDJ1, pDJ2, pDJ3 and pPSM1 plasmids.	104
4.12	Graphs illustrating the relative fluorescence of <i>E. coli</i> transformants harboring pDJ1, pDJ2, pDJ3 and pPSM1 (A) at different pH and (B) at different temperatures, in exponential and stationary phase (*p < 0.05, **p < 0.01).	106
4.13	Enzyme-ligand complexes showing binding of malic enzyme with (a) malate and (b) pyruvate and CO <sub>2</sub> in the presence of co-factor NAD <sup>+</sup> and NADH, respectively. The dashed line represents hydrophobic interaction, cyan-colored line represents hydrogen bond, and yellow-colored line represents van der Waals interactions.	109
4.14	(a) Schematic representation of pRK1 construction strategy. (b) Agarose gel images showing (A) PCR amplification of <i>Ecmae</i> gene, (B) digestion of pET-29a (+) with NdeI and HindIII enzymes (2) and undigested plasmid (1), and (C) digestion of pRK1 with NdeI and HindIII for clone confirmation.	110
4.15	SDS-PAGE showing expression of malic enzyme under T7 promoter. M, Protein marker; 1, 3 h after IPTG induction; 2, 5 h after IPTG induction; 3, overnight after IPTG induction.	111
4.16	Schematic representation of construction of pRK2 and pRK4 plasmids.	113
4.17	Agarose gel images showing (a) digestion of pRK1 with SphI and NdeI for vector preparation, (b) PCR amplification of <i>Pchap</i>	114

- (106 bp) and *Ppdc* (199 bp) promoters, (c) confirmation of *Pchap-mae* clone, and (d) confirmation of *Ppdc-mae* clone by colony PCR. M1, 1 kb DNA ladder; M2, 100 bp DNA ladder.
- 4.18 (a) Schematic representation of construction of pRK3 plasmid. 115  
 (b) Agarose gel images showing (A) digestion of pPM8 with NdeI and HindIII for vector preparation, (B) PCR amplification of *mae* gene, and (C) clone confirmation by digestion with NdeI and HindIII restriction enzymes. M, 1 kb DNA ladder.
- 4.19 (a) Schematic representation of PCR-amplification of Promoter-*mae-kanR* fragment, and (b) fragments of ~3.9 kb length were obtained by PCR using Phusion polymerase. Lane M: 1 kb DNA ladder, (1) *Pchap-mae-kanR* (2), *Ppdc-mae-kanR*, and (3) *Ptac-mae-kanR* fragment. 117
- 4.20 Confirmation of putative *mae*<sup>+</sup> recombinants by colony PCR. (a) 119  
 Schematic showing the Promoter-*mae-kanR* fragment integrated into genome and the annealing of primers used to verify the respective recombinant, and (b) agarose gel images confirming the *Ppdc-mae* (1-4), *Pchap-mae* (5-8) and *Ptac-mae* (9-10) recombinants.
- 4.21 RT-PCR profile of *mae*<sup>+</sup> recombinants showing mRNA 120  
 transcript levels of *mae* gene in comparison with that in wild type (WT) *Z. mobilis*.
- 4.22 SDS-PAGE gel showing expression of malic enzyme (marked 121  
 with arrow) in *mae*<sup>+</sup> recombinants. Lane M: Protein marker; 1 and 2: wild type *Z. mobilis* at stationary and exponential phase;

3 and 4: *Pchap-mae* recombinant at stationary and exponential phase; 5 and 6: *Ptac-mae* recombinant at stationary and exponential phase; 7 and 8: *Ppdc-mae* recombinants at stationary and exponential phase, respectively.

- 4.23 Phase-contrast microscopic images showing cellular morphology of (a) *Pchap-mae*, (b) *Ptac-mae*, and (c) *Ppdc-mae* recombinant in aerobic and anaerobic conditions. The oil immersion objective with 100x was used for imaging. 122
- 4.24 Growth profile of *Ppdc-mae* recombinant in media supplemented with antibiotic (●) and media without antibiotic (■). 124
- 4.25 (a) The circular genome map of *Ppdc-mae* recombinant showing the adjacent genes to the integration site. The Promoter-*mae-kanR* fragment was integrated close to the origin. Parts of genome sequences of (b) wild type, and (c) *Ppdc-mae* mutant of *Z. mobilis* are shown in enlarged form. The target site and integrated dsDNA sequence have been marked with red and magenta colors, respectively. Genes on both sides have been shown with different colors. Numerical values represent the coordinates of corresponding genes in the genomes. *trmB*, tRNA (guanine(46)-N(7))-methyltransferase TrmB; *gnat*, GNAT family N-acetyltransferase; *pyk*, pyruvate kinase; *ZMO\_RS00645*, YebC/PmpR family DNA-binding transcriptional regulator; *ruvC*, crossover junction endodeoxyribonuclease RuvC; *ruvA*, Holliday junction branch migration protein RuvA. 125

4.26	Growth of and metabolites' production by (a) <i>Pchap-mae</i> , (b) <i>Ptac-mae</i> , and (c) <i>Ppdc-mae</i> recombinants of <i>Zymomonas mobilis</i> in aerobic and anaerobic conditions.	127
4.27	LC-MS (a) chromatogram showing peak at 2.67 min and (b) spectrum showing peak at $m/z = 135.0288$ corresponding to malic acid. Peaks are marked with arrows for easier interpretation.	129
4.28	Growth profile and production of metabolites in (a) wild type and (b) <i>Ppdc-mae</i> recombinant under aerobic conditions in a 3 L batch fermenter with 1.5 L working volume.	133
4.29	Fed-batch fermentation profile of <i>Ppdc-mae</i> recombinant.	135



## LIST OF TABLES

Table No.	Title	Page No.
2.1	Malic acid production by wild-type microorganisms	21
2.2	Malic acid production by genetically engineered microorganisms	26
2.3	Production of malic acid from lignocellulosic biomass	30
2.4	Production of polymalic acid/malic acid from renewables	32
2.5	Native plasmids of <i>Z. mobilis</i>	34
2.6	Heterologous and shuttle plasmids used in <i>Z. mobilis</i>	35
2.7	Heterologous promoters that are functional in <i>Z. mobilis</i>	36
2.8	Genome engineering methods used for strain improvement in <i>Z. mobilis</i>	37
3.1	Bacterial strains procured/constructed in the present study	45
3.2	Plasmids procured/constructed in the study	46
3.3	Primers used in the study	47
3.4	Reaction mixture composition for PCR with Taq DNA polymerase / Phusion® High-Fidelity DNA polymerase	52
3.5	Reaction mixture composition for cDNA synthesis from RNA	59
3.6	Reaction mixture composition for RT-PCR	60
3.7	Composition of reaction mixture for malic enzyme assay	64
3.8	Composition of resolving gel used for SDS-PAGE analysis of protein samples	66
3.9	Composition of stacking gel used for SDS-PAGE	67
3.10	Reaction mixture composition for digestion of genomic DNA	71
3.11	Composition of DIG labeling reaction mixture	76

4.1	LC/Q-TOF data showing proteins found in abundance	97
4.2	Growth and fluorescence of <i>E. coli</i> recombinants harboring pDJ1, pDJ2, pDJ3 and pPSM1 plasmids during exponential and stationary phases. The mentioned values are the average of experiments performed in triplicates and repeated twice	103
4.3	Production of different metabolites by wild type and recombinants of <i>Z. mobilis</i>	128

## 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 {1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1 <sup>3,7</sup> ]decan}-4-yl
DIG	Digoxigenin

DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
DTT	Dithiothreitol
<i>Ecmae</i>	Gene encoding malic enzyme in <i>E. coli</i>
EDTA	Ethylenediamine tetraacetic acid
EtBr	Ethidium bromide
g/L	Gram per liter
GFP <sub>UV</sub>	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
<i>mae</i>	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 <sup>+</sup>	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NCBI	National Center for Biotechnology Information
ng	Nanogram(s)
OD <sub>600</sub>	Optical density at 600 nm wavelength
PAP2	Phosphatidic acid phosphatases
PBS	Phosphate buffer saline
<i>Pchap</i>	Promoter transcribing gene encoding chaperonin
PCR	Polymerase chain reaction
<i>pdc</i>	Pyruvate decarboxylase encoding gene
<i>Ppap</i>	Promoter transcribing gene encoding phosphatase PAP2 family protein
<i>Ppdc</i>	Promoter transcribing gene encoding pyruvate decarboxylase enzyme
<i>Ptac</i>	<i>E. coli</i> promoter hybrid between the <i>trp</i> and <i>lac</i> UV5 promoters
<i>recA</i>	<i>recombinase A</i>
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
<i>Zmmae</i>	Gene encoding malic enzyme in <i>Z. mobilis</i>