



Statistical optimization and formulation of microalga cultivation medium for improved omega 3 fatty acid production

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Abstract

Microalgae are considered a rich source of high-value metabolites with an array of nutraceutical and pharmaceutical applications. Different strategies have been developed for cultivating microalgae at large-scale photobioreactors but high cost and low productivity are the major hurdles. Optimizing the composition of media for the cultivation of microalgae to induce biomass production and high-value metabolite accumulation has been considered as an important factor for sustainable product development. In this study, the effect of plant growth regulators together with basal microalgal cultivation medium on biomass, total lipid, and EPA production was studied using the Plackett–Burman model and Response surface methodology. The traditional one-factor-at-a-time optimization approach is laborious, time-consuming, and requires more experiments which makes the process and analysis more difficult. The Designed PB model was found to be significant for biomass (396 mg/L), lipid (254 mg/L), and EPA (5.6%) production with a P value < 0.05 . The major objective of this study is to formulate a medium for EPA production without compromising the growth properties. Further, we had formulated a new media using RSM to achieve the goal and the significant variables selected were NaNO_3 , NaH_2PO_4 , and IAA and was found to be significant with 16.72% EPA production with a biomass production of 893 mg/L with a P value < 0.05 . The formulated medium can be used in large-scale cultivation systems which can enhance biomass production as well as the omega 3 fatty acid production in marine microalgae *Nannochloropsis oceanica*.

Keywords Microalgae · Omega 3 fatty acids · EPA · Plackett–Burman · Response surface methodology · Plant growth regulators

Introduction

Microalgae are unicellular photosynthetic organisms capable of producing high-value energy-rich compounds like carbohydrates, lipids, proteins, polyunsaturated fatty acids, vitamins, etc. Microalgae have been considered as promising bio-factories for nutrition for almost four decades. Omega 3 fatty acids (Eicosapentaenoic acid, EPA, and

Docosahexaenoic acid, DHA) are essential polyunsaturated fatty acids (PUFAs) necessary for the growth and metabolism of humans. They are incorporated in many parts of the body including cell membranes and play a role in anti-inflammatory processes and the viscosity of cell membranes [1, 2]. EPA and DHA are essential for proper fetal development and healthy aging. DHA is a key component of all cell membranes and is found in abundance in the brain and retina. EPA and DHA are also the precursors of several metabolites that are potent lipid mediators, considered by many investigators to be beneficial in the prevention or treatment of several diseases [3, 4]. These fatty acids are considered essential fats because humans cannot synthesize omega 3 fatty acids due to the lack of enzymes necessary for the synthesis of long-chain fatty acids and these fatty acids must obtain through diet. Currently, fish and fish-derived products are the current major dietary sources of omega 3 fatty acids. But fish oil has many disadvantages like overexploitation of fish stocks leads to ecological imbalances, presence of

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toxic contaminants like mercury, etc., and is not suitable for infants and pregnant women. Moreover, microalgae are considered as the primary producers of omega 3 fatty acids in the aquatic ecosystem and fish obtain these essential fatty acids in their bodies through the consumption of microalgae. Hence microbial oil rich in omega 3 and omega 6 fatty acids produced in a controlled environment is one of the recent topics of research because of its advantages over fish oil. *Nannochloropsis oceanica* is a unicellular non-motile marine microalga and has been considered as a potential producer of omega 3 LC PUFAs (Long-chain polyunsaturated fatty acids) specifically EPA. Due to its benefits and advantages over fish oil, many researchers are focused to improve EPA production from microalgae and making the process industrially feasible.

Microalgal metabolite induction can be achieved by changing the growth conditions or through the modification of nutrient composition. Increased accumulation of starch or lipids can be correlated with microalgal survivor mechanisms in response to different stress conditions such as temperature, pH, UV radiation, or nutrient limitation [5]. During stress conditions or nutrient limitation, microalgal growth will shift towards the accumulation of high-energy-rich compounds like lipids and unsaturated fatty acids [6]. Omega 3 fatty acid production can be increased by modification of nutrient conditions and environmental stresses like light intensity, temperature, pH, and UV radiation. The major drawbacks associated with these methods are lower biomass and product yield with high operational costs in large-scale cultivation systems. In this scenario, developing a cost-effective and efficient large-scale cultivation system for the commercial production of EPA and DHA would address a significant global demand.

Plant growth regulators (phytohormones) (PGRs) are small molecules involved in the growth, metabolism, and development of plants and active at very low concentrations [7]. Auxins, cytokinins, gibberellins, ethylene, Brassinosteroids, Jasmonic acid, and salicylic acid are the major

phytohormones produced in higher plants. Algae share similar with terrestrial land plants [8]. Chae et al. 2014, reported growth of some green microalgae can be enhanced by the addition of plant growth regulators [9]. Supplementation of brassinosteroids to *Chlorella vulgaris* showed improved stress tolerance [10]. Methyl Jasmonate and Gibberellic acid also increased the astaxanthin accumulation in *H. pluvialis* [11]. Increased concentration of Kinetin caused a gradual decrease in the growth of *Dunaliella salina* and *H. pluvialis* [12]. The addition of Gibberellic acid has been increased the growth of *Chlorella* [13]. In our previous studies, we had tested the effect of exogenously supplemented PGRs on biomass, lipid, and omega 3 fatty acid production in *Nannochloropsis oceanica* CASA CC201 [4, 14, 15]. Udayan et al., 2018 reported fourfold increase in EPA production in *N.oceanica* CASA CC201 [14]. Treatment with 40 ppm Indole-3-acetic acid also promoted EPA production [14]. Biomass and lipid production was decreased by the addition of Gibberellic acid with a significant increase in lipid accumulation [14]. We had also analyzed the effect of stress-associated PGRs like Methyl jasmonate and Salicylic acid on *N.oceanica* growth and metabolite production. Oleic acid production was found to be increased with the addition of methyl jasmonate and EPA production was found to be increased with treatment with salicylic acid in *N. oceanica* CASA CC201 [4] (Table 1).

The conventional ‘one-factor-approach-at-a-time’ for media optimization has several drawbacks like time consumption, a greater number of experiments which often lead to confusion in understanding the process parameters [16]. For the industrial production of high-value metabolites from microalgae, there are different combinatorial interactions of media components and culture conditions. Optimization of single variables excludes the interaction among the variables which is incapable of reaching the correct optimum point. An experimental setup based on statistical models helps to evaluate the relationship between a group of controllable experimental conditions and specific responses [17].

Table 1 Higher (+) and lower (–) limits of variables selected for the Plackett–Burman experimental screening

Factor	Name	Low level (– 1)	High level (+ 1)
A	NaNO ₃	37.5 g L ⁻¹	112.5 g L ⁻¹
B	NaH ₂ PO ₄ ·2H ₂ O	2.5 g L ⁻¹	7.5 g L ⁻¹
C	NaEDTA	2.18 g L ⁻¹	6.54 g L ⁻¹
D	FeCl ₃	1.58 g L ⁻¹	4.73 g L ⁻¹
E	CuSO ₄ ·5H ₂ O + ZnSO ₄	0.5 + 1.1 g 100 mL ⁻¹	1.5 + 3.3 g 100 mL ⁻¹
F	CoCl ₂ + MnCl ₂	0.5 + 0.9 g 100 mL ⁻¹	1.5 + 2.7 g 100 mL ⁻¹
G	NaMoO ₄	0.315 g 100 mL ⁻¹	0.945 g 100 mL ⁻¹
H	IAA	10 ppm	30 ppm
I	GA	10 ppm	30 ppm
J	Kinetin	5 ppm	10 ppm
K	Citric acid	5 ppm	10 ppm

Statistical tools like the Plackett–Burman design were used for the screening of parameters to detect large main effects. The selected parameters were further optimized using Response Surface Method (RSM). Plackett–Burman design is a well-established and commonly used statistical tool to optimize the media parameters and to screen which media component gives the maximum effects. RSM is an effective method for the screening of key factors from the multiple factors for the optimization of culture conditions. Studies using RSM helps to avoid the errors that occurred during single parameter optimization [18]. The application of RSM has been successfully utilized in the chemical industry, engineering, and biological studies. But only a few reports are available on the application of RSM for the optimization of biomass and lipid accumulation in microalgae. The main objective of this study is to use Plackett–Burman and RSM for developing an optimized medium using PGRs along with D-Walnes media components to increase the growth, lipid, and EPA production from marine microalgae *N. oceanica* CASA CC201.

Materials and methods

Selection of plant growth regulators and stock solution preparation

Three major plant growth regulators (PGRs) used in this study were Indole-3-acetic acid (IAA), Kinetin, and Gibberellic acid (GA), purchased from Sigma-Aldrich. Each PGR was dissolved in the specific solvent and diluted with autoclaved distilled water (IAA in ethanol, Kinetin in 1 N NaOH, and GA in ethanol), and sterilized using 0.22 μm syringe filters. Kinetin and GA were added at a concentration of 10 ppm to 30 ppm and IAA was 5 ppm to 10 ppm.

Plackett–Burman experimental design for screening of variables

Plackett–Burman design was used for screening the effect of major plant growth regulators like Kinetin, Gibberellic acid (GA), and Indole-3-Acetic acid (IAA). The parameters selected from the D-Walnes medium are macronutrients like Sodium nitrate (NaNO_3), Sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$), Ethylene diamine tetraacetic acid (EDTA, disodium salt), Ferric chloride (FeCl_3), and micronutrients like Copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), Zinc sulfate (ZnSO_4), Cobalt chloride ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$), Manganese chloride (MnCl_2) and Sodium molybdate (NaMoO_4).

Citric acid is also selected for the study. Citric acid is a major intermediate in the Krebs cycle, by which organisms degrade organic fuel molecules in the presence of oxygen to harvest the energy for growth and division. Citric acid also

plays an important role in lipid production and metabolism. Each variable was set at higher (+) and lower values to identify their significant role in specific responses.

An experimental model of twelve runs was designed for the eleven factors by Design-Expert Software Version 12 (Stat Ease Inc. Minneapolis, MN, USA) based on the range of variables given in Table 2. The experiments were carried out in 1000 mL Erlenmeyer flasks containing 400 mL D-Walnes medium and incubated in a growth chamber under the illumination of 40.5 $\mu\text{mol photons m}^{-2}\text{S}^{-1}$ light intensity and 14:10 light: dark photoperiod. The temperature was maintained at 25 ± 2 °C. 10% inoculums containing $3 \times 10^6 \text{ mL}^{-1}$ of cells in the exponential growth phase were added to the culture medium and incubated for 21 days. Three responses were measured in terms of the dry weight of biomass (mg/L) Total lipid content (mg/L), and EPA content (%). The results obtained were analyzed using ANOVA and the significant parameters with $P < 0.05$ were used for further studies.

Response surface methodology

Response surface methodology (RSM) will help to study the expression for a variable based on the response values obtained with specific combinations and concentrations of variables in the experimental model. Central Composite Design (CCD) was conducted to evaluate the main effects of the parameters studied and their interactions with each other (Table 3). To evaluate the influence of significant factors, three independent variables were selected based on the Pareto analysis of Plackett–Burman's experimental design. The selected variables are NaNO_3 , NaH_2PO_4 , and IAA. The ranges of factors were determined based on the path of the steepest ascent in the Pareto chart. A total of twenty experiments were conducted using Design Expert Software Version 12 (Supplementary Table 1).

The experiments were conducted in 500 mL Erlenmeyer flasks containing 200 mL medium which is prepared according to the design. 10% inoculums containing 3×10^6 cells/mL in the log phase were added to the culture medium aseptically and incubated for 21 days. The responses were measured in terms of biomass, total lipid, and EPA content. 3D plots were created using RSM to study the interactions among different variables and to optimize the medium components suitable for the responses. The optimized results were further confirmed using point prediction.

Statistical analysis of the data

The data was processed and analyzed using the statistical software, Design-Expert version 12 (Stat-Ease, Inc., Minneapolis, USA) to estimate the coefficient of regression and to plot the model graphs. ANOVA was done to study the

Table 2 Range of factors and responses in Plackett–Burman experimental design

Std	Run	A	B	C	D	E	F	G	H	J	K	L	Response 1 Bio-mass (mg L ⁻¹)	Response 2 Lipid (mg L ⁻¹)	Response 3 EPA (%)
7	1	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	327	222.75	2
8	2	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	359.5	229.7	1.8
12	3	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	329.5	210	0.74
6	4	-1.00	-1.00	-1.00	1.00	1.00	1.00	1.00	-1.00	1.00	1.00	1.00	241	214.75	2.82
3	5	1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	352	250.25	5.55
1	6	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	382.5	225	2.95
11	7	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	359.25	233.25	4.32
10	8	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	346.5	250.75	2
4	9	-1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	352.5	254.25	2.66
2	10	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	1.00	1.00	396.5	238	0.55
9	11	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	344.25	225.25	2.96
5	12	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	282	203.7	1.78

significance of variables in the fitted equations and to estimate the goodness of fit in each case.

Results

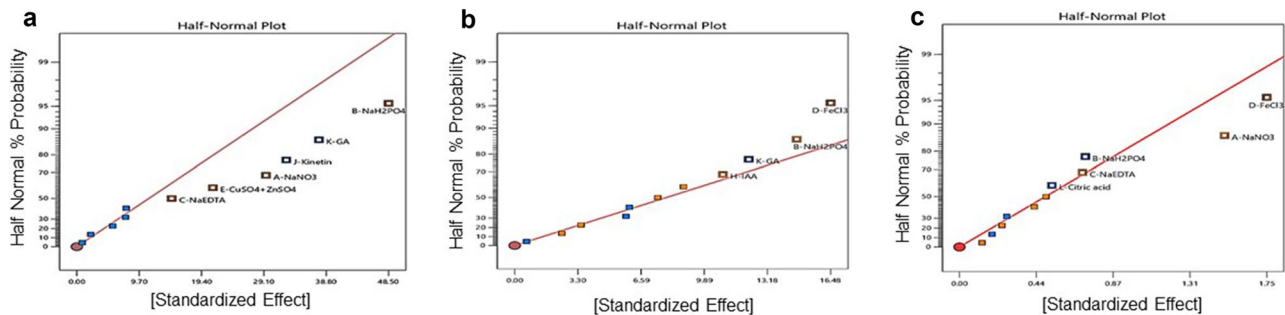
Screening of variables using Plackett Burman design for optimization of biomass, lipid, and EPA production

Plackett–Burman statistical experiment was conducted to screen out positive parameters influencing biomass, total lipid, and EPA production. This will serve as a guide in developing an effective microalgal cultivation medium for enhanced lipid and EPA production without compromising growth and biomass production. PGRs like Kinetin, GA, and IAA were used for the optimization of biomass, lipid, and EPA production together with media components (NaNO₃), NaH₂PO₄·2H₂O, EDTA, FeCl₃, CuSO₄·5H₂O + ZnSO₄, CoCl₂·6H₂O + MnCl₂NaMoO₄, and Citric acid. Results showed that the highest biomass production of 396 mg/L was observed in run number 8, the highest lipid production of 254 mg/L was observed in run number 9 and the highest EPA production of 5.6% was observed in run number 11 (Supplementary Table 2). Optimization of nutrient sources for biomass production based on Plackett–Burman experimental design. In the case of biomass production, out of the 11 parameters tested, 6 parameters were significantly contributed to increasing the growth. Figure 1 represents the half-normal plot of the standardized effects of significant nutrients showing the magnitude and direction of their significant effects.

Figure 1a. reveals that NaH₂PO₄ has the highest influence on biomass production by the microalgae *N. oceanica* CASA CC201 because of its effect positioned the furthest to the right of the response line. Other factors that contribute to the significant effects on growth and biomass productions are GA, Kinetin, NaNO₃, CuSO₄·5H₂O + ZnSO₄, and Na-EDTA. FeCl₃ exerts the lowest effects on biomass production. The Analysis of variance (ANOVA) was done to find out the significant variables on biomass production (Supplementary Table 3). The results confirm that NaH₂PO₄ is the most significant factor in biomass production (*P* value 0.003, *P* < 0.005) by its very large *F* value. Figure S1 shows the one-factor analysis graph of the significant variables. Kinetin and Citric acid give the highest biomass production at lower concentrations, while higher concentrations decrease biomass production. The linear regression coefficient of determination gives an adjusted *R*² of 94.84% which indicates that the model equation given below in coded units is significant and can explain 94.84% of the variability in the responses. The equation for biomass production also reveals that NaH₂PO₄ has the largest coefficient that is indicated by a

Table 3 Actual factor levels of CCD of the Response Surface optimization showing biomass, lipid and EPA production by *N. oceanica* CASA CC201

Std	Run	A:NaNO ₃ (g L ⁻¹)	B:NaH ₂ PO ₄ (g L ⁻¹)	C:IAA (ppm)	Response 1 Bio-mass (mg L ⁻¹)	Response 2 Lipid (mg L ⁻¹)	Response 3 EPA (%)
6	1	220	2.5	70	393	103	13.38
14	2	170	5.0	80	305	106	14.54
12	3	170	9.2	55	662	243	14.76
7	4	120	7.5	70	348	107	13.39
13	5	170	5.0	29	427	172	13.64
17	6	170	5.0	55	319	66.5	13.56
4	7	220	7.5	40	398	101	9.14
1	8	120	2.5	40	893	320	16.83
10	9	254	5.0	55	421	106	11.41
11	10	170	0.79	55	338	56.5	14.53
9	11	85	5.0	55	398	52	9.10
2	12	220	2.5	40	440	58	15.80
18	13	170	5.0	55	501	200	7.86
5	14	120	2.5	70	477	51	9.65
20	15	170	5.0	55	303	125	13.35
15	16	170	5.0	55	427	175	11.80
19	17	170	5.0	55	574	225	11.14
8	18	220	7.5	70	492	111	15.64
16	19	170	5.0	55	419	120	13.58
3	20	120	7.5	40	484	27	9.41

**Fig. 1** Half normal plot representing significant nutrient factors for (a) biomass production; (b) lipid production; (c) EPA production from *N. oceanica* CASA CC201

positive sign proving once again that it has a strong enhancement on biomass production (Supplementary Table 4).

Equation for biomass production is given below:

$$\begin{aligned} \text{Biomass} = & 339.375 + 14.7083 * \text{NaNO}_3 + 24.25 \\ & * \text{NaH}_2\text{PO}_4 + 7.375 * \text{Na} - \text{EDTA} \\ & + 10.5833 * \text{CuSO}_4 + \text{ZnSO}_4 + \\ & - 16.2917 * \text{Kinetin} + - 18.8333 * \text{GA} \end{aligned}$$

Optimization of nutrient sources for lipid production based on Plackett–Burman experimental design

In contrast to the results obtained in biomass production, FeCl₃ placed right side of the response line has the major effects on total lipid production by *N. oceanica* CASA

CC201 (Fig. 1b). The other factors with significant effects on lipid production by the microalgae are NaH₂PO₄, GA and IAA. Here we can see that the added PGRs also contribute to significant lipid production along with the micronutrients in the algal growth medium. The results of ANOVA of the model, which also prove that FeCl₃ is the most significant variable on lipid production with a *P* value of 0.0220 and has the highest *F* value. The model is found to be significant with a *P* value of 0.0205. Figure S2 shows the one-factor analysis graph of the significant variables for total lipid production. Here we can see the lipid production increases as the concentration of NaH₂PO₄, FeCl₃, and IAA increases. In contrast, a lower concentration of GA favors higher lipid production.

The equation for lipid production is

$$\text{Total lipid} = 229.804 + 7.35417 * \text{NaH}_2\text{PO}_4 + 8.2375 * \text{FeCl}_3 + 5.42917 * \text{IAA} + -6.10417 * \text{GA}.$$

Optimization of significant nutrient factors for EPA production using Plackett–Burman experimental design

A half-normal plot for EPA production (Fig. 1c) also reveals that FeCl_3 contributes to a significant role for omega 3 fatty acid production by *N. oceanica* CASA CC201. The other positive significant parameters are NaNO_3 , NaH_2PO_4 , Na-EDTA, and Citric acid. The ANOVA analysis for EPA production also supports this result. FeCl_3 has the most significant effect on EPA production with a *P* value of 0.0015 (largest *F* value of 30.28) compared to the other factors. The Model *F* value of 13.09 indicates that the designed model is significant. There is only a 0.35% chance that an *F* value this large could occur due to noise. Here A and D are significant variables in the model with a *P* value less than 0.05. Adjusted *R*² of 84.61% indicates that the given model equation is significant for the response data. From Figure S3, it is clear that FeCl_3 , NaNO_3 , and Na-EDTA will enhance the EPA production when its concentration increases. While NaH_2PO_4 and Citric acid facilitate EPA production in lower concentrations applied in the experimental model. The equation for EPA production also proves that FeCl_3 is the most significant parameter for EPA production.

$$\text{EPA} = 2.51083 + 0.7525 * \text{NaNO}_3 + 0.3575 * \text{NaH}_2\text{PO}_4 - + 0.349167 * \text{NaEDTA} + 0.8725 * \text{FeCl}_3 + - 0.2625 * \text{Citric acid}$$

Response surface methodology

The main objective of this study is to develop an optimized medium for the large-scale cultivation of *N. oceanica* CASA CC201 for enhanced EPA production cost-effectively and to find a solution for scale-up problems associated with microalgal cultivation. The major concern associated with industrial microalgal cultivation is lower biomass production. PGRs can be used for modulating the product accumulation without compromising the growth characters. Based on the results obtained from the Plackett–Burman design sodium

nitrate, sodium dihydrogen phosphate, and Indole-3-acetic acid and their suitable concentrations were selected for biomass, lipid and omega 3 fatty acid production from *N. oceanica* CASA CC201. The primary goal of this study is to enrich EPA production in *N. oceanica* using the optimized medium. Based on the results obtained from Plackett–Burman experimental model, we had observed that NaNO_3 and NaH_2PO_4 have a key role in biomass production. It is also clear that as the primary plant growth regulators which are involved in all the metabolic functions in higher plants, IAA contributes to the growth and biomass production of *N. oceanica*. Sustaining or maintaining biomass production in large-scale cultivation is a major challenge. Therefore, it is very important to include a growth-promoting substance in the optimized medium to overcome this challenge. The other two selected parameters will help to increase lipid and EPA production, while IAA enhances the growth as well as metabolite accumulation. The three-factor five-level Central Composite Design (CCD) with twenty experiments were used to detect the optimum concentration of NaNO_3 , NaH_2PO_4 , and IAA and thereby to develop a mathematical correlation between the three important variables and the responses.

The results of experiments conducted using CCD showed that maximum EPA production of 16.72% (Fig. 2) with high biomass (893 mg/L) and high lipid content 320 (mg/L) was obtained in run number 8. The optimum concentration of variables in run number 8 is NaNO_3 -120 g/L,

NaH_2PO_4 -2.5 g/L and IAA-40 ppm (Table 3). These results presented about a 2.98-fold increase in EPA production when compared with the maximum production obtained from the Plackett–Burman model. This reflects the importance and value of the optimization process. ANOVA analysis revealed that the model is significant only for EPA production with Prob. > *F* less than 0.05 even though the biomass and lipid production increased significantly.

The quadratic equation provides the level of EPA production which can be presented in terms of actual factors

$$\begin{aligned} \text{EPA} = & 45.6369 + 0.0343111 * \text{NaNO}_3 + -5.24692 * \text{NaH}_2\text{PO}_4 \\ & + -0.887615 * \text{IAA} + -0.00083 * \text{NaNO}_3 * \text{NaH}_2\text{PO}_4 \\ & + 0.001195 * \text{NaNO}_3 * \text{IAA} + 0.0665667 * \text{NaH}_2\text{PO}_4 * \text{IAA} \\ & + -0.000244653 * (\text{NaNO}_3)^2 + 0.150475 \\ & * (\text{NaH}_2\text{PO}_4)^2 + 0.00330776 * \text{IAA}^2 \end{aligned}$$

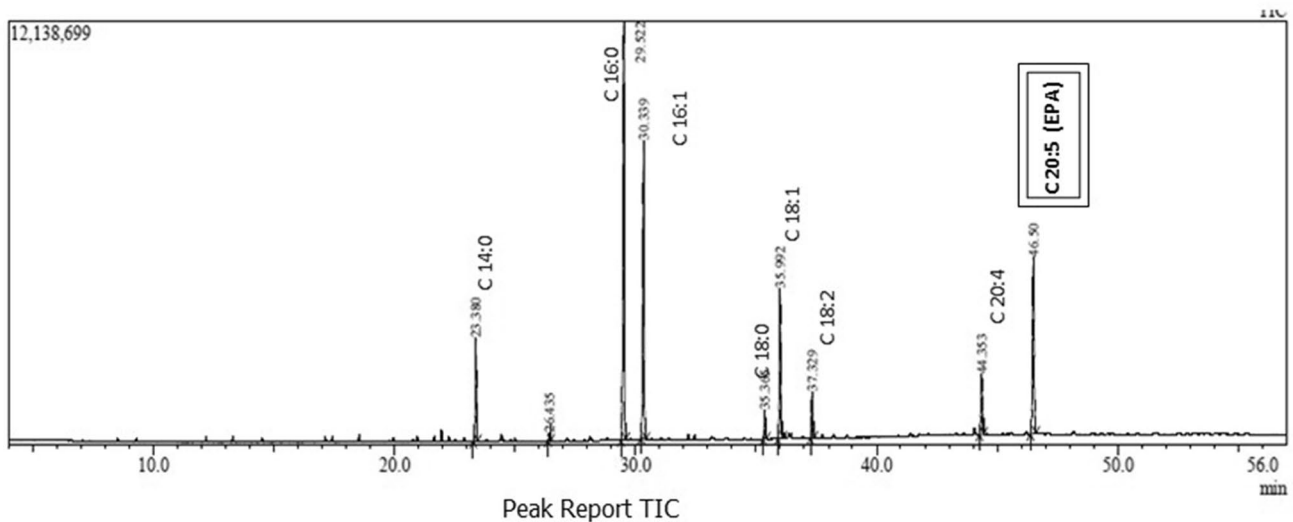


Fig. 2 GC–MS chromatogram showing fatty acid profile for run number 8 of central composite design

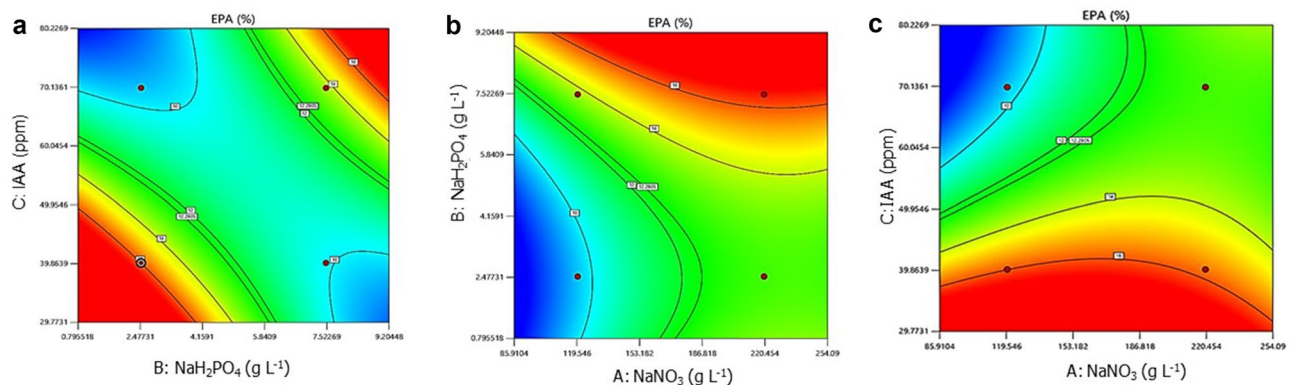


Fig. 3 Contour plots of two-way interactions of independent variables (a) BC; (b) AB; and (c) AC on maximum EPA production by *N. oceanica* CASA CC201

The model was found to be significant for EPA production with a p value of 0.0295. From the ANOVA results, it is evident that BC is the only significant model term. Lack of fit was found to be non-significant which makes the model more significant and accurate. The Model F value of 3.59 implies the model is significant. There is only a 2.95% chance that an F value this large could occur due to noise. P values less than 0.0500 indicate model terms are significant. In this case, BC is a significant model term. The Lack of Fit F value of 0.18 implies the Lack of Fit is not significant relative to the pure error. There is a 95.68% chance that a Lack of Fit F value this large could occur due to noise. Non-significant lack of fit is good which makes the model fit.

Three contour surfaces were plotted based on the model equation, which identifies the interactions among the variables and determines the optimum concentration of each factor for maximum response (Fig. 3). Each figure represents the effect of two variables while keeping the concentration of other variables at zero level. Figure 3 a, shows the interaction effect of NaH_2PO_4 and IAA (BC) concentrations on EPA production by *N. oceanica* CASA CC201. The figure shows that very high and very low concentrations of IAA and NaH_2PO_4 facilitate EPA production and give the maximum production. An interactive effect of NaNO_3 and NaH_2PO_4 can be seen in Fig. 3b. The response is expected to increase with increased NaNO_3 and NaH_2PO_4 concentrations provided when IAA concentrations are lower. The

interaction plot of IAA and NaNO_3 (AC) can be seen in Fig. 3c. Here also we can observe higher EPA production with higher concentrations of AC when NaH_2PO_4 concentration is at its maximum.

Discussion

Nannochloropsis oceanica, non-motile marine microalgae belonging to the family of Eustigmataceae, has been considered a prolific producer of an essential omega-3 fatty acid Eicosapentaenoic acid (EPA, C20:5) [19]. Among the two omega 3 fatty acids, EPA has a significant role in memory and cognitive processes [20]. EPA has shown many important health effects throughout the body for maintaining a proper immune system and balancing different body functions [21]. Currently, due to the covid-19 pandemic situation, the food industry mainly focused on the products that boost the immune system which has gained more attention recently. Fish and fish-derived oils are the major sources of omega 3 fatty acids which have many disadvantages, which has created more attention towards the development of sustainable and green sources of omega 3 fatty acids. *Nannochloropsis sp.* is widely used as feed-in mariculture and domestic hen farming. Lemahieu et al. showed that *Nannochloropsis sp.* supplemented in the diet of hens lead to the enrichment of omega 3 fatty acids in egg yolk, thereby increasing the nutritional value of egg [22]. Reports are also available on reduced cholesterol levels in male rats supplemented with *Nannochloropsis sp.* [23]. This result shows the importance of *Nannochloropsis sp.* as a potential source of Omega 3 fatty acids in human nutrition.

Different methods like modification of cultivation conditions and genetic engineering techniques have been adopted to increase the omega 3 fatty acid production from microalgae [24]. In this context, different strategies have been developed such as nutrient stress (nitrogen, phosphorous, etc.), irradiance, temperature, and salinity, application of growth-promoting substances, heavy metal exposure, and other chemicals [25, 26]. Induction of nitrogen stress in culture medium has led to increased lipid production in *N. oculata* [27]. Moderated use of UV radiation for 7 days leads to increased production of fatty acids in *Nannochloropsis sp.* [28]. Another study in *Haematococcus pluvialis* also proves that nitrogen starvation and Fe treatment leads to increased production of omega 3 fatty acids [29].

Plant Growth Regulators (PGRs) are attracted nowadays attracted more research attention due to their growth-promoting mechanisms [30]. PGRs are active even at very low concentrations which could increase the growth of microalgae at low concentrations which could eventually reduce the cultivation cost. Microalgae being primitive eukaryotic plants share a close evolutionary relationship with higher

plants, hence PGRs are also expected to play similar roles in microalgae. Different studies showed the effect of PGRs on the growth and biomass production of different microalgal species [4, 14, 15, 31]. PGRs also have an important role in regulating physiological processes such as germination, rooting, growth, flowering, fruit ripening, foliage, and senescence of crop plants. Among the different PGRs, auxin is an active ingredient in the rooting mixtures, which helps the vegetative propagation of plants. Moreover, auxins are applied exogenously to unpollinated flowers to enhance fruit development in horticultural plants [32]. Different classes of Gibberellins are widely used to induce fruit set in horticultural crops and exogenous applications of GA inhibitors prevent fruit growth [33]. Ethylene is the most commonly used agent in fruit ripening on a commercial scale. Hence PGRs are widely used in the agricultural sector to improve the quality of crop plants and for food applications. Therefore, PGRs can be used as a green and sustainable agent to increase the nutraceutical value of different microalgal species together with improved biomass production.

Optimizing the composition of media for the cultivation of microalgae to induce biomass production and high-value metabolite accumulation has been considered as an important factor for sustainable product development. Omega 3 fatty acids are widely used in nutraceutical and pharmaceutical industries and increasing biomass productivity is also considered as a crucial bottleneck in economic concern. In the present study, major PGRs like IAA, Kinetin, and GA were used to develop an optimized media together with selected components from the D-Walne's medium used for the cultivation of *N. oceanica* CASA CC201. The overall production costs of omega 3 fatty acids from microalgae are very expensive and significant research is required to reduce the production cost [34]. The traditional microalgal cultivation methods to increase lipid and high-value metabolite production include the introduction of different stress conditions like nutrient stress, high temperature, high salinity, the addition of heavy metals, antioxidants, etc. But the application of these methods often leads to decreased growth, which will eventually decrease the product accumulation and increase the cost of production.

We had screened the effect of PGRs together with D-Walnes media components on biomass, total lipid, and EPA production using the Plackett–Burman model. The traditional one-factor-at-a-time optimization approach is laborious, time-consuming, and requires more experiments which makes the process and analysis more difficult. The statistical experimental design provides an effective method to develop a suitable media for product development. Plackett–Burman design helps to identify the significant components for the formulation of a specific medium. RSM is a convenient and effective tool for the identification of significant variables from multiple variables and can solve the defects that

occurred in single factor optimization. Till now various studies have been conducted to enhance biomass and high-value metabolite production in microalgae by optimizing different media components [35]. But there is no results are available for statistical optimization of PGRs together with D-Walsh's media components for the cost-effective production of omega 3 fatty acids from *N. oceanica* CASA CC201. In our study, the designed Plackett–Burman model was found to be significant for biomass, lipid, and EPA production with a *p* value less than 0.05 (Supplementary Table 3).

Significant factors that contribute to higher biomass production are NaH_2PO_4 , GA, Kinetin, NaNO_3 , $\text{CuSO}_4 + \text{ZnSO}_4$, and Na-EDTA [1]. Sheek et al., in 2016 reported that phosphate was one of the most significant factors affecting the growth of *N. oculata* [36]. For industrial production of any high-value metabolite, it is very important to obtain sufficient biomass production. Nitrogen, phosphorous, and sulfur are very essential nutrients for the growth of microalgal cells. Yang et al., (2018) reported that nitrogen deficiency and phosphorous deficiency will inhibit microalgal growth and cell division [37]. Micronutrients like Cu and Zn which are required in small amounts have a strong impact on microalgal growth because they mediate and control many enzymatic activities in the cell [38]. Our results indicate that NaH_2PO_4 is the major significant factor that contributes to biomass production [1]. The role of phosphorous as an essential nutrient for nitrate absorption, photosynthetic respiration, signal transduction, and energy transfer is already well established [38]. Nowadays one of the most common methods to increase lipid production from microalgae for biodiesel applications are nutrient limitation or nutrient stress and the major nutrient that comes into play is nitrogen. But it is reported that even though nitrogen limitation has increased intracellular lipid accumulation but showed 80% decrease in biomass production and also caused failure in final lipid product improvement [39]. It has also been reported that phosphorous addition is an effective strategy to enhance microalgal biomass production under nitrogen limitation [40]. So phosphorous can be considered as a major nutrient for microalgal growth which is the reason behind enhanced biomass production in Plackett–Burman experimental design. Moreover, we had also observed that treatment with GA and Kinetin has a significant role in biomass production. From our study, it is evident that treatment with lower concentrations of Kinetin significantly enhances the biomass production of *N. oceanica* CASA CC201. Park et al. also reported that treatment with optimum concentrations of GA and Kinetin has increased biomass production in the selected microalgal species [41]. Similar results were observed in *Aurantiochytrium* Sp. YLH70 where biomass production was increased by 14.4% under gibberellin induction [42].

In the case of lipid production, the designed model was found to be significant by using ANOVA analysis

(Supplementary Table 4) and the significant factors observed are FeCl_3 , NaH_2PO_4 , GA, and IAA. FeCl_3 was found to be the major significant factor that contributes to lipid production. Liu et al., (2008) reported that high iron concentration increased lipid accumulation in the marine strain of *Chlorella vulgaris* with decreased biomass production [43]. We had also observed similar effects in our study. In the case of EPA production also FeCl_3 contributes to a major significant factor followed by NaNO_3 . Vu et al. in 2016 reported that the percentage of EPA production has decreased 50% during nitrogen limitation [44]. Similar results were observed in *Rhodomonas baltica* where nitrogen limitation decreases DHA and EPA production in the marine microalgae. This result explains the significance of nitrogen as a major player in EPA production.

The major objective of this study is to formulate a medium for EPA production without compromising the growth properties. For achieving the goal, we had formulated a new media using RSM and the significant variables selected are NaNO_3 , NaH_2PO_4 , and IAA. The model was found to be significant with 16.72% EPA production. Several reports are available on the effect of nitrogen and phosphate on biomass and lipid production. The reason behind the selection of IAA is IAA is the most common naturally occurring phytohormones coming under auxin class, which regulates various aspects of plant growth and development [45]. Moreover, PGRs can be activated at very low concentrations which can be effectively used in large-scale cultivation. Here we had selected IAA because of growth and lipid promoting activity. It is very necessary to maintain higher biomass productivity in large-scale cultivation and IAA can be successfully used to achieve this goal. In our study, we had observed the designed CCD is significant for EPA production with high biomass and lipid production. In our previous study, we had observed that treatment with IAA significantly enhances biomass production with 60.9% lipid and 10.76% EPA production [15]. Salama et al. also demonstrated 1.9-fold increases in PUFA content when treated with IAA [46]. Treatment with IAA resulted in a 39% increase in total lipid accumulation by *C. vulgaris* [47]. Hence all these results are in agreement with our study. Currently, most of the PUFAs especially EPA and DHA used as food supplements and medicines are derived from fish oil [1]. These oils are accumulated in fish through the consumption of omega 3 fatty acid-rich microalgae in the aquatic ecosystem. Fish oil is not safe as it contains heavy metals and the extraction involves costly downstream processing steps. The present study proves a green and sustainable process for enhancing omega 3 fatty acid specifically EPA production in marine microalgae *Nannochloropsis oceanica*, which can be used for edible applications without any toxic effects or contamination problems.

Conclusion

Achieving adequate nutrition is very essential in the current global scenario for which it is necessary to develop efficient and cost-effective sources of essential nutrients. Omega 3 fatty acids are essential dietary fats that have an important role in proper brain development and cognitive functions. *Nannochloropsis oceanica* is considered as the potential feedstock for the production of EPA for food and feed applications. The major challenge associated with the production of high-value metabolites from microalgae is lower biomass productivity which in turn decreases the product accumulation. In the present study, the newly formulated medium using RSM together with IAA will help to overcome the challenges associated with traditional microalgal cultivation methods. Most of these methods are not feasible in large-scale applications and will increase the cost of production also. Thus, the newly formulated medium using sodium nitrate, sodium dihydrogen phosphate and IAA will give high EPA production with improved growth in large scale cultivation systems.

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Author contributions MA planned and designed the experiments. AU conducted the experiments; collected the primary data and analyzed. NS helped in statistical analysis. AU prepared the manuscript draft; corrected and communicated by MA. All authors read and approved the final manuscript for submission.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The article does not contain any studies with human participants or animals performed by any of the authors.

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