

TABLE OF CONTENT

Chapter – 1	An introduction on approaches used in engineering methyl-D-erythritol 4-phosphate (MEP) pathway of <i>S. elongatus</i> UTEX 2973 for sustainable production of isoprene	1
1.1	Introduction	2
1.2	Approach	8
1.3	Objectives	11
1.4	Thesis outline	12
Chapter – 2	A comprehensive review of literature on engineering microorganisms for isoprene production	15
2.1	Background	16
2.2	Metabolic pathways for isoprene biosynthesis	16
2.3	Recent toolboxes for synthetic biology in cyanobacteria	19
2.3.1	Promoters	20
2.3.2	Ribosome binding sites	24
2.3.3	Riboswitches	25
2.3.4	CRISPR based techniques	26
2.3.5	Vectors	28
2.4	Transformation techniques	31
2.5	Engineering and regulatory strategies for microbial isoprene production	32
2.5.1	Modulating IspS enzyme activity	32
2.5.2	Genome mining	34
2.5.3	RNA based regulatory tools	35
2.5.4	Optogenetic tools	35
2.6	Isoprene production by engineered microorganisms	36
2.6.1	<i>Bacillus subtilis</i>	37
2.6.2	<i>Escherichia coli</i>	38
2.6.3	<i>Saccharomyces cerevisiae</i>	39
2.6.4	Cyanobacteria	30
2.7	Techno-economic assessment of microbial isoprene production	43
2.8	Identified research gaps	44
Chapter – 3	Engineering of MEP pathway of <i>S. elongatus</i> UTEX 2973 for isoprene production	47
3.1	Background	48
3.2	Materials and methods	48
3.2.1	Strains and culture conditions	48
3.2.2	Preparation of plasmid constructs	49
3.2.3	Conjugal transfer of plasmid into <i>S. elongatus</i> UTEX 2973	52
3.2.4	Genomic DNA isolation and PCR analysis of recombinant strains of <i>S. elongatus</i> UTEX 2973	54
3.2.5	Growth profile and dry cell weight determination	55
3.2.6	Expression analysis of <i>IspS</i> and the <i>IDI</i> genes in recombinant <i>S. elongatus</i> UTEX 2973 strains through semi-quantitative RT-PCR	55
3.2.7	Analysis of protein by SDS-PAGE	56
3.2.8	Isoprene production conditions and quantification	57
3.3	Results and discussions	58
3.3.1	Construction of plasmids and recombinant <i>S. elongatus</i> UTEX 2973 strain	58

3.3.2	Expression of <i>IspS</i> and <i>IDI</i> transgenes and the growth profile of recombinant <i>S. elongatus</i> UTEX 2973 Strains	62
3.3.3	Isoprene production by recombinant <i>S. elongatus</i> UTEX 2973 Strains	63
3.4	Conclusion	66
Chapter – 4	Enhancement of Isoprene Production in Engineered <i>Synechococcus elongatus</i> UTEX 2973 by Metabolic Pathway Inhibition and Machine Learning-Based Optimization Strategy	67
4.1	Background	68
4.2	Materials and methods	69
4.2.1	Strain and seed culture conditions	69
4.2.2	Growth and isoprene production analysis	69
4.2.3	Experimental workflow	71
4.2.4	Geranyl diphosphate synthase (CrtE) inhibition studies: Structure preparation, molecular docking simulations and analysis	72
4.2.5	Effect of alendronate concentrations on isoprene production and cell growth	73
4.2.6	Statistical optimization of process factors for maximization of isoprene production	74
4.2.7	Artificial neural network-genetic algorithm (ANN-GA) model development to maximize isoprene production	74
4.3	Results and discussions	77
4.3.1	In silico studies on CrtE enzyme inhibition by alendronate	77
4.3.2	Isoprene production by recombinant <i>S. elongatus</i> UTEX 2973 strains and effect of alendronate on production	80
4.3.3	Effect of alendronate dose on growth rate and isoprene production	83
4.3.4	Optimization of production parameters to maximize isoprene production	86
4.3.5	Model validation of the RSM and ANN-GA	94
4.4	Conclusion	98
Chapter – 5	Design and simulation of isoprene production plant using flue gas as sole carbon source and its techno-economic analysis	99
5.1	Background	100
5.2	Materials and methods	101
5.2.1	Assumptions and process description	101
5.2.2	Gas supply unit	102
5.2.3	Isoprene production unit	104
5.2.4	Isoprene recovery	106
5.2.5	Biomass recovery	106
5.2.6	Economic evaluations	107
5.3	Results and discussions	109
5.3.1	Base case process description	109
5.3.1	Capital cost expenditure (CapEx)	110
5.3.3	Operating expenditure (OpEx)	111
5.3.4	Minimum isoprene selling price	113
5.3.5	Sensitivity analysis	115
5.4	Conclusion	117
Chapter – 6	Conclusion and Future Prospects	119
References		121
Appendix		137

LIST OF FIGURES

Figure 1.1	Engineering Methyl-D-erythritol 4-phosphate (MEP) pathway in cyanobacteria and inhibition of geranyl diphosphate synthase enzyme (CrtE) by bisphosphonate for enhanced production of isoprene.	9
Figure 2.1	Biosynthesis of isoprene through methyl-D-erythritol-4 phosphate (MEP) and mevalonic acid (MVA) pathways.	18
Figure 3.1	Schematic plasmid maps showing the major DNA inserts in constructed integrative vectors (A) pAM2991IspS and (B) pBbE1k-IDI-NSIII.	59
Figure 3.2	The homologous recombination process is used for the genomic DNA integration of the IspS and IDI genes at neutral site I (NSI) and neutral site III (NSIII), respectively.	61
Figure 3.3	Gene expression analysis and growth profiles of <i>S. elongatus</i> UTEX 2973, <i>S. elongatus</i> UTEX IspS, and <i>S. elongatus</i> UTEX IspS.IDI strains.	64
Figure 3.4	Isoprene production profiles of recombinant <i>S. elongatus</i> UTEX 2973 strains. (A) <i>S. elongatus</i> UTEX IspS (B) <i>S. elongatus</i> UTEX IspS.IDI.	65
Figure 4.1	(A) Schematic representation of work flow of experimental design and optimization of process variables. (B) A schematic representation of the feedforward artificial neural network (ANN) architecture consisting of three neuron input layer, two hidden layers of six neurons each, and one neuron output layer integrated with genetic algorithm (GA) model.	72
Figure 4.2	Docked pose of isopentenyl diphosphate (IPP) and alendronate in the cavity of SeCrtE. The surface line represented stereo views of docked poses of (A) IPP and (B) Alendronate with SeCrtE. The hydrogen bonds have been represented as the dashed yellow stick, and the interacting residues are labeled, thus demonstrating higher binding in the case of alendronate as compared to IPP. SeCrtE – CrtE of <i>Synechococcus elongatus</i> PCC 7942.	79
Figure 4.3	Growth and isoprene production ($\mu\text{g/L}$ of culture broth) profiles of recombinant <i>S. elongatus</i> UTEX 2973 strains. (A) and (B) <i>S. elongatus</i> UTEX IspS. (C) and (D) <i>S. elongatus</i> UTEX IspS.IDI.	81
Figure 4.4	Effect of alendronate concentration for enhanced isoprene production in engineered <i>S. elongatus</i> UTEX 2973-IspS.IDI.	84
Figure 4.5	Three-dimensional response surface plot for isoprene productivity ($\mu\text{g/L/h}$) with (A) Light ($\mu\text{mol photon/m}^2/\text{s}$) vs NaHCO_3 (mM). (B) Light ($\mu\text{mol photon/m}^2/\text{s}$) vs temperature ($^{\circ}\text{C}$). (C) NaHCO_3 (mM) vs temperature ($^{\circ}\text{C}$).	89
Figure 4.6	The maximum response of isoprene productivity at optimized input factors by statistical BBD tool.	89
Figure 4.7	Artificial neural network model performance with 5-fold cross validation. Consistent values of R^2 , RMSE and MAPE in all folds demonstrate that the model is performing consistently well on different subsets of the data.	92
Figure 4.8	ANN regression plots. (A) Training validation and test model regression plots with regression coefficient values (B) Validation performance plot. (C) The validation of ANN model by gradient and validation checks plots.	93

Figure 4.9	Cumulative isoprene production profile at unoptimized, statistical and artificial neural network-genetic algorithm optimized conditions in 4 days in presence of 20 µg/mL alendronate. (Inset) Isoprene productivity after 4 days of isoprene production studies at different process conditions. The experiments were conducted in triplicates and values were represented as mean ± SD.	95
Figure 5.1	Simplified process flow diagram of cyanobacterial isoprene production plant. (S1) Purified flue gas compressor stream, (S2) Purified flue gas photobioreactor stream, (S3) Nutrient feed pump stream, (S4) Nutrient feed photobioreactor stream, (S5) Off gas vent condenser stream, (S6) Cyanobacterial culture pump stream, (S7) Cyanobacterial culture decanter stream, (S8) Wastewater stream, (S9) Biomass stream, (S10) O ₂ stream, (S11) Isoprene stream.	102
Figure 5.2	Cost breakdown of the isoprene production plant. (A) Annual operating expenses including both fixed (supervision, labor burden and maintenance) and variable costs (chemicals and electricity) (B) Capital cost contains the installed cost of operational units and other major expenses.	111
Figure 5.3	Effect of isoprene productivity on minimum isoprene selling price (MISP).	114
Figure 5.4	Impact of single point variations in parameters on the minimum isoprene selling price (MISP) (MISP is 4.85 \$/kg in base case scenario) for the isoprene production process from flue gas.	116

LIST OF TABLES

Table 1.1	Comparison of fuel properties of hydrogenated isoprene dimers with various advanced fuels.	7
Table 2.1	Cyanobacterial host organisms used for synthetic biology approach.	20
Table 2.2	List of native and foreign promoters used in cyanobacteria.	23
Table 2.3	List of commercially available replicative plasmid.	30
Table 2.4	Integrative plasmids available commercially.	31
Table 2.5	Isoprene synthase (IspS) from different sources and their kinetic parameters.	34
Table 2.6	Microbial isoprene production and corresponding volumetric productivities in different production hosts.	41
Table 3.1	List of primers used cloning and semiquantitative PCR.	51
Table 3.2	Amplification of gene/DNA segment by PCR.	52
Table 3.3	List of plasmids and strains used in this study.	60
Table 4.1	Types of intermolecular interactions between isopentenyl diphosphate (IPP)-SeCrtE and alendronate-SeCrtE as obtained from docking simulations.	79
Table 4.2	Statistical Box-Behnken design matrix in uncoded and coded (in parenthesis) values with experimental isoprene productivity.	86
Table 4.3	Determination of model coefficient by multiple regression analysis of isoprene productivity.	87
Table 4.4	Analysis of variance for isoprene productivity.	88
Table 4.5	Different Artificial neural network models and their performance.	91
Table 4.6	Comparison of isoprene production in different cultivations conditions.	96
Table 5.1	Financial and productivity baseline assumptions for techno-economic analysis of isoprene production plant.	103
Table 5.2	Cost of raw materials used in the base case isoprene production study.	107
Table 5.3	Total project capital expenditure (CapEx) with costs of individual equipment and other costs associated with isoprene production plant.	112
Table 5.4	Annual operating expenditure (OpEx) including fixed operating costs (FOC) and variable operating costs (VOC) for a plant of 1000-tonne isoprene production capacity.	113

ABBREVIATIONS

AIC	Akaike information criterion
ANN-GA	Artificial neural network-genetic algorithm
ANOVA	Analysis of variance
BBD	Box-Behnken Design
BGC	Biosynthetic gene clusters
BOM	Basis of mobility
CapEx	Capital expenditure
CO	Carbon monoxide
CO ₂	Carbon dioxide
<i>cpcB</i>	C-phycocyanin β subunit
CRISPR	Clustered regularly interspaced short palindromic repeats
<i>CrtE</i>	Geranyl diphosphate synthase
DCF	Discounted cash flow
DCW	Dry cell weight
DMAPP	Dimethylallyl diphosphate
DMCO	Dimethylcyclooctanes
DNA	Deoxyribonucleic acid
DXP	Deoxy-D-xylulose 5-phosphate
DXR	Deoxy-d-xylulose-5-phosphate reductoisomerase
DXS	Deoxy-D-xylulose-5-phosphate synthase
EDTA	Ethylene diamine tetra acetic acid
EMP	Embden-Meyerhof pathway
eYFP	Yellow fluorescence protein
FFBP	Feedforward backpropagation
FGD	Flue gas desulfurization unit
FID	Flame ionization detector
FOC	Fixed operating cost
FPP	Farnesyl diphosphate
FPPS	Farnesyl diphosphate synthase
GFP	Green fluorescent protein
GGPP	Geranylgeranyl diphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hFPPS	Human farnesyl diphosphate synthase
HID	Hydrogenated isoprene dimer
HMBPP	Hydroxy-2-methyl-2-butenyl-4-diphosphate
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
IATA	International Air Transport Association
<i>IDI</i>	Isopentenyl diphosphate isomerase
IPP	Isopentenyl diphosphate
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IRR	Internal rate of return
ISBL	Inside battery limit
<i>IspS</i>	Isoprene synthase
LB	Luria-Bertani
LD	Light and dark
LM	Levenberg Marquardt
LOD	Limit of detection

LOQ	Limit of quantification
MAPE	Mean absolute percentage error
MEP	Methyl-D-erythritol-4-phosphate
MISP	Minimum isoprene selling price
Moco	Molybdenum cofactor
MOE	Molecular Operating Environment
MSE	Mean square error
MVA	Mevalonic acid
NaHCO ₃	Sodium bicarbonate
nblA	Nonbleaching protein A
NHOC	Net heat of combustion (Gravimetric)
NO _x	Nitrogen oxides
NPV	Net present value
NREL	National renewable energy laboratory
NSI	Neutral site I
NSIII	Neutral site III
O ₂	Oxygen
OpEx	Operating expenditure
PAM	Protospacer adjacent motif
PAR	Photosynthetically active radiation
PBR	Photobioreactor
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulphonyl fluoride
PTFE	Polytetrafluoroethylene
RBS	Ribosome binding sites
RFP	Red fluorescent protein
RP-1	Rocket propellant
RSM	Response surface methodology
SAFs	Sustainable aviation fuels
SAH	S-adenosyl-l-homocysteine
SD	Shine-Dalgarno
sgRNA	Single-guide RNA
TEA	Techno-economic analysis
THF	Tetrahydrofolate
VOC	Variable operating cost
GDP	Gross domestic product

NOTATIONS

\$	Dollar
\$/kg	Dollar per kilogram
%	Percent
°C	Degree Celsius
°C/min	Degree Celsius/minute
µg/L/h	Microgram per liter per hour
µmol photon/m ² /s	Micromole photons per meter square per second
bp	Boiling point
g/L	Gram per liter
g/L/day	Gram per liter per day
g/m ³ /day	Gram per meter cube per day
g/mL	Gram per milli liter
h	Hour
K _{cat}	Turnover number
kDa	Kilo Dalton
K _m	Michaelis constant
m ² /s	Square meter per second
m ³	Cubic meter
mg/g DCW	Milligram per gram dry cell weight
mg/L	Milli gram per liter
min	Minute
MJ/kg	Megajoule per kilogram
MJ/L	Megajoule per liter
mL	Milli liter
mL/min	Milli liter per minute
mM	Millimolar
ng	Nanogram
pH	Potential of hydrogen
rpm	Revolution per minute
s	Second
T _m	Melting temperature
v/v	Volume by volume
γ	Gamma
µg	Microgram
µg/g DCW/h	Microgram per gram dry cell weight per hour
µg/L	Microgram per liter
µL	Microliter