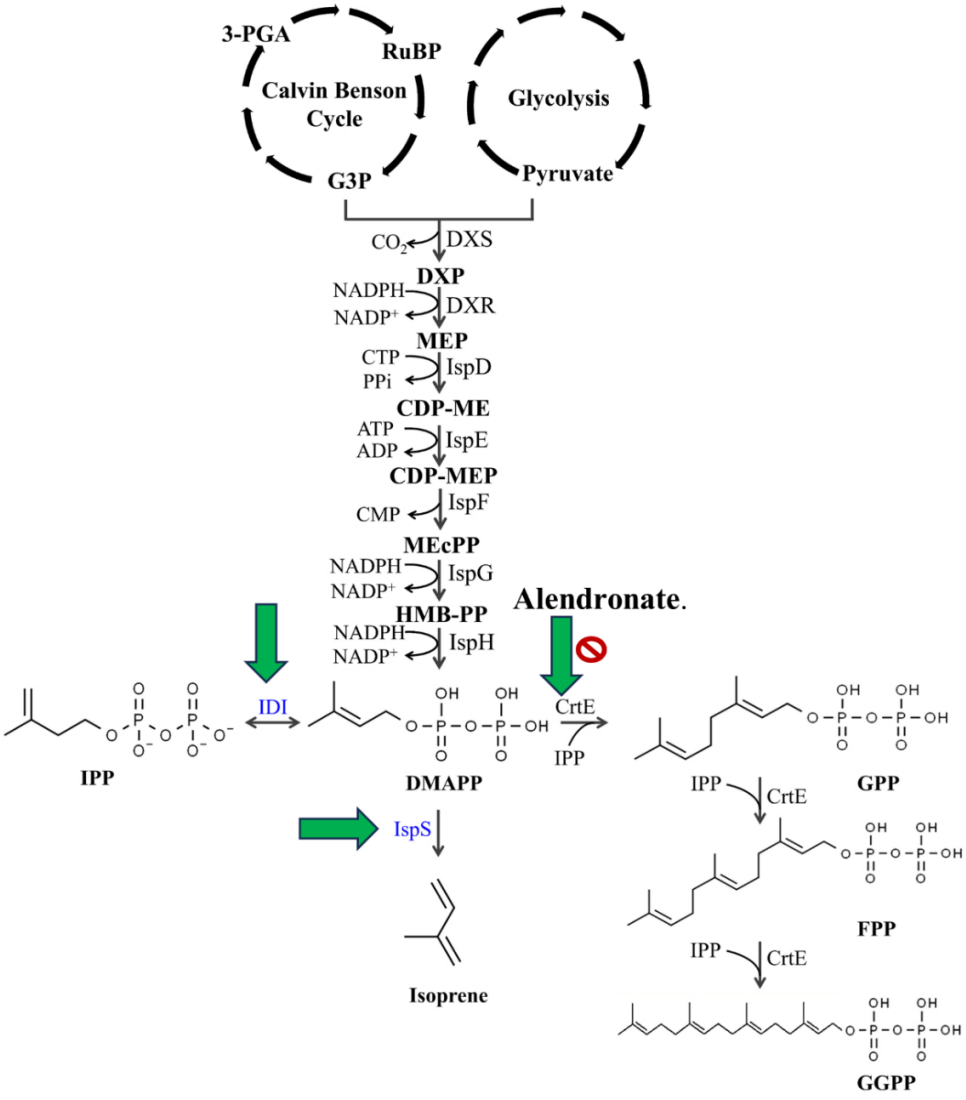
Chapter – 1

An introduction on approaches used in engineering methyl-D-erythritol 4-phosphate (MEP) pathway of *S. elongatus* UTEX 2973 for sustainable production of isoprene



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1.1 Introduction

Industrialization and the human population explosion have triggered a global energy crisis due to the dependence on non-conventional fossil-based fuels like petroleum, coal, and natural gases (Andrews et al., 2021; Angermayr et al., 2015; Yadav et al., 2021). Moreover, the extensive utilization of fossil fuels leads to the emission of significant quantities of detrimental greenhouse gases, resulting in their accumulation in the environment and subsequent adverse impacts on planetary life (Yadav et al., 2021). The depletion of fossil fuel reserves and rising uncertainties regarding global warming have emphasized the researchers to devise sustainable processes primarily based on renewable sources like solar energy, wind energy, bioenergy and hydro energy (Zhang et al., 2023). The International Air Transport Association (IATA) emission reduction roadmap has expected a 50% decrease in aviation CO_2 emissions by 2050 when compared to the levels recorded in 2005 (Rana et al., 2022). Meeting the IATA goal of reducing CO_2 emissions becomes more challenging in rapidly expanding economies where air traffic growth is notably steep. Achieving this goal necessitates technological advancements and the adoption of sustainable aviation fuels that go beyond the current usage of biofuels derived from biomass cultivation and processing (Rana et al., 2022; Yadav et al., 2023b). Sustainable biofuel production technologies have been highly explored in past years due to their outstanding importance in developing a green and circular biobased economy (Velmurugan and Incharoensakdi, 2022). Solar radiation and CO_2 are the primary energy and carbon sources, respectively, that are abundantly available and widely used in manufacturing of bioenergy and economically valuable products using photosynthetic microbial systems (Andrews et al., 2021; Bentley and Melis, 2012). The harvesting of solar energy and utilization of CO_2 to produce biofuels through photosynthesis is one of noteworthy achievements of nature that could also be a solution for the future generation of energy and various platform chemicals

(Rautela and Kumar, 2022; Yadav et al., 2021). Cyanobacteria offers extreme potential for bioengineering achievements, powered by their efficient light-harvesting capabilities and adaptable metabolism for biotechnological applications compared to microalgae which open ample opportunities to modify complex biosynthetic pathways using synthetic biology approaches (Azevedo et al., 2019; Lin et al., 2021). Cyanobacteria have appeared as promising cellular production systems for the sustainable generation of carbon-neutral biofuels to mitigate problems caused by the excessive consumption of petroleum-based fuels (Andrews et al., 2021; Lin and Pakrasi, 2019). Engineered cyanobacteria strains, with their superior lipid production capabilities, offer a promising biofuel alternative like biodiesel (Eungrasamee et al., 2019; Quintana et al., 2011). The concept of converting CO_2 into a desirable fuel gave rise to genetically engineered cyanobacteria for biofuel production (Knoot et al., 2018). The first model cyanobacterium was *Synechocystis sp.* PCC6803 for which the complete genome was sequenced in 1996 (Kaneko et al., 1996). Some cyanobacterial model strains, *Synechocystis sp*. PCC 6803, *Synechococcus elongatus* sp. PCC 7942, *Synechococcus* sp. PCC 7002, *Synechococcus elongatus* UTEX 2973, *Synechococcus elongatus* PCC 11801, and *Synechococcus elongatus* PCC 11802 have been used in synthetic biology and metabolic engineering studies. In addition, cyanobacteria show a higher photosynthetic efficiency and growth rate than plants; they are capable to divert a large sum of absorbed carbon into the synthesis of industrially important biofuels and chemical feedstocks (Bentley and Melis, 2012). Cyanobacteria are the preferred photosynthetic bio-cellular factories to produce biofuels and chemicals due to the ease of genetic manipulation. Some attributes of cyanobacteria like high cell density growth, ability to grow on unfertile land, consumption of water from different waterbodies (fresh, marine and waste water) and capability to produce both biofuels and other economically important products qualifies them a suitable cell factory (Zahra et al., 2020). Efforts have been made

to produce various biofuel-related metabolites, such as isoprene, α-farnesene, ethanol, isobutanol, limonene, and alkane, using recombinant cyanobacterial systems (Ahmed et al., 2021; Bentley and Melis, 2012; Chaves and Melis, 2018; Lindberg et al., 2010; Rautela and Kumar, 2022; Sengupta et al., 2019). These biofuel molecules produced from biological routes could be used to substitute or blend conventional petroleum-based fuels to overcome the fossil fuel crisis and environmental concern. To fulfill these objectives, different types of synthetic biology tools have been established such as engineered promoters (constitutive and inducible), ribosome binding sites library (RBS), riboswitches, CRISPR/Cas system and vectors (Li et al., 2021; Sengupta et al., 2023). These tools come under the common term "BioBricks" which stands for the part of the DNA.

Isoprene is a 5-carbon simplest terpenoid volatile molecule which serves a variety of industrial purposes, including as a precursor in the production of chemicals used in the manufacture of synthetic rubber, pesticides, lubricants, and adhesives (Kim et al., 2016; Pade et al., 2016; Wang et al., 2017). Production of isoprene at industrial levels is carried out by thermal cracking of crude oil at high temperatures, followed by fractional distillation which is an energy intensive and environment unfriendly process (Janke et al., 2020; Li et al., 2018). In nature, isoprene is produced by various plants including *Eucalyptus globulus*, *Pueraria montana*, *Populus alba*, and *Populus canescens* in response to heat stress and drought through methyl-D-erythritol-4-phosphate (MEP) and mevalonic acid (MVA) pathway and emitted into atmosphere in large quantities (Fini et al., 2017). Due to its high volatile character, it is tedious to collect from plant production system. Additionally, plant farming is seasonal and needs extensive terrestrial capitals, which limits isoprene production at larger scale using plant systems (Li et al., 2018). Lately, efforts have been made to produce isoprene by heterotrophic microbial systems such as yeast and bacteria (Kim et al., 2016; Ye et al., 2016). Although heterotrophic recombinant microorganisms

offer a remarkable isoprene yield, their reliance on expensive, intricate, and environmentally unsustainable nutrient sources poses a significant barrier to large-scale, cost-effective production, prompting the search for alternative strategies. However, there is an increasing interest in developing substitute processes for sustainable isoprene production using photosynthetic microbial sources such as cyanobacteria and microalgae (Zahra et al., 2020). The sustainable and renewable production of isoprene, using recombinant cyanobacteria from CO_2, could be a promising model production system. Compared to the conventional method of isoprene production from crude oil, these alternative methods have the potential to be more sustainable and eco-friendly due to carbon neutrality (Gao et al., 2016; Kim et al., 2016; Rautela and Kumar, 2022). Archaea, animals, yeast and some bacteria use the MVA pathway whereas bacteria, cyanobacteria, microalgae use the MEP pathway for the synthesis of a variety of terpenoid molecules (Englund et al., 2018). Although cyanobacteria lack the key enzyme isoprene synthase (*IspS*) gene, they can be genetically modified with a plant-origin *IspS* gene and bottleneck gene overexpression for the sustainable production of isoprene by using CO_2 and light (Yadav et al., 2021).

Dimethylallyl diphosphate (DMAPP), the main precursor of isoprene, is biologically produced by two main pathways, i.e., MEP and MVA pathways (Rautela and Kumar, 2022). Studies on metabolic pathway engineering showed that isoprene synthesis mainly depends on the intracellular concentrations of isopentenyl diphosphate (IPP), DMAPP, and the activities of *IspS*, isopentenyl diphosphate isomerase (*IDI*), and geranyl diphosphate synthase (CrtE) (Englund et al., 2018; Zhao et al., 2013). The initial step of isoprene synthesis by the MEP pathway utilizes pyruvate and glyceraldehyde 3-phosphate in a condensation reaction to form deoxy-D-xylulose 5-phosphate (DXP), which is catalyzed by deoxy-D-xylulose-5-phosphate synthase (DXS). Following a series of reactions, two

final products, DMAPP and IPP, are synthesized. DMAPP is used as a precursor for isoprene synthesis by *IspS* (Zhao et al., 2013).

Jet fuel stands as a pivotal resource in modern-day society, with a staggering global consumption of 95 billion gallons in 2019. The aviation industry contributes over 2 trillion $ (dollars) to the worldwide GDP (Doliente et al., 2020). However, prevailing jet fuels are presently derived from non-renewable petroleum sources, and their combustion significantly elevates atmospheric CO_2 levels, thereby worsening global warming (Doliente et al., 2020). Furthermore, petroleum-based fuels contain aromatic compounds, culminating in the generation of particulates that increase radiative forcing, thereby compounding the impacts of global warming. In response to these challenges, researchers worldwide are diligently endeavouring to devise efficient methodologies for renewable production of sustainable aviation fuels (SAFs) from biomass and CO_2, that burn cleanly and maintain full performance characteristics (Xu et al., 2022). Isoprene holds the potential to not only serve as a raw material in the synthetic chemistry industry, particularly for rubber production, but also demonstrates considerable promise in generating renewable, readily integrable biofuels. Due to its double bond and branched chain configuration, isoprene lends itself readily to the formation of polymers with ring structures. This characteristic has enabled the oligomerization of isoprene monomers, yielding improvised fuel molecules that can be blended to gasoline, aviation fuel, and diesel. Isoprene-derived biofuels and chemicals offer potential alternatives to petroleum-based fuels due to its properties like elevated energy density and lower viscosity (Rana et al., 2022; Wang et al., 2017). Recently, the hydrogenated isoprene dimers ($C_{10}H_{20}$ compounds—derivatives of cyclobutene, cyclohexane, and cyclooctane) from recombinant cyanobacteria have been characterized as ideal drop-in jet fuel (Rana et al., 2022; Woodroffe and Harvey, 2022). The

fuel properties of isoprene dimers have been presented in Table 1.1 and compared with various advanced fuels.

Table 1.1 Comparison of fuel properties of hydrogenated isoprene dimers with various advanced fuels.

Fuel properties	HID	DMCO	Jet-A	*p*-Menthane	RP-1
NHOC, MJ/kg	43.34	43.82	>42.8	43.20	43.37
Volumetric NHOC, MJ/L	34.94	36.22	>33.17	34.72	34.96
Density (15°C), g/mL	0.806	0.827	>0.775	0.804	0.806
Kinematic viscosity (−20 °C), m^2/s	3.10	4.17	<8.0	2.98	-
Flash point, °C	42	50	>38	-	57
Freezing point, °C	< (-78)	< (-78)	<−40	-	-

HID – hydrogenated isoprene dimer; DMCO – dimethylcyclooctanes; RP-1 – rocket propellant; NHOC – Gravimetric net heat of combustion (Rana et al., 2022; Woodroffe and Harvey, 2022).

In the industrial domain, isoprene functions as a pivotal precursor for the synthesis of both natural and synthetic rubbers. Specifically, polyisoprene, demonstrates extensive application across diverse sectors, including automotive and healthcare industries (Batten et al., 2021). This polymer manifests outstanding attributes such as exceptional elasticity, robust resilience, and heightened flexibility at low temperatures. These characteristics render polyisoprene an indispensable material in the fabrication of a wide spectrum of products and components (Batten et al., 2021). Furthermore, polymers derived from isoprene play a prominent role in the adhesives and sealants sector. These substances are highly valued for their notable properties of tackiness, adhesion strength, and robust resistance to environmental influences.

1.2 Approach

In the present work, we have focused on the heterologous expression and enhancement of isoprene production in a fast-growing cyanobacterial strain, *Synechococcus elongatus* UTEX 2973, using a genetic engineering approach in combination with the inhibition of the downstream of the MEP pathway reaction by an inhibitor (alendronate). To accomplish the objective, the *IspS* and *IDI* genes are integrated at neutral site I (NSI) and neutral site III (NSIII) in the *S. elongatus* UTEX 2973 genome, respectively. We have performed further in silico studies to check the inhibition of the SeCrtE (CrtE of *S. elongatus* PCC 7942) enzyme which catalyzes the DMAPP and IPP condensation in *S. elongatus* strains by alendronate using molecular docking tools. An alendronate inhibitor was further applied during isoprene production by *S. elongatus* UTEX *IspS* and *S. elongatus* UTEX *IspS.IDI* recombinant strains in a closed cultivation system to see the effect on yield improvement. To the best of our knowledge, no inhibition strategy has yet been used in a recombinant cyanobacterial system to enhance isoprene production. Metabolic studies on downstream MVA/MEP pathways in animal, human, and plant systems suggested that the farnesyl diphosphate synthase (FPPS which is similar CrtE of *S. elongatus* UTEX 2973) enzyme can be inhibited by alendronate and other bisphosphonates (Park et al., 2021; Suva et al., 2021). We hypothesized that the inhibition of cyanobacterial CrtE could lead to improved production of isoprene. Bisphosphonates are bioisosteres of pyrophosphates and used as potent inhibitors of enzymes that act downstream of the MEP pathway as well as utilize substrates containing a pyrophosphate moiety, such as IPP (Rasulov et al., 2015). The synthesis of various terpenes is carried out by the condensation of prenyl pyrophosphate (IPP and DMAPP) precursors catalyzed by the prenyl transferase enzyme. The DMAPP acts as a priming molecule for the addition of IPP in a condensation reaction for the synthesis of various terpenoids, such as monoterpenes

(C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), and tetraterpenes (C_{40}) (Feng et al., 2020; Rautela and Kumar, 2022) (Figure 1.1). This study presents a first-hand explanation of the enhanced production of isoprene by the inhibition of the reactions downstream of the MEP pathway by alendronate in recombinant *S. elongatus* UTEX 2973 strains.

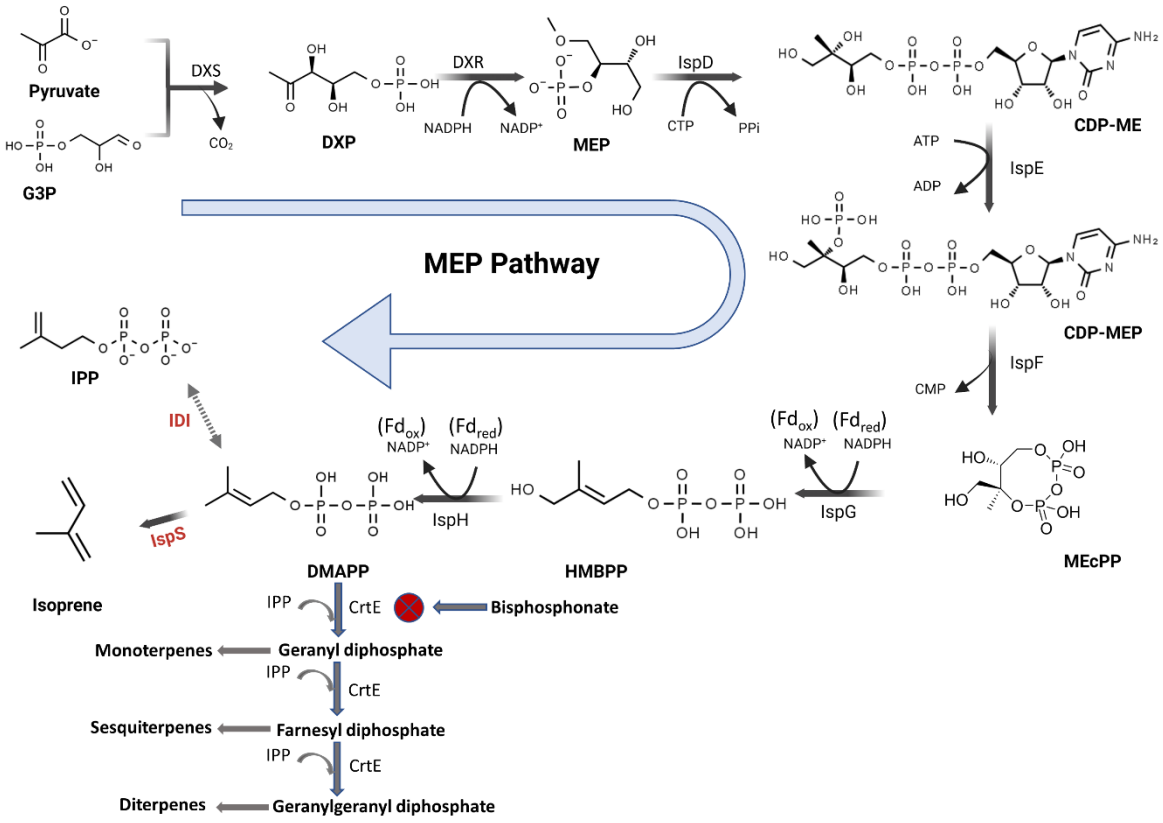

Figure 1.1 Engineering Methyl-D-erythritol 4-phosphate (MEP) pathway in cyanobacteria and inhibition of geranyl diphosphate synthase enzyme (CrtE) by bisphosphonate for enhanced production of isoprene. Heterologously expressing genes IspS and IDI are denoted in red. Abbreviations used (metabolites): G3P—glyceraldehyde 3-phosphate; DXP—deoxy-D-xylulose-5-phosphate; MEP—methyl-D-erythritol 4-phosphate; CDP-ME—diphosphocytidylyl methylerythritol; CDP-MEP—diphosphocytidylyl methylerythritol phosphate; MEcPP—methyl erythritol-2,4- cyclodiphosphate; HMBPP—hydroxymethylbutenyl diphosphate; DMAPP—dimethylallyl diphosphate; IPP—isopentenyl diphosphate; NADPH—nicotinamide adenine dinucleotide phosphate; CTP—cytidine triphosphate; PPi—diphosphate; ATP—adenosine triphosphate; ADP—adenosine diphosphate; CMP—cytidine monophosphate; Fd_{red}—ferredoxin reduced; and Fd_{ox}—ferredoxin oxidized. Enzymes: DXS—deoxy-D-xylulose-5-phosphate synthase; DXR—DXP reductoisomerase; IspD—CDP-ME synthase; IspE—CDP-ME kinase; IspF—ME-cPP synthase; IspG—HMBPP synthase; IspH—HMBPP reductase; IDI—isopentenyl diphosphate isomerase; and IspS—isoprene synthase; CrtE — geranyl diphosphate synthase.

The photosynthetic production of isoprene using engineered cyanobacterial systems still has a few challenges like low-yield of isoprene, stability of the modified strain/production process and economic viability of production process (Kant et al., 2023; Rana et al., 2022). These challenges require to be resolved before this technology could be implemented on a larger production scale (Pade et al., 2016; Rodrigues et al., 2023b). The productivity of isoprene is influenced by process operating parameters such as growth temperature, light intensity and concentration of carbon source present (Gao et al., 2016; Rodrigues et al., 2023b). The optimization of production process parameters for enhanced isoprene productivity would be vital for the isoprene production at industrial level. To the extent of available information, no study has reported for the process parameter optimization for enhancing photosynthetic production of isoprene by the engineered cyanobacterial system using machine learning and statistical tools. Furthermore, the recombinant strain *S. elongatus* UTEX 2973-*IspS.IDI*, was used for maximized isoprene production studies by optimizing growth and nutrient conditions (Yadav et al., 2023b). To maximize isoprene production in the engineered cyanobacterium *S. elongatus* UTEX 2973-*IspS.IDI*, we optimized inhibitor concentration and key process parameters like light intensity, $NaHCO_3$ concentration, and growth temperature. We used a combination of statistical and machine learning tools, including a Box-Behnken design for experimentation and both statistical modelling and an artificial neural network-genetic algorithm (ANN-GA) for analysis. By feeding in real data from the experiments, we trained the ANN model in a feedforward back propagation manner, which then guided a genetic algorithm to find the optimal parameter combinations for enhancing isoprene yield. We then validated the predictions of both models through further experiments.

Furthermore, to test the economic viability of the sustainable isoprene production process at larger scale a conceptual plant for isoprene production of 1000 tonnes annual capacity

was designed. It was assumed that the plant utilizes industrial flue gas as sole CO_2 source and base case productivity and other parameters based on the preliminary data obtained from lab scale experimental results and literatures. Moreover, an early-stage techno-economic analysis was performed to assess potential financial feasibility of plant. The isoprene production facility comprising of tubular photobioreactors (PBRs) may achieve an isoprene productivity of 74.4 g/m^3/day in which industrial flue gas as sole source of CO_2 would be supplied to reduce the production cost. In the current isoprene production facility design, it is assumed that produced isoprene and other gases including unconverted CO_2 and oxygen would be transferred to isoprene recovery unit continuously passing through an amine solution. This target productivity serves as a key research milestone, suggesting a significant step forward beyond existing theoretical boundaries. The Inside Battery Limit of isoprene manufacturing facility comprises five main operational units: gas supply, isoprene production, isoprene recovery, biomass separation and wastewater treatment and recycle units.

1.3 Objectives

Based on the identified research gaps after comprehensive literature review presented in chapter 2, following objectives were determined to fill these gaps and contribute new information to the field of study.

- Selection of suitable cyanobacterial host for genetic modification and isoprene production.

  (a) Preparation of integrative plasmids for *IspS* and *IDI* genes.

  (b) Genomic integrations of heterologous *IspS* and *IDI* genes using triparental conjugation.

  (c) Characterization of genetically modified cyanobacterial strains.
- Enhancement of isoprene production in engineered cyanobacteria

(a) Identification and effect of potential inhibitor for isoprene yield improvement.

(b) Optimization of process parameters using statistical and machine learning approaches.

- Techno-economic analysis of isoprene production process using modified cyanobacterial strain.

1.4 Thesis outline

The thesis has been presented in six chapters which are briefly mentioned below.

Chapter 1. This section delivers an overview of the fundamental structure, underlying motivation, and goals of the current study, as well as outlining the approach taken to address the main challenges.

Chapter 2. This section offers an extensive examination of the current state and recent biotechnological breakthroughs in the domain of cyanobacterial metabolic engineering for biofuel production, synthetic biology tools used for microbial engineering, cyanobacterial transformation techniques, isoprene production using microbial systems, various advanced engineering strategies aimed at enhancing cyanobacterial isoprene production as well as technoeconomic assessment of microbial isoprene production process at larger scales.

Chapter 3. This section encompasses the procedures which involves choosing a fast-growing cyanobacterial host for genetic modification for isoprene production. This is followed by the construction of integrative plasmids consisting of *IspS* and *IDI* genes. Subsequently, the heterologous *IspS* and *IDI* genes are inserted into the genome of host cyanobacteria via homologous recombination using triparental conjugation process. Finally, the genetically modified cyanobacterial strains undergo comprehensive characterization for heterologous gene expressions and isoprene production.

Chapter 4. This section focuses on enhancing isoprene production in engineered cyanobacteria through the identification and evaluation of potential inhibitors for improved isoprene yield. Additionally, it involves optimizing process parameters using both statistical and machine learning approaches.

Chapter 5. This section deals with conducting a techno-economic evaluation of the isoprene production process at larger scale employing the modified cyanobacterial strain.

Chapter 6. This section outlines the importance of the current study and provides insights into future perspectives.