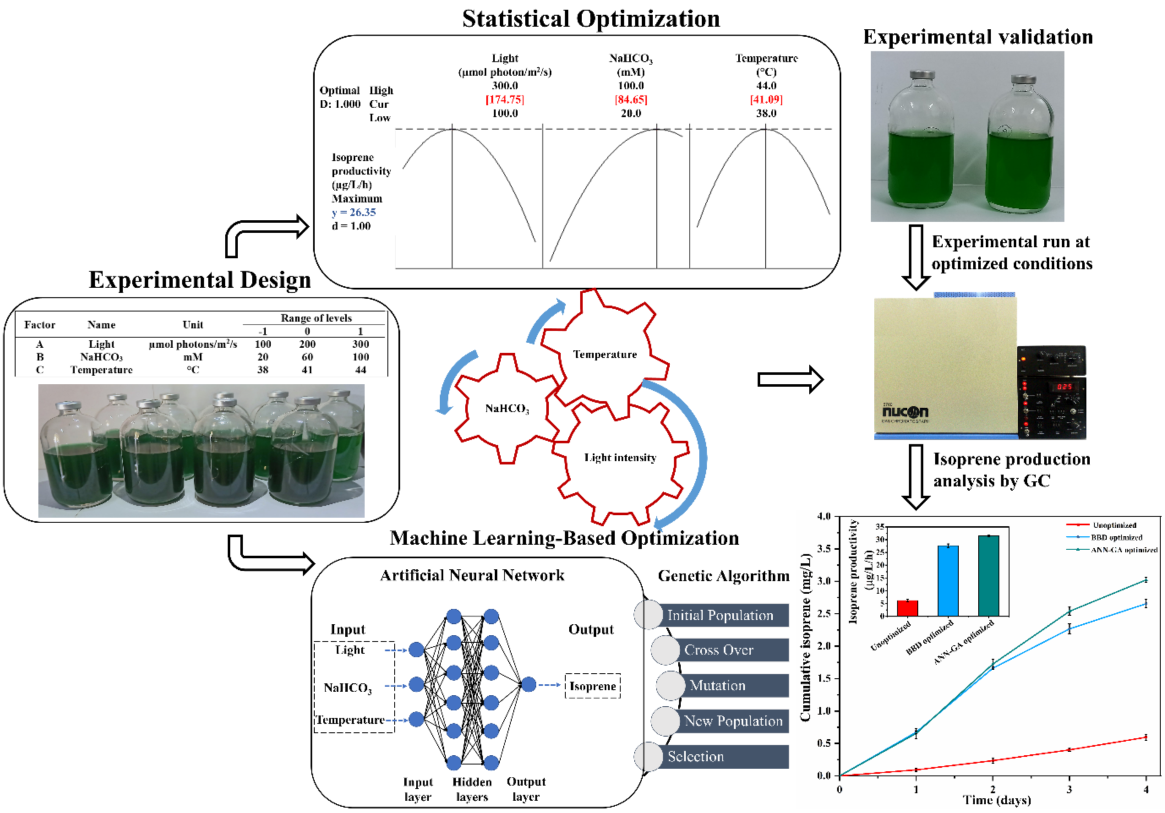
Chapter – 4

Enhancement of Isoprene Production in Engineered *Synechococcus elongatus* UTEX 2973 by Metabolic Pathway Inhibition and Machine Learning-Based Optimization Strategy



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4.1 Background

An engineered *S. elongatus* UTEX 2973-*IspS.IDI* is used to enhance isoprene production through geranyl diphosphate synthase (CrtE) inhibition which catalyzes the side reactions of MEP pathway and process parameters such as light intensity, $NaHCO_3$ and growth temperature optimization approach. Engineered cyanobacterial systems are promising microbial hosts for sustainable and photosynthetic isoprene production. However, several challenges hinder its widespread implementation, including low yield, strain/process stability, and economic feasibility. Therefore, addressing these issues is essential for large-scale commercialization. The productivity of isoprene is influenced by several process operating conditions, such as growth temperature, light intensity, and carbon source availability (Gao et al., 2016; Rodrigues et al., 2023b). Therefore, optimizing production process parameters is crucial for the industrial-scale production of isoprene. Consequently, optimization of these production conditions is essential for achieving industrial-scale isoprene production. Remarkably, no prior research has investigated for the optimization of process parameters for enhancing photosynthetic isoprene production utilizing machine learning and statistical methodologies. In this study, a genetically modified strain, *S. elongatus* UTEX 2973-*IspS.IDI*, was employed for the enhancement of isoprene production by utilizing an inhibitor (alendronate) to block the MEP pathway side reactions as well as optimization of production parameters (Yadav et al., 2023b). A potent inhibitor of CrtE enzyme was used to downregulate the CrtE activity for enhancing isoprene production. Furthermore, the concentration of the inhibitor and various process parameters including light intensity, $NaHCO_3$ concentration, and growth temperature were optimized to enhance the production of isoprene. To facilitate this optimization, response surface methodology (RSM) based on Box-Behnken Design (BBD) was implemented to generate experimental data sets. These data sets were then utilized to optimize process parameters using a combination of statistical techniques and artificial neural network-genetic algorithm (ANN-GA) models, with the aim of maximizing isoprene yield. The input variables and experimental outcomes gathered from real experiments were employed to train an artificial neural network (ANN) algorithm using a feedforward backpropagation (FFBP) methodology. Thus, the generated ANN model was employed as a driving force for a single-objective genetic algorithm model, aimed at optimizing process parameters to enhance isoprene production in recombinant *S. elongatus* UTEX 2973-*IspS.IDI*. Furthermore, the developed models from RSM and ANN-GA studies have been validated experimentally.

4.2 Materials and methods

4.2.1 Strain and seed culture conditions

The cyanobacterium *S. elongatus* UTEX 2973 was engineered with heterologous *IspS* and *IDI* genes in its genome for isoprene production as described in chapter 3 (Yadav et al., 2023a). The modified cyanobacteria is named as *S. elongatus* UTEX 2973-*IspS.IDI*. The engineered cyanobacteria were maintained and cultivated in BG-11 media (pH 7.5) for seed culture preparation with 50 μg/mL spectinomycin/streptomycin and 50 μg/mL kanamycin antibiotic as selection markers. The *S. elongatus* UTEX 2973-*IspS.IDI* cultures were incubated at 38 °C under continuous 100 μmol photon/m^2/s white fluorescent light and 180 rpm in an orbital shaking photo incubator (Scigenics Biotech Private Limited, Chennai, TN, India).

4.2.2 Growth and isoprene production analysis

Growth studies of engineered *S. elongatus* UTEX 2973 strains were performed using a spectrophotometric method previously explained by (Yadav et al., 2023a). The DCW was determined using the method described by (Bentley and Melis, 2012). The growth rate of cyanobacteria was calculated using Equation (4.1).

$$\text{Growth rate} = \frac{(DCW_2 - DCW_1)}{t_2 - t_1} \quad (4.1)$$

Where, DCW_2 and DCW_1 are biomass at time t_2 and t_1, respectively. The equation for growth rate using DCW is commonly applied in cyanobacteria studies because it directly quantifies biomass increase over time. It is especially useful during the exponential growth phase, where the rate of growth is nearly linear. This method, requiring only measurements at two time points, makes it practical for routine monitoring in lab. This equation helps optimize growth conditions and compare growth rates across different strains or treatments, providing valuable insights for research.

For isoprene production, 100 mL BG-11 media with suitable antibiotics were inoculated with active seed cyanobacterial culture in wheaton glass serum bottles of volume 160 mL (Sigma-Aldrich, St. Louis, MI, USA). Initial optical density (OD_{730}) of production culture was maintained at ~ 0.1 in cotton-plugged serum bottles. Then, *S. elongatus* UTEX 2973-*IspS.IDI* cells were grown up to OD_{730}~0.70 at 38 °C, 100 µmol photons/m^2/s continuous white light and 180 rpm. Further, cultures were supplemented with 50 mM $NaHCO_3$, 1 mM IPTG and 10 mM HEPES (pH 7.5) buffer and alendronate (25 µg/mL) for isoprene production and all culture bottles were sealed with an aluminium crimp and septa (Sigma-Aldrich, St. Louis, MI, USA). The septa were composed of polytetrafluoroethylene (PTFE)/silicone. The PTFE side of the septa was placed at inner side of the culture bottles to avoid the isoprene sorption into silicone material. All the culture bottles of triplicate experiments were incubated at the same conditions as mentioned above for isoprene production and estimation.

Quantification and estimation of isoprene was performed using a gas chromatography (Model: 5765, Nucon Engineers Private Limited, Delhi, India) equipped with flame ionization detector (FID) and Porapack Q packed column (80/100) as mentioned by (Yadav et al., 2023a). Isoprene was estimated at every 24 h interval by injecting 1 mL gas sample from headspace gases of the culture bottles in GC. The total volume of headspace gases was measured using water displacement method for quantification of isoprene and the produced gases were released at every 24 h interval. To measure the volume of gas produced during 24-hour incubation, a silicone tube, connected to a needle at one end, was inserted into the septa, with the other end submerged in a water-filled 200 mL measuring cylinder placed inside a 5-liter water container. As gas was produced, it passed through the silicone tube, displacing water in the cylinder. The displaced water volume, equivalent to the gas produced, was recorded. This setup ensures accurate measurement of gas production over the incubation period. Further, the septa were removed aseptically and rest of the isoprene and other gases from the headspace of culture bottles were released and culture bottles were re-sealed with sterile septa. Isoprene concentrations were determined by referencing a standard calibration curve constructed using known amounts of pure vaporised isoprene (Catalog number: I19551; Sigma-Aldrich, St. Louis, MI, USA) mixed in the air.

4.2.3 Experimental workflow

The isoprene production studies in *S. elongatus* UTEX 2973-*IspS.IDI* were conducted in presence of 1 mM IPTG and 10 mM HEPES buffer (pH 7.5) at unoptimized process conditions (100 µmol photon/m^2/s light intensity, 50 mM $NaHCO_3$ and 38 °C temperature). In silico studies were conducted to test the potential of alendronate as CrtE inhibitor. The effect of alendronate, acting as a CrtE inhibitor, on both isoprene production and the growth rate of cyanobacteria was evaluated. Consequently, the concentration of alendronate was optimized to achieve optimal levels for maximizing isoprene production. Furthermore, a range of production process factors, light intensity (100 to 300 µmol photon/m^2/s), $NaHCO_3$ (20 to 100 mM) and growth temperature (38 to 44 °C) were selected for the optimization.

Utilizing Minitab software (version 19.1), an experimental design was executed in triplicates employing BBD by varying the process factors. The experimental input variables and responses were used for the optimization of process factors using RSM and ANN-GA model. Both the models were compared based on Akaike information criterion (AIC) value, R^2 and isoprene production results on optimized values of process variables obtained from RSM and ANN-GA models. A process flow diagram of the experimental design, process optimization and validation has been represented in Figure. 4.1A.

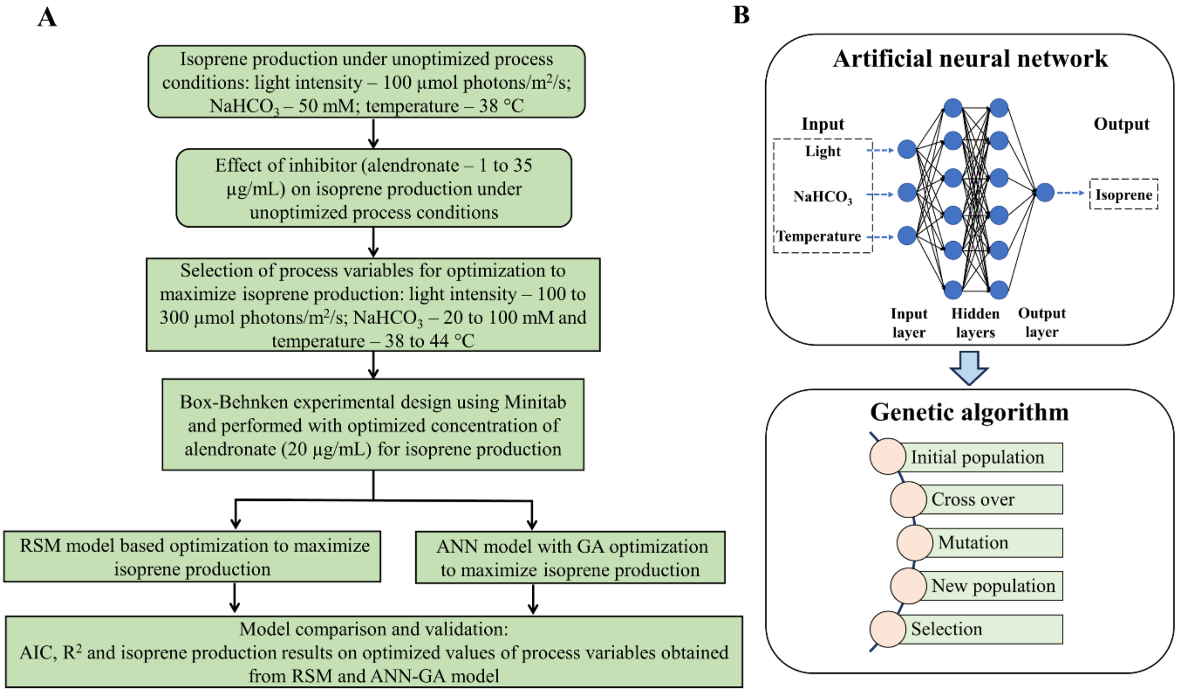


Figure 4.1 (A) Schematic representation of work flow of experimental design and optimization of process variables. (B) A schematic representation of the feedforward artificial neural network (ANN) architecture consisting of three neuron input layer, two hidden layers of six neurons each, and one neuron output layer integrated with genetic algorithm (GA) model.

4.2.4 Geranyl diphosphate synthase (CrtE) inhibition studies: Structure preparation, molecular docking simulations and analysis

The X-ray crystal structure of SeCrtE (PDB ID: 7MY7) was taken as the initial coordinate for the molecular docking of alendronate (Satta et al., 2022). Before docking, the structure was prepared using Molecular Operating Environment (MOE), v2022.03. To evaluate the binding and energetics of alendronate and IPP, molecular docking simulations were carried out using CB-Dock2 (Liu et al., 2022; Yang et al., 2022). The CB-Dock2 is an improved protein-ligand docking method with cavity detection, ligand docking, and homologous template fitting features. CB-Dock2 computes the (i) ligand-binding sites using a curvature-based cavity detection method, followed by the (ii) identification of the center and size of the ligand-binding site, (iii) adjusting the docking grid box size and then utilizing Autodock Vina for molecular docking. In addition, it integrates the template-based docking engine to improve the identification of the binding site and binding pose prediction of the ligand. To perform the docking, the MOE-prepared monomeric structure of apo SeCrtE was considered. Twenty cavities were generated per molecule (IPP and alendronate) and molecular docking was performed for each reaction. The template-based docking was performed using the crystal structure of SeCrtE C-term His-tag with IPP (PDB ID: 7MY6) as a reference. The docked poses were then used for interactions and energetic analysis.

To check the effect of alendronate on the activity of other key enzymes IspS, IspH and IDI, molecular docking of these enzymes was performed with their respective natural substrates and alendronate. Modelled AlphaFold structure of IspS (UniProt ID: Q6EJ97, AF-Q6EJ97-F1) from *Pueraria montana* was docked with DMAPP and alendronate to compare the binding site and binding energies. Similarly, the modeled AlphaFold structure of the IspH enzyme (UniProt ID: Q31S64, AF-Q31S64-F1) was docked with HMBPP and alendronate to compare the binding site and binding energy. Finally, as the crystal structure of IDI, was available (PDB ID: 1NFZ), it was directly used for docking with IPP and alendronate to evaluate the binding and energetics.

4.2.5 Effect of alendronate concentrations on isoprene production and cell growth

To estimate the detailed effect of alendronate concentrations on isoprene production and cyanobacteria cell growth different concentrations of alendronate from 1 to 35 μg/mL with an interval of 5 μg/mL was used with 1 mM IPTG and 50 mM $NaHCO_3$ in isoprene production cultures with a negative control. All culture bottles of triplicate experiments were sealed with an aluminium crimp and septa and incubated at 38 °C temperature, 100 μmol photons/m^2/s continuous white light and 180 rpm agitation. For statistical analysis, Tukey's test was applied using RStudio software (version 4.3.0) at the 5% significance level to determine whether there was a significant difference between the mean values of isoprene production and growth rate.

4.2.6 Statistical optimization of process factors for maximization of isoprene production

The BBD experimental design for three process parameters (light intensity, $NaHCO_3$, growth temperature) was created using Minitab software for maximizing the isoprene production in engineered *S. elongatus* UTEX 2973-*IspS.IDI*. A total of 15 experiments, including 12 base runs and 3 centre points, were conducted in triplicates for 4 days. The other conditions of growth and isoprene production were kept the same as mentioned earlier. Minitab software was employed to perform an analysis of variance (ANOVA) aimed to assess the presence of statistically significant differences among the process variables affecting isoprene productivity. ANOVA was performed on the raw process variables without normalization and transformation. Validation experiments were also performed at optimized conditions where the response optimizer predicted maximum isoprene productivity.

4.2.7 Artificial neural network - genetic algorithm (ANN-GA) model development to maximize isoprene production

In this work, the generated experimental data sets from BBD, input factors (light, $NaHCO_3$, and temperature) and response (isoprene productivity) were employed for training, testing, and validation in ANN model. The construction of the ANN model followed the approach of previous studies that utilized a complete RSM-BBD matrix. This choice was preferred due to the advantages offered by ANN optimization, including efficient sampling, decreased computational burden, interpretability, and rapid initial evaluation (Saini et al., 2021). The model development was executed in MATLAB software (version R2023a). The data were normalized using Z-score normalization before training of the ANN model. The mean and standard deviation of input and target values were stored to scale back to the original values. Z-score normalization can improve optimization algorithm's convergence speed in model training. When the features are standardized, the optimization process is less likely to get stuck in elongated, skewed or irregularly shaped data space, resulting in faster convergence. The Z-score normalization has been represented as following (Equation 4.2).

$$z = \frac{x-\mu}{\sigma} \tag{4.2}$$

Where Z is normalized value, x is original value, μ is mean of datasets, σ is standard deviation of the data sets.

Here, a FFBP network technique for the ANN with the Levenberg Marquardt (LM) algorithm was used. Forward propagation calculates the amount of error in this ANN model by summing the inputs, multiplying randomized weights with bias, and then applying it to the tangent sigmoid activation function. The iterative process of the algorithm stops when the mean square error (MSE) reaches its minimum value. The derivative function of the inputs, the weights, and the function of transfer affect how the LM algorithm is trained. A high-performance ANN model will exhibit a minimum MSE and a high regression value (R). A k-fold cross-validation trial was executed to assess potential overfitting in the developed model. The same dataset was used for modelling across five distinct data partition schemes (5-fold). This involved training on four subsets of the data while testing on the remaining one. This process is repeated 5 times, rotating the test set. The performance of each trial was assessed by comparing them against each other and against the average of the five trials. Furthermore, Akaike information criterion (AIC) was used to compare the performance of BBD and ANN models using SAS Studio online tool (https://welcome.oda.sas.com/).

The constructed ANN model was further connected with a GA for the optimization of input factors to maximize isoprene production (Fig.4.1B). Genetic algorithms are typically used to minimize objective functions, but they can be easily modified to maximize objective functions. The fitness function was altered by incorporating a negative sign to transform it into a maximization problem. By taking the negative of the neural network output, the optimization algorithm will try to find the candidate solution that gives the largest output value from the neural network. The GA process typically involves generating an initial population of potential solutions randomly. Each solution in the population is then evaluated using a fitness function that measures its quality. The solutions exhibiting higher fitness scores are chosen as parents for the subsequent generation. These selected solutions undergo genetic operations such as crossover and mutation, simulating the process of reproduction and mutation in natural selection. The offspring produced by the genetic operators are added to the next generation, and the process is repeated until a termination criterion is reached. The objective function shall be defined as the trained model, and local target could be defined as the maximization of the objective function and thereby to find out the optimal parameters at which this objective function gives the maximum value, i.e., the maximum value of isoprene productivity is given as Equation (4.3).

$$f = \max\ [\mathrm{ANNGA}] \tag{4.3}$$

Subject to: A∈(100, 300); B∈(20, 100); C∈(38, 44)

The *f* signifies enhanced isoprene productivity. A, B, and C denote input factors light intensity, $NaHCO_3$, and temperature, respectively. Experiments were performed in triplicates at optimized conditions at which isoprene production was predicted maximum by the ANN-GA model to validate the model.

4.3 Results and discussions

4.3.1 In silico studies on CrtE enzyme inhibition by alendronate

Bisphosphonates are structural analogue of IPP and could inhibit the IPP binding to DMAPP blocking the downstream reactions to the MEP and MVA pathways that catalyze the synthesis of various terpenoid molecules (Gao et al., 2010). Previously, phosphonates and bisphosphonates have been used as potent inhibitors of human FPPS (hFPPS/CrtE) to treat bone-related diseases, inhibition of hFPPS for the development of pancreatic cancer drugs and inhibition of *Leishmania* and *Giardia* FPPS to inhibit cell proliferative activity. Alendronate showed one of the most effective drugs in such studies (Gadelha et al., 2020). In the present work using an in-silico approach, we have reported inhibition of the CrtE enzyme by alendronate to divert the carbon pool towards isoprene synthesis in recombinant *S. elongatus* UTEX 2973 through engineered MEP pathway. CrtE enzyme catalyzes three condensation reactions downstream to MEP pathway making various terpenoids. Here, we have used the SeCrtE enzyme of *S. elongatus* PCC 7942 for docking studies to verify the activity of the alendronate inhibitor.

To evaluate the (i) binding site and interactions of alendronate and IPP in the binding pocket of SeCrtE and (ii) the difference in binding energies between alendronate and IPP with SeCrtE, docking studies were performed, and results were analyzed. First, alendronate was docked into the twenty identified cavities of SeCrtE, and it was found to be docked into a cavity with dimensions of 260 Å^3, center: 42,41,17 (x,y,z), and size: 18,18,18 (x,y,z). A high binding affinity of -4.7 kcal/mol (AutoDock Vina score) was obtained for the best pose. In contrast, when the SeCrtE structure was used for the IPP docking, 20 cavities were identified into which the IPP was docked. It was found that IPP docked into one of the cavities in SeCrtE with an AutoDock Vina score of -3.2 kcal/mol, having a cavity size of 79 Å^3, center: 38,45,23 (x,y,z) and size: 19,19,19 (x,y,z) (Figure. 4.2). This result shows that alendronate binds more tightly than IPP in the cavity of SeCrtE with a higher number of intermolecular interactions and energy (Table 4.1). Other important MEP pathway enzymes such as IspS, IspH, and IDI were also checked for the molecular interaction and binding affinity with alendronate using molecular docking studies. The DMAPP, HMBPP and IPP are the natural substrate of the IspS, IspH and IDI enzymes, respectively. A high binding affinity of -6.5 kcal/mol (AutoDock Vina score) was obtained when IspS was docked with DMAPP whereas a score of -5.0 kcal/mol was obtained when IspS was docked with alendronate. Similarly, a binding affinity of -4.1 kcal/mol was obtained when IspH was docked with HMBPP, whereas it was found to be -3.3 kcal/mol when docked with alendronate. Moreover, the binding affinity of IDI with IPP was found to be -4.2 kcal/mol, as opposed to -3.4 kcal/mol when docked with alendronate. The results obtained in the terms of binding energy and interacting sites indicate that alendronate does not inhibit the activity of these enzymes. Thus, a comparative evaluation of docking poses and scores of CrtE, IspS, IspH and IDI enzymes with their respective substrates and alendronate clearly demonstrates that alendronate significantly inhibits the binding of IPP to CrtE enzyme whereas it does not affect the binding of DMAPP, HMBPP and IPP with IspS, IspH and IDI enzyme, respectively.

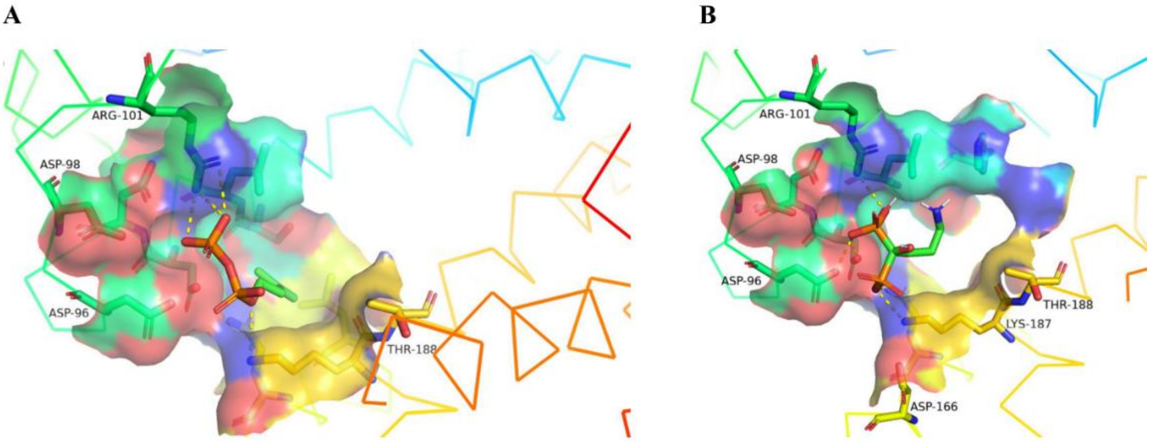


Figure 4.2 Docked pose of isopentenyl diphosphate (IPP) and alendronate in the cavity of SeCrtE. The surface line represented stereo views of docked poses of (A) IPP and (B) Alendronate with SeCrtE. The hydrogen bonds have been represented as the dashed yellow stick, and the interacting residues are labeled, thus demonstrating higher binding in the case of alendronate as compared to IPP. SeCrtE – CrtE of *Synechococcus elongatus* PCC 7942.

Table 4.1 Types of intermolecular interactions between isopentenyl diphosphate (IPP)-SeCrtE and alendronate-SeCrtE as obtained from docking simulations.

Residue (SeCrtE)	Types of interaction (IPP)	Residue (SeCrtE)	Types of interaction (alendronate)
Ser86	Hydrogen bond	Ser86	Hydrogen bond
Leu87	Hydrophobic	Leu87	Hydrophobic
Asp90	Hydrophobic	Asp90	Hydrophobic
Asp91	Hydrogen bond	Asp91	Hydrogen bond
Asp96	Hydrogen bond	Asp96	Hydrogen bond
Asp98	Hydrogen bond	Asp98	Hydrogen bond
Arg101	Salt bridge	Arg101	Salt bridge
Lys187	Salt bridge	Leu159	Hydrophobic
		Gln163	Hydrophobic
		Lys187	Salt bridge

The molecular interactions between SeCrtE residues and IPP exhibited a binding energy of -3.2 kcal/mol, while the interactions between SeCrtE and alendronate demonstrated a binding energy of -4.7 kcal/mol.

4.3.2 Isoprene production by recombinant *S. elongatus* UTEX 2973 strains and effect of alendronate on production

All the production studies were performed in the closed serum bottles. Culture bottles were incubated in inverted position to avoid the evaporation of produced isoprene, as it is highly volatile. Isoprene concentration was measured in the gaseous phase of bottle headspace every 24 h after inducer and/or inhibitor addition. Previous studies showed that isoprene and oxygen accumulation in the closed vessel inhibits cell growth and isoprene productivity (Gao et al., 2016; Rana et al., 2022). Therefore, headspace gases of culture bottles were vented at every 24 h to remove the accumulated isoprene and oxygen during production phase. The isoprene level in recombinant strain UTEX *IspS* could not be quantified in presence of IPTG, since the isoprene concentration was detected below LOQ of the GC system (Figure. 4.3A,B).

Downstream to the MEP pathway, enzyme CrtE catalyzes the sequential condensation of 5-carbon allylic substrate DMAPP with three molecules of IPP forming geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP) in cyanobacteria (Rautela and Kumar, 2022). These intermediates GPP, FPP and GGPP are used for the synthesis of various monoterpenes, sesquiterpenes, diterpenes and triterpenes in cyanobacteria (Satta et al., 2022). In the first condensation reaction, IPP is added with DMAPP to form GPP. Here, we hypothesized that CrtE enzyme inhibition can downregulate or stop the synthesis of GPP, FPP and GGPP in MEP pathway. Thus, accumulated DMAPP will be available for isoprene synthesis by a heterologous expressed IspS enzyme. During the same time, free IPP will also be converted into DMAPP by the IDI enzyme increasing the intracellular DMAPP concentration. For the first time, we reported the strategy of CrtE enzyme inhibition to enhance isoprene productivity by alendronate inhibitor in a cyanobacterial system.

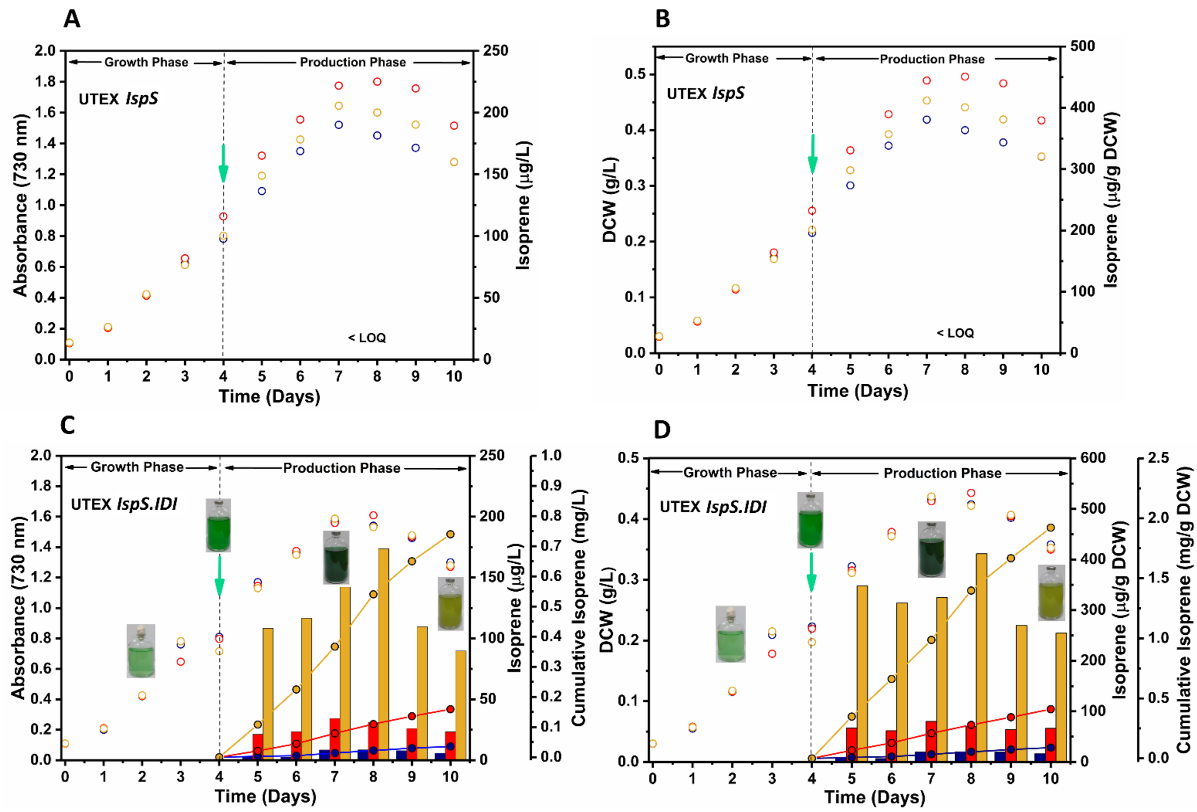


Figure 4.3 Growth and isoprene production (µg/L of culture broth) profiles of recombinant *S. elongatus* UTEX 2973 strains. (A) and (B) *S. elongatus* UTEX IspS. (C) and (D) *S. elongatus* UTEX IspS.IDI. Cotton-plugged cultures were grown (100 µmol photons/m^2/s, 38 °C, ambient CO_2, and 180 rpm) for 4 days. On 4th day, cultures were supplemented with 50 mM $NaHCO_3$, 10 mM HEPES buffer, and inducer (1 mM IPTG) and/or inhibitor (25 µg/mL), and sealed to entrap the produced isoprene in the bottle headspace. Cultures supplemented only with inhibitor are depicted in blue color, cultures supplemented only with IPTG are depicted in red color, and cultures supplemented with IPTG and inhibitor are depicted in orange color. Hollow circles in the scatter plot show the growth profile of recombinant UTEX 2973 strains, the bar diagrams show the isoprene production, and the line plots show the cumulative isoprene production. The green arrow denotes the addition of an inducer and/or inhibitor. All data are expressed as the average values of triplicate experiments with <3% standard deviation. Limit of detection (LOD), 0.5 µg isoprene/L of gas phase; limit of quantification (LOQ), 2.0 µg isoprene/L of the gas phase.

Isoprene production could not be quantified in presence of alendronate and IPTG by *S. elongatus* UTEX *IspS* culture because the level of isoprene was below the LOQ. Whereas, maximum isoprene level was achieved in this study using *S. elongatus* UTEX *IspS.IDI* strain in presence of alendronate and IPTG. The maximum production was observed 173.62 µg/L of culture equivalent to 411.51 µg/g DCW biomass in a single day (Figure. 4.3C,D).

In similar conditions, a cumulative isoprene was achieved 0.74 mg/L culture equivalent to 1.92 mg/g DCW in 6 days and productivity was found to be 5.14 µg/L/h equivalent to 13.34 µg/g DCW/h (Figure. 4.3C,D). When the strain *S. elongatus* UTEX *IspS.IDI* was cultured only in presence of 25 µg/mL inhibitor without IPTG, a maximum isoprene 8.50 µg/L equivalent to 20.04 µg/g DCW and a cumulative isoprene 0.04 mg/L was achieved in 6 days with an average production rate of 0.25 µg/L/h equivalent to 0.63 µg/g DCW/h (Figure. 4.3C,D). Isoprene production in cultures without IPTG induction by *S. elongatus* UTEX *IspS.IDI* strain supports the leaky nature of the P_{trc} promoter.

Studies reporting the isoprene production from CO_2 using recombinant cyanobacteria in closed cultivation and open continuous culture system have been previously performed. Pioneering work reported 50 µg/g DCW isoprene production per day by integrating codon-optimized *IspS* gene under P_{psbA2} promoter in *Synechocystis sp.* PCC 6803 from the *Pueraria montana* plant (Lindberg et al., 2010). This study was followed by several researchers utilizing different engineering strategies and culture conditions to enhance isoprene yield. In our study using alendronate inhibitor in *S. elongatus* UTEX *IspS.IDI* strain harboring *IspS* from *Pueraria montana* and *IDI* from *E coli* DH5α, we surpassed isoprene production levels in closed system from previously reported isoprene production studies. We found a cumulative isoprene yield 0.41 mg/g DCW when induced only with IPTG. The yield was enhanced to 1.92 mg/g DCW when the culture was supplemented with alendronate inhibitor, a 4.7-fold increase in isoprene yield was achieved. Since closed system cultivation of recombinant strain accumulates the isoprene and oxygen in the headspace, causing a negative impact on cell growth and isoprene production. Isoprene production level could be further enhanced by culturing in an open system by continuous bubbling with air or CO_2 as reported in previous studies (Gao et al., 2016; Rana et al., 2022).

4.3.3 Effect of alendronate dose on growth rate and isoprene production

Alendronate inhibits the binding of IPP with CrtE, which catalyzes the condensation of DMAPP with IPP to form geranyl diphosphate. Results of in-silico study showed the binding affinity of CrtE of engineered *S. elongatus* for alendronate and IPP. The results showed the first-time application of alendronate (bisphosphonates) in cyanobacterial CrtE inhibition for improved hemiterpene synthesis. This study also concluded that alendronate binds to CrtE more strongly than IPP and carbon pool fluxed towards DMAPP, leading to enhanced isoprene synthesis in engineered *S. elongatus*. The effect of alendronate doses has been determined on the growth and isoprene production. Different concentrations of alendronate (1-35 µg/mL) were supplemented in a series of cultures. Isoprene assay and growth studies were performed to see the effect of alendronate. The results indicated that the 4 days cumulative isoprene yield enhances on increasing the concentration of alendronate in culture from 1 to 20 µg/mL. The maximum isoprene production of 1.21 µg/gDCW was observed in 4 days in cultures supplied with 20 µg/mL alendronate, which is 5.76-fold higher in comparison to without alendronate added sample (Figure. 4.4). Further increasing the concentration of alendronate, the negative effect was observed on growth rate and cumulative isoprene yield. Supplementation of culture media with alendronate up to 25 µg/mL shows that the non-significant variation in the growth rate of the cyanobacterial culture. However, if alendronate concentration was increased further, the growth rate of *S. elongatus* UTEX 2973-*IspS.IDI* culture was decreased along with a significant reduction in cumulative isoprene production (Fig. 4.4). Tukey's test ($\alpha = 5\%$) was applied to compare the significant differences between mean values of cumulative isoprene production which revealed statistically significant differences among various mean values. Thus, adding alendronate at optimized doses to the engineered cyanobacterial cultures could significantly increase the isoprene production without considerably affecting the growth. Therefore, 20 µg/mL optimized dose of alendronate was used for further process parameters optimization experiments. This study is the first to use alendronate in an optimized dose to improve the production of hemiterpene, isoprene in an engineered cyanobacterial system.

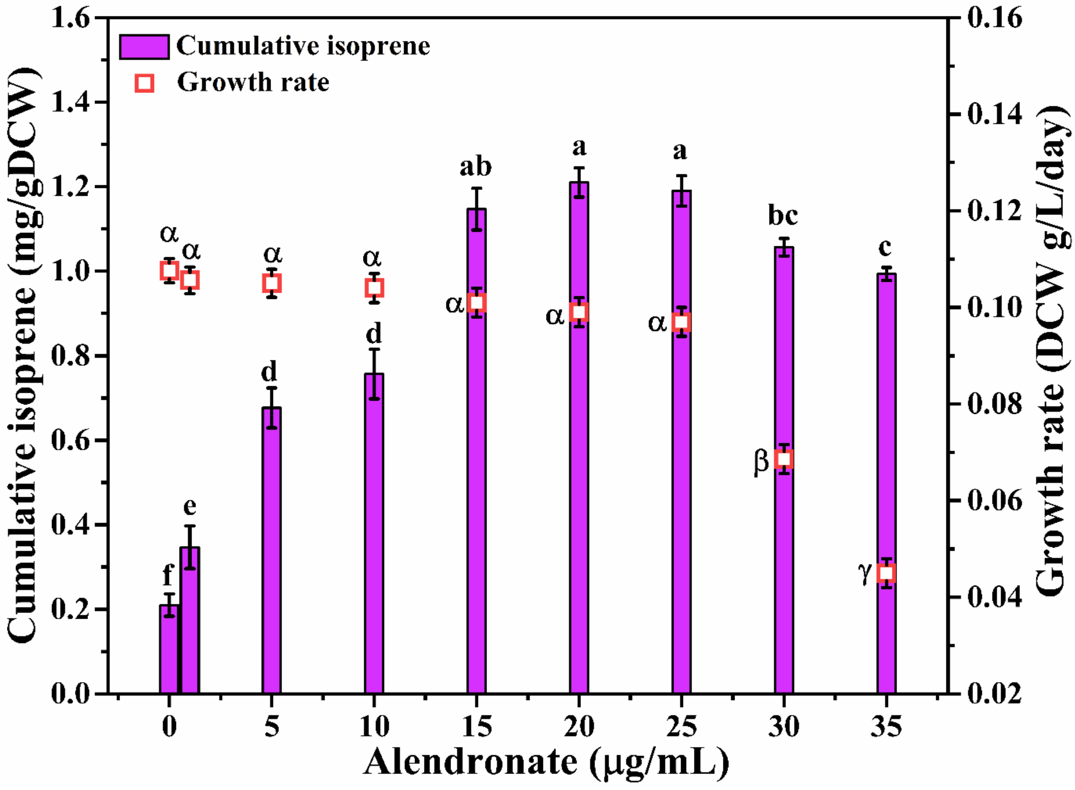


Figure 4.4 Effect of alendronate concentration for enhanced isoprene production in engineered *S. elongatus* UTEX 2973-IspS.IDI. The bar chart represents the cumulative isoprene production in 4 days at different concentrations of alendronate whereas scatter plot represents the effect of alendronate doses on growth rate of *S. elongatus* UTEX 2973-IspS.IDI. All experiments were performed in triplicates in serum bottles sealed with septa (PTFE/silicon) and incubated at 38 °C temperature, 100 µmol photons/m^2/s continuous white light and 180 rpm agitation. The values are presented as mean ± SD. Tukey's test was performed at 5% significance level for comparison of the means ($p < 0.05$). Tukey's test confirms significant differences between the groups. Different Greek letters (α, β, γ) indicate significant differences within the same experimental group, while different English letters (a, b, c, d, e, f, ab, bc) represent significant differences in another experimental group. Groups that share the same are not significantly different from each other. Groups that are assigned different letters are significantly different from each other. Group "ab": This group is not significantly different from groups labelled with either "a" or "b". Similarly, "bc": there is no significant difference between the "bc" group and either "b" or "c".

4.3.4 Optimization of production parameters to maximize isoprene production

4.3.4.1 Response surface methodology

RSM-based BBD approach was utilized to maximize the isoprene production in recombinant *S. elongatus* UTEX 2973-*IspS.IDI* strain, by optimizing three process variables defined as, light intensity, $NaHCO_3$ and growth temperature. The BBD offers to maximize the desired response by considering a wide range of input variable values (Kanaga et al., 2016). The range of input variables was selected based on the data obtained from previous studies (Bentley et al., 2014; Rana et al., 2022; Yu et al., 2015). The statistical analysis of the tested parameters and response were conducted in 15 sets of experiments in triplicates for a 4-day experimental run. The experimental results showed a considerable variation in isoprene productivity (4.17 to 25.44 µg/L/h) which indicates the importance of the optimization of process variables (Table 4.2). The relationship between process variables and response were determined by multiple regression analysis (Table 4.3). The data were analyzed for fitness with a second-order polynomial equation for the isoprene productivity (response factor). The quadratic polynomial equation was established for the isoprene productivity in coded terms as presented below in Equation (4.4).

$$Y_{isoprene\ productivity} = 24.49 - 2.51\,A + 5.90\,B + 0.01\,C - 7.54\,A^2 - 5.19\,B^2 - 9.35\,C^2 - 2.32\,AB + 0.54\,AC + 1.24\,BC \qquad (4.4)$$

Where, Y is response factor (isoprene productivity), A is Light intensity, B is $NaHCO_3$, and C is growth temperature.

The equation coefficient, student's *t*-value and corresponding probability *P*-values are shown in Table 4.3. Multiple regression analysis suggests that the *P*-values of all linear and quadratic relationships between process variables and isoprene productivity are statistically significant ($P < 0.05$) except for temperature. The effect of interactions of light intensity

Table 4.2 Statistical Box-Behnken design matrix in uncoded and coded (in parenthesis) values with experimental isoprene productivity.

Run	Light (µmol photon/m^2/s) *A*	$NaHCO_3$ (mM) *B*	Temperature (°C) *C*	Isoprene Productivity (µg/L/h)	Isoprene Cumulative Production (mg/L)	Isoprene Cumulative Yield (mg/gDCW)
1	100 (-1)	20 (-1)	41 (0)	5.4 ± 0.43	0.5 ± 0.04	1.5 ± 0.08
2	300 (1)	20 (-1)	41 (0)	4.6 ± 0.13	0.4 ± 0.01	1.5 ± 0.13
3	100 (-1)	100 (1)	41 (0)	23.6 ± 0.28	2.3 ± 0.03	3.3 ± 0.17
4	300 (1)	100 (1)	41 (0)	13.5 ± 0.63	1.3 ± 0.06	1.9 ± 0.28
5	100 (-1)	60 (0)	38 (-1)	11.1 ± 0.72	1.1 ± 0.07	2.6 ± 0.16
6	300 (1)	60 (0)	38 (-1)	5.5 ± 0.21	0.5 ± 0.02	1.4 ± 0.05
7	100 (-1)	60 (0)	44 (1)	8.7 ± 0.39	0.8 ± 0.04	3.2 ± 0.13
8	300 (1)	60 (0)	44 (1)	5.1 ± 0.84	0.5 ± 0.08	1.8 ± 0.31
9	200 (0)	20 (-1)	38 (-1)	5.4 ± 0.39	0.5 ± 0.04	1.8 ± 0.07
10	200 (0)	100 (1)	38 (-1)	13.0 ± 0.94	1.3 ± 0.09	1.9 ± 0.13
11	200 (0)	20 (-1)	44 (1)	4.3 ± 0.29	0.4 ± 0.03	1.5 ± 0.07
12	200 (0)	100 (1)	44 (1)	16.9 ± 0.62	1.6 ± 0.06	3.9 ± 0.08
13	200 (0)	60 (0)	41 (0)	24.3 ± 0.81	2.3 ± 0.08	5.2 ± 0.28
14	200 (0)	60 (0)	41 (0)	25.2 ± 0.22	2.4 ± 0.02	5.2 ± 0.14
15	200 (0)	60 (0)	41 (0)	24.0 ± 0.74	2.3 ± 0.07	5.1 ± 0.13

Experiments were performed in triplicates and represented with mean value and standard deviation (mean ± SD). All responses (productivity/production/yield) were calculated based on the 4 days of isoprene production experimental run after addition of 1mM IPTG and 20 µg/mL alendronate. Coded values (0, 1, -1) of process variables have been represented in parenthesis.

with sodium bicarbonate, light intensity with temperature and sodium bicarbonate with temperature were highly significant ($P < 0.05$). The correlation coefficients (R^2) value of 98.45% shows that the model for the measured response was attributed to the tested process variables and presented the goodness of fit for the developed model.

Table 4.3 Determination of model coefficient by multiple regression analysis of isoprene productivity.

Term	Coefficient	Standard Error	*t*-value	*P*-value
Constant	24.49	0.37	66.15	0.001
A	-2.52	0.23	-11.10	0.001
B	5.90	0.23	26.04	0.001
C	0.01	0.23	0.04	0.965
A^2	-7.54	0.33	-22.60	0.001
B^2	-5.19	0.33	-15.55	0.001
C^2	-9.35	0.33	-28.00	0.001
AB	-2.32	0.32	-7.25	0.001
AC	0.54	0.32	1.68	0.103
BC	1.24	0.32	3.87	0.001

A – Light, B – $NaHCO_3$, C – temperature, isoprene productivity model: $R^2 = 98.45\%$.

The ANOVA results were summarized after analyzing response variable as per BBD experiments (Table 4.4). The null hypothesis assumes that the response variable does not depend on the factors, and any observed differences are merely due to random variation or chance. The ANOVA was used to test statistical significance effects of the factors as well as interactions between the factors upon the response variable. The p-values (< 0.0001) obtained from the ANOVA indicate that they are statistically highly significant, it suggests that the null hypothesis is unlikely, and confirms the correlation between factors and response variables. The derived quadratic model for isoprene productivity is highly significant, with an F-value of 246.7 coupled with a lowest P-value (<0.0001). The linear, quadratic and interactions of all model terms were significant (P-value < 0.05) for isoprene productivity (see supplementary materials). This analysis suggested that all process variables for the created model contribute positively to maximize isoprene productivity. The three-dimensional surface diagrams also confirm the positive interactions between process variables, light intensity, $NaHCO_3$ and growth temperature in the X-Y axis against response, isoprene productivity on Z- axis while keeping remaining variable at center level for each plot (Figure 4.5).

Table 4.4 Analysis of variance for isoprene productivity.

Source	df	SS	MS	*F*-value	*P*-value
Model	9	2739.5	304.4	246.7	0.001
Linear	3	988.6	329.5	267.1	0.001
Square	3	1664.0	554.7	449.5	0.001
Interaction	3	86.8	28.9	23.4	0.001
Residual error	35	43.2	1.2		
Lack-of-Fit	3	31.3	10.4	28.2	0.001
Pure Error	32	11.86	0.4		
Total	44	2782.6			

df – degree of freedom, SS – sum of square, MS – mean square.

The parameters obtained from RSM optimization using equation 4 were found to be 172.72 μmol photons/m^2/s light, 85.45 mM $NaHCO_3$, and 41.09 °C temperature. The predicted isoprene productivity was observed to be 26.69 μg/L/h (Figure 4.6). The maximization of cumulative isoprene production in engineered *S. elongatus* UTEX2973-*IspS.IDI* at statistically optimized process variables was found to be 5.22 ± 0.14 mg/gDCW (2.63 ± 0.04 mg/L) with productivity of 54.4 μg/gDCW/h (27.40 ± 0.44 μg/L/h) with 20 μg/mL alendronate in 4 days. An overall 24.85-fold improvement in isoprene production was achieved compared to isoprene production at unoptimized process conditions without alendronate.

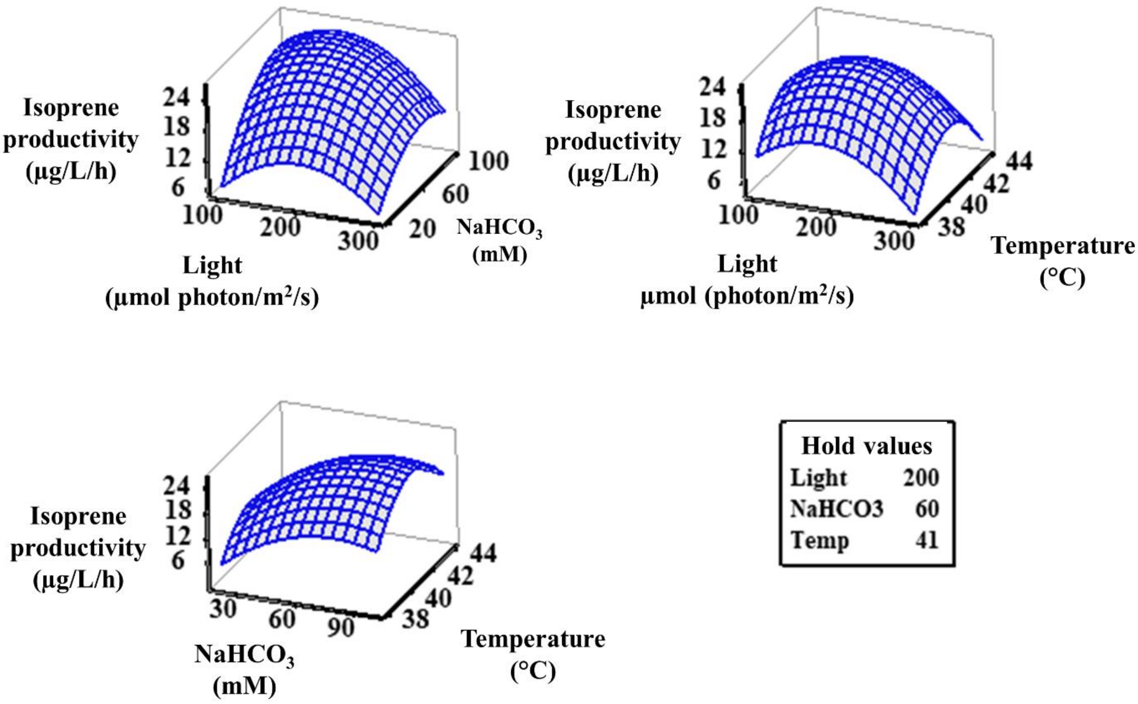


Figure 4.5 Three-dimensional response surface plot for isoprene productivity (µg/L/h) with (a) Light (µmol photon/m^2/s) vs $NaHCO_3$ (mM). (b) Light (µmol photon/m^2/s) vs temperature (°C). (c) $NaHCO_3$ (mM) vs temperature (°C).

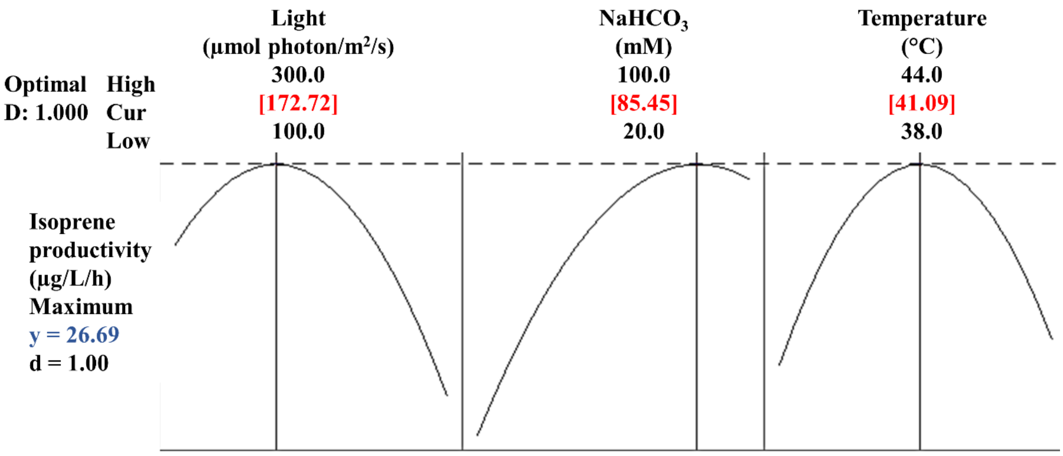


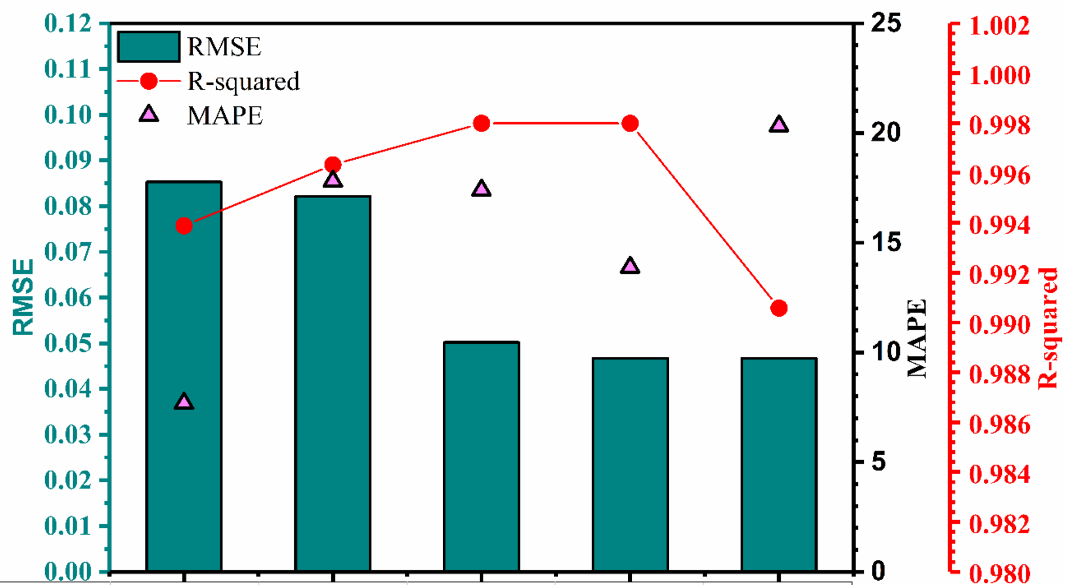
Figure 4.6 The maximum response of isoprene productivity at optimized input factors by statistical BBD tool.

4.3.4.2 ANN and hybrid ANN-GA model development

The maximization of the production of various value-added products and biomass in cyanobacteria using RSM and machine learning approaches by changing the micronutrients, culture media components, and incubation conditions has been extensively investigated (del Rio-Chanona et al., 2016; Saini et al., 2021). In the current study, BBD experimental datasets, including input factors and response, were employed for training, testing and validation of the ANN model. The ANN model was developed to maximize isoprene production by optimizing input factors such as light intensity, concentrations of $NaHCO_3$ and growth temperature. The ANN model functions as a driving engine in machine learning method and predicts maximum isoprene productivity by optimizing input factors. Several FFBP ANN models by varying the size of hidden layers were constructed and tested for their accuracy. The ANN architecture consists of three input nodes representing light intensity, temperature, and sodium bicarbonate concentration. These inputs are processed through two hidden layers, each containing 6 neurons, which apply activation functions to learn non-linear patterns. The network culminates in a single output node representing isoprene productivity. The ANN is trained using backpropagation to minimize error and optimize the relationship between input variables and the output. This architecture allows for predictive modeling of isoprene productivity based on the given environmental factors. The model with an input layer of three neurons, two hidden layers with six neurons each, and a single-neuron output layer (3,6,6,1) was chosen for its superior accuracy, demonstrated by a high R-squared value and low RMSE (Table 4.5). To check the fitness of model, a k-fold cross validation test was performed. Overfitting occurs when a model shows significantly better performance on the training set compared to the validation set. However, in this case, the average R^2 value on the validation set (0.99511) is remarkably close to the average R^2 value on the training set (0.99686). The minor difference between these values indicates that the model is generalizing well to unseen data. Furthermore, upon examining the individual R^2 values for both the validation and training sets across all folds, it is evident that they are consistently close to each other. This observation reinforces the notion that the model is not overfitting. The consistent R^2, RMSE and mean absolute percentage error (MAPE) values across folds demonstrate that the model is performing consistently well on different subsets of the data which is supported by previously reported study (Vu et al., 2022) (Figure 4.7).

Table 4.5 Different Artificial neural network models and their performance.

Model	Layer architecture	Hidden layers	R- squared (Overall)	Performance (MSE)
1	3,3,3,1	2	0.9880	0.0242
2	3,3,3,2,1	3	0.9856	0.0236
3	3,4,4,1	2	0.9617	0.0498
4	3,5,5,1	2	0.9933	0.0157
5	3,6,6,1	2	0.9951	0.0081



Fold (K)	1	2	3	4	5
R- squared	0.99388	0.99633	0.99799	0.99799	0.99058
RMSE	0.0853	0.0822	0.05023	0.04675	0.04675
MAPE	7.68342	17.8039	17.406	13.8804	20.3276

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.

Figure 4.8(A) shows the regression plot derived from the ANN model using the tangent sigmoid activation function. The current ANN model showed an overall R-value of 0.99759 (R^2 value = 0.9951) which signifies that the constructed ANN model is 99.51% accurate. Other R^2-values, such as those for training, testing, and validation showed 99.68%, 99.10% and 99.53% accuracy, respectively. The best validation performance was 0.0081 at epoch 75 with lowest MSE Figure 4.8(B). The iterations end at 81 epochs while achieving minimum MSE, as evidenced by the validation checks and gradient Figure 4.8(C). When ANN and BBD models were compared using SAS Studio, it was observed that ANN model performs better with a lower AIC value (1.9875) than the BBD model (AIC value: 2.2057).

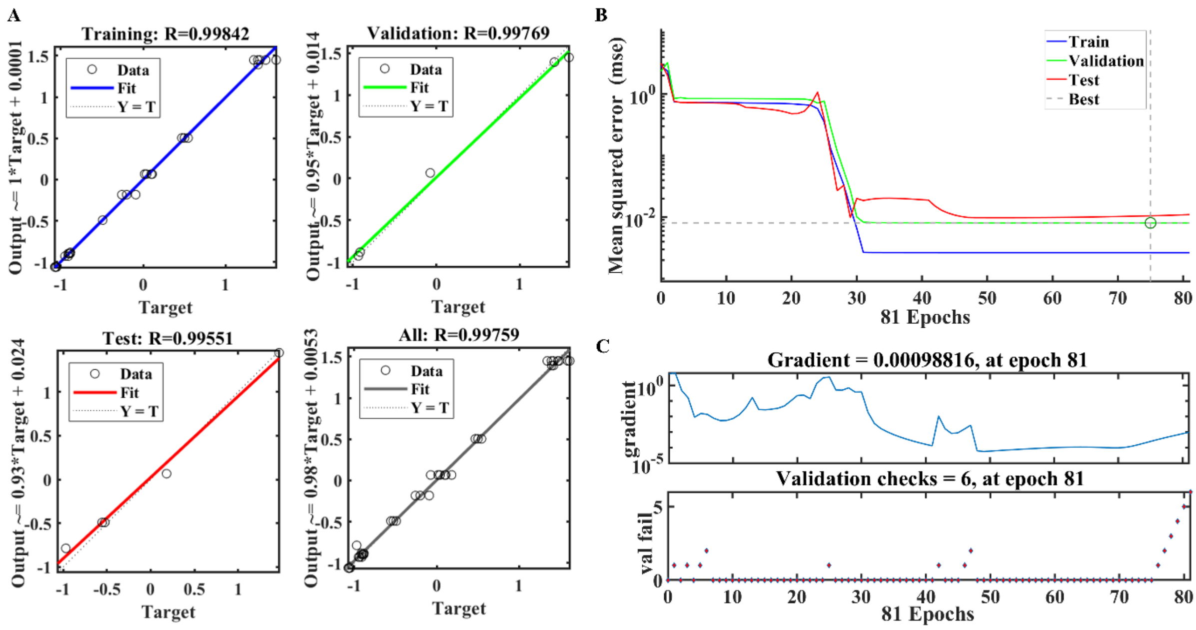


Figure 4.8 ANN regression plots. (A) Training validation and test model regression plots with regression coefficient values (B) Validation performance plot. (C) The validation of ANN model by gradient and validation checks plots.

To maximize isoprene productivity in recombinant *S. elongatus* UTEX-*IspS.IDI* strain, a GA function was combined with ANN resulting in a hybrid model termed ANN-GA. The trained ANN model was used to evaluate the fitness of GA solutions, completing the architecture of a hybrid machine learning model to maximize isoprene productivity. The ANN-GA algorithm was executed in MATLAB through multiple cycles to find the best combination of light, $NaHCO_3$, and growth temperature to maximize isoprene productivity. Using a creation function, the genetic algorithm populates the input factors as a population within a defined range. In this population, the chromosome size represents the combination of multiple input factors with in the specified range to be optimized. The population size and the chromosome size are essential parameters in a genetic algorithm that impact its performance and ability to find optimal solutions. The random population for the ANNGA-based optimization technique is formed by setting the population size to 200. Each chromosome size is utilized as an input for an ANN model established through the LM and FFBP approach, with the objective of achieving optimal regression performance and minimizing the MSE. Additionally, a random population was employed to generate offspring through uniform mutation and two-point crossover techniques. Other GA factors, such as mutation rate, crossover fraction and elite count were kept as 0.01, 0.8, and 2, respectively. The termination function dictates the number of generations required for the proposed method to reach convergence. The ANN-GA model produced the best-optimized results by considering all the above characteristics.

The maximum isoprene productivity of 30.6 µg/L/h was predicted with ANN-GA model at optimized process factors: light intensity – 159.40 µmol photon/m^2/s, $NaHCO_3$ – 97.5 mM, growth temperature – 42 °C. The predicted values were experimentally validated at given optimized process factors. An improved isoprene production of 6.20 mg/gDCW (2.98 ± 0.06 mg/L) with productivity of 64.6 µg/gDCW/h (31.0 ± 0.58 µg/L/h) was observed in the presence of 20 µg/mL alendronate in engineered *S. elongatus* UTEX2973-*IspS.IDI* in 4 days (Figure 4.9). An overall 29.52-fold increase in isoprene production was observed compared to unoptimized process conditions without alendronate.

4.3.5 Model validation of the RSM and ANN-GA

The experiments were performed in triplicates at RSM-based BBD optimization process factors (light intensity –172.72 µmol photons/m^2/s, $NaHCO_3$ – 85.45 mM, and growth temperature – 41.09 °C) to verify the validity of the model. The experimental results showed an improved isoprene productivity of 27.40 ± 0.44 µg/L/h in 4 days which is in a slightly higher than the predicted value of 26.69 µg/L/h by the RSM model (Table 4.6). The verification of the model showed that it is highly accurate, with an accuracy of over 97.4%. This evidence supports the validation of the model under the investigated conditions.

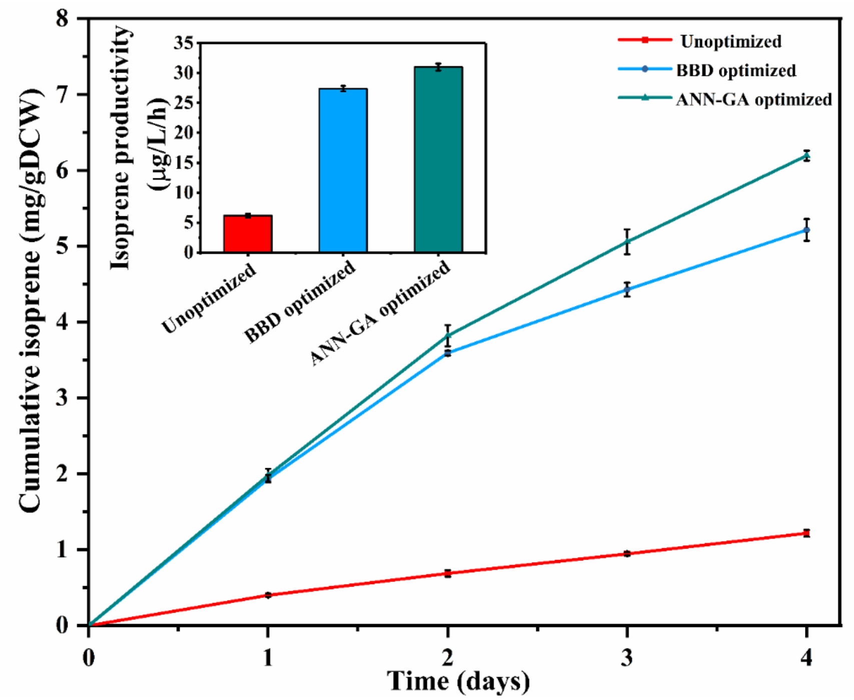


Figure 4.9 Cumulative isoprene production profile at unoptimized, statistical and artificial neural network-genetic algorithm optimized conditions in 4 days in presence of 20 µg/mL alendronate. (Inset) Isoprene productivity after 4 days of isoprene production studies at different process conditions. The experiments were conducted in triplicates and values were represented as mean ± SD.

A cumulative isoprene production of 0.21 mg/g DCW (0.12 mg/L) was achieved in 4 days with a productivity of 2.18 µg/g DCW/h (1.25 µg/L/h) in culture supplemented with IPTG at unoptimized process conditions. Isoprene production was increased 24.85-fold by culturing under RSM-optimized conditions in the presence of alendronate, compared to unoptimized conditions without alendronate (Table 4.6). Model validation was also performed for the optimized process factors to maximum isoprene productivity predicted by the ANN-GA model. The experiments were carried out in triplicates at optimum conditions (light intensity – 159.4 µmol photon/m^2/s, $NaHCO_3$ – 97.5 mM, growth temperature – 42 °C) to confirm the accuracy of the constructed model. An isoprene productivity of 31.0 ± 0.58 µg/L/h was achieved in 4 days of experimentation which is very close to the value predicted by ANN-GA model. The verification of the model's accuracy showed that it is over 98.7%, which is a strong indicator that the model is valid under the investigated conditions. The isoprene production at ANN-GA optimized conditions increased up to 29.52-fold compared to unoptimized conditions. The validation of the model showed that the ANN-GA model was able to predict the response with more accuracy than the RSM-based BBD model. A comparative summary of isoprene production in different process conditions has been presented in Table 4.6.

Table 4.6 Comparison of isoprene production in different cultivations conditions.

Process condition	Process parameters			Isoprene			Improvement (Fold)
	Light (μmol photon/m^2/s)	$NaHCO_3$ (mM)	Temperature (°C)	Productivity (μg/L/h)	Cumulative production (mg/L)	Cumulative yield (mg/gDCW)	
Unoptimized (IPTG- 1mM)	100	50.0	38.0	1.25 ± 0.28	0.12 ± 0.02	0.21 ± 0.03	1.0
Unoptimized (IPTG 1mM + Alendronate 20 μg/mL)	100	50.0	38.0	6.16 ± 0.30	0.59 ± 0.03	1.21 ± 0.05	5.76
Statistical optimized (IPTG 1mM + Alendronate 20 μg/mL)	172.7	85.5	41.1	27.40 ± 0.44	2.63 ± 0.04	5.22 ± 0.14	24.85
ANN-GA optimized (IPTG 1mM + Alendronate 20 μg/mL)	159.4	97.5	42.0	31.00 ± 0.58	2.98 ± 0.06	6.20 ± 0.07	29.52

The majority of isoprene synthases are recognized for their optimal activity at elevated temperatures, reaching up to 50 °C (Yeom et al., 2018). Because *S. elongatus* UTEX 2973 grows well at elevated temperatures, it was chosen as the production host of this study. The optimum growth temperature for *S. elongatus* UTEX 2973 was previously determined as 41 °C (Yu et al., 2015). In current study, isoprene production was studied using *S. elongatus*

UTEX-*IspS.IDI* in temperature range between 38 to 44 °C to see the effect of temperature on isoprene production. It was observed that the maximum level of isoprene was produced at 42 °C temperature at ANN-GA optimized conditions. The results from a previous study using *Synechocystis sp.* PCC 6803 confirm the finding that higher temperatures (with an optimum of 40°C) lead to the highest levels of isoprene production (Rodrigues et al., 2023b). Similarly, the effect of light intensity was tested on isoprene production in *S. elongatus* UTEX-*IspS.IDI.* The *S. elongatus* UTEX 2973 demonstrates robust growth under elevated light intensities, the investigation for isoprene production was carried out within the range of 100 to 300 µmol photons/m^2/s. The results indicated that the optimum light intensity for maximum isoprene production was 159.4 µmol photon/m^2/s. A slight increase in isoprene production has been reported in *Synechocystis sp.* PCC 6803 on increasing the light intensity from 50 to 100 µmol photon/m^2/s which supports the findings of current study (Rodrigues et al., 2023b).

The hybrid ANN-GA model helps to optimize process parameters for maximized isoprene production using a cyanobacterial production system. In current study, ANN-GA model-based process factors optimization has given better isoprene productivity results in comparison to the RSM-based BBD optimization technique which was supported by recently reported studies (Muhammad et al., 2022; Okewale et al., 2017; Sharma et al., 2023). Sharma et al. (2023) evaluated the use of ANN FFBP based model and RSM for predicting the extraction yield of bio-oil from wood sawdust pyrolysis of *Mangifera indica*. The advantage of the ANN-FFBP model (3,9,1) over the RSM-central composite design (CCD) was reported. In another study, ANN-GA and RSM-BBD techniques were used to maximize production of ethanol using sawdust. The achieved ethanol yield was 56.968 wt. % for RSM and 57.387 wt. % for ANN model, which showed higher predictive ability of ANN-GA model than RSM (Okewale et al., 2017). Similarly, another study employed

BBD and ANN to optimize the individual and interactive effects of factors (temperature, time, solvent-to-wet biomass ratio, and hydrochloric acid concentration) for biodiesel production using *Chlorella pyrenoidosa*. The precision of the outcomes reveals that both the ANN and BBD models effectively predict the experimental data for fatty acid methyl ester yields, with high R^2 value of 0.94 and 0.92, respectively (Muhammad et al., 2022). Thus, optimizing process parameters using a hybrid machine learning methodology could be preferred for enhancing biofuels and chemical commodity production in microbial systems.

4.4 Conclusion

Cyanobacteria are possible photosynthetic microbial cell factories for isoprene and other value-added products in biorefinery systems for developing circular bio-economy. This study showed that engineered *S. elongatus* UTEX 2973 harboring *IspS* ans *IDI* gene as well as employing an inhibitor have the potential to produce isoprene in a sustainable way. The application of alendronate significantly improved isoprene production in engineered cyanobacteria. A 24.85-fold improvement in isoprene productivity was achieved when isoprene production was carried out at statistical RSM optimized production process conditions in presence of inducer and optimized concentration of inhibitor. Whereas, the process parameter optimization using an ANN-GA model was found to be superior to statistical RSM-based model for maximized production of isoprene. A 29.52-fold increase in isoprene production was achieved in ANN-GA-optimized process conditions with alendronate compared to unoptimized process conditions without alendronate. This study provides a foundation for further studies on the scalability and economic viability of isoprene and other commodity chemicals production using engineered cyanobacteria.