

Production of biodiesel from cyanobacteria (*Oscillatoria annae*) by alkali and enzyme mediated transesterification

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This study presents lipids from *Oscillatoria annae* BDU6, a freshwater cyanobacterium, to generate biodiesel. Efficient oil extraction from *O. annae* was achieved by ultrasonication in combination with organic solvents (50.9%) than homogenization (40.4%). Efficient conversion of triglycerides of *O. annae* into biodiesel was obtained by alkali mediated transesterification (86% w/v) when compared with lipase-mediated transesterification (76.5% w/v). RSM and CCRD were employed to optimize the growth medium to improve biomass of *O. annae*, which resulted in 3.2-fold increase in biomass yield (5.3 g⁻¹ dry wt/g of inoculum) compared to initial level (1.6 g⁻¹ dry wt/g of inoculum).

Keywords: Biodiesel, *Oscillatoria annae*, Response surface methodology, Transesterification

Introduction

At today's consumption level, earth's oil and gas reserves has been estimated to deplete in 40 and 64 years, respectively¹. Use of fossil petroleum fuels becoming unsustainable due to over exploitation and being a great source of toxic and green house gases that aggravates global climatic change and becomes the key factor to global warming issue². Transesterification is a chemically catalyzed reaction transforming triglycerides into fatty acid alkyl ester in the presence of an alcohol (methanol or ethanol) and a catalyst (alkali or acid) with glycerol as a byproduct³. Currently, many alternative oil sources from algae are used for biodiesel production than the plant crops as their photosynthetic productivity is greatly higher and are exceedingly rich in oil than other biomass⁴. Microalgae are sunlight-driven miniature biochemical factories that are photosynthetically efficient than terrestrial plants, easily grown in non-arable land, efficient CO₂ fixers and their oil content exceeds 80% of dry weight of biomass⁵. Moreover, average biodiesel production from microalgae is 10-20 folds higher than

the yield obtained from other plant sources⁶. Among microalgae, *Cyanophyceae* (blue green algae), *Chlorophyceae* (green algae), *Chrysophyceae* (golden-brown algae) and *Bacillariophyceae* (diatoms) are extensively utilized for biodiesel production. Cyanobacteria have received considerable attention in recent years as excellent organisms for renewable biofuel production because they are the only known prokaryotes capable of oxygenic photosynthesis, having high-yield oil, and easily grown in non-arable land.

This study presents biodiesel production from *Oscillatoria annae* and optimization of growth medium to improve the biomass yield in order to obtain higher biodiesel yield using response surface methodology (RSM) and central composite rotatable design (CCRD).

Experimental Section

Organism and Growth Conditions

O. annae BDU 6 was obtained from Department of National Facility for Marine Cyanobacteria, Bharathidasan University, Tiruchirappalli, India. Culture was sub cultured into NPK media (NaNO₃, 1.54; and K₂HPO₄·3H₂O, 0.4 g/l; pH 6.8±0.2), which was sterilized at 12°C at 15 lbs for 15 min to that 1.5 g/l of *O. annae* was inoculated. Culture flask was incubated in light

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(1500 lux) at room temperature (RT) for two weeks. Nutritional sources like sugarcane molasses (5%) were also added to increase biomass.

Lipid Extraction and Analysis

Total lipids were extracted with a mixture of methanol-chloroform-water (1:1:0.9, v/v). Chloroform phase was removed and evaporated to dry at RT. Dried extracts were dissolved in a known volume of ethanol-benzene (1:4 v/v) and stored under N_2 at -80°C . *O. annae* (10 g, dry wt basis) was ground with 10 ml chloroform and 20 ml methanol (monophasic system). Solution was rehomogenized with 10 ml chloroform, following which 10 ml of either distilled water or weak salt solution (0.88% NaCl or KCl) was added. Final biphasic system was allowed to separate into two layers and lower (chloroform) phase was collected. Lipid content was determined by gravimetric method.

Extracted lipid was dissolved in acetonitrile (conc., 10 mg/ml) and compounds present in extract were identified by high performance liquid chromatography (HPLC, PerkinElmer 200) equipped with refractive index detector C-18 column (220 mm x 4.6 mm; particle size, 5 μm) was used for separation. Sample injection volume was 20 μl and methanol/water was used as a mobile phase. Retention times of cyanobacterial lipids standard were compared with olive oil.

Alkali mediated Transesterification (AMT)

Amount of catalyst required for AMT was determined as reported⁷. A solution of lye (1 g/l) was made up with distilled water and was used as a reference tester for titration process. Isopropyl alcohol (10 ml) was mixed with sample oil (1 ml). After titration, two drops of phenolphthalein was added. Using a graduated eye dropper, required amount of lye/water solution was added drop by drop into the oil/isopropyl/phenolphthalein solution. In cold weather, oil might congeal and not work so that titration was performed in a hot room. If conditions are right, eventually the solution turns pink (magenta), and stays for 10 s that indicates change of pH (8-9). The process was repeated to obtain concordant values. Every liter of fresh oil needs 3.5 g of lye for the reaction. So for every liter of oil to be transesterified, an additional 3.5 g of lye was added. To prepare sodium methoxide, methanol (20%) was mixed into a solution with calculated amount from the titration of sodium hydroxide (lye) in an exothermic reaction. Extracted oil was preheated in order to remove any water molecules.

Sodium methoxide was added to oil (4:1) and it was kept in shaker for 2-3 h. After shaking, it was allowed for at least 2 h for settle down. Then upper biodiesel layer was separated from lower glycerin layer. Yield (%) was calculated in terms of weight of alkyl esters obtained per unit weight of oil⁸.

Lipase mediated Transesterification (LMT)

For lipase production, microorganisms were isolated from soil and wastewater samples rich in fat and oil. A well grown culture (0.1 ml) was transferred to double agar⁹. Double layer agar media is the same as the enrichment media but supplied with 2% (w/v) agar. Bottom layer has no olive oil while top layer was supplemented with olive oil. Single colony was screened based on the ability to hydrolyze lipids¹⁰. Production media for lipase was as follows: $(\text{NH}_4)_2\text{SO}_4$, 1.3; K_2HPO_4 , 0.9; KH_2PO_4 , 0.6; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; yeast extract, 0.1; and CaCO_3 , 0.5 g/l; and olive oil, 10 ml/l. Selected isolates were inoculated in to the liquid medium and that was incubated at 30°C on rotary shaker with 250 rpm. The supernatant of the culture was assayed for lipase activity¹¹.

For lipase assay, solutions (cell lysate: 100% ice-cold acetone: 100 % TCA) were mixed in a ratio of 1:8:1 and precipitated at -20°C for 1 h. Contents were centrifuged at 11,500 rpm. Supernatant was discarded. Pellet was washed with 1 ml ice-cold acetone, and centrifuged at 11,500 rpm for 15 min at 4°C . Supernatant was discarded and protein pellet was dissolved in appropriate volume of buffer. Sample was vortexed at RT for 1 h at every 10 min intervals and then contents were transferred to eppendorf tube and centrifuged at 14,000 rpm for 10 min at RT. Immediately, supernatant was transferred into a new eppendorf tube and stored at -80°C . Olive oil emulsion was prepared by mixing (1:3) of olive oil (25 ml) with polyvinyl alcohol (75 ml) and was homogenized at 11,000 rpm for 5 min. Standardized 0.02 N sodium hydroxide was used as titrant. 0.1 M of phosphate buffer (pH 7.0) was prepared by mixing 1 M K_2HPO_4 (61.5 ml) and 1 M KH_2PO_4 (38.5 ml). Emulsion (5 ml) and phosphate buffer (4 ml; pH 7.0) was added to 100 ml Erlenmeyer flasks and mixed well. Flasks were preincubated at 37°C for 10 min. After preincubation, enzyme (500 μl) was added and incubated at 37°C for 20 min. Acetone (10 ml) was added to stop the reaction. Release of fatty acid was estimated by titration against 0.02 N NaOH using phenolphthalein as indicator. Appearance of permanent pale pink color was the end

Table 1—Experimental levels of media components (pH, MgSO₄, NaNO₃, molasses conc.) on biomass and lipid production

Variables	-α	-1	0	+1	+α
pH	5	6	7	8	9
MgSO ₄ .7H ₂ O	0.025	0.050	0.075	0.100	0.125
NaNO ₃	1.0	1.25	1.5	1.75	2.0
Molasses	1	2	3	4	5

point¹². One unit of lipase activity is defined as one micromole of free fatty acid liberated per ml of enzyme per minute.

For immobilization of lipase, sodium alginate (2%) solution was prepared by dissolving sodium alginate in boiling water and that was autoclaved at 121°C for 15 min. Both alginate slurry and protein suspension were mixed and stirred for 10 min to get a uniform mixture. Alginate/protein mixture was extruded drop by drop into a cold sterile 0.2 M CaCl₂ solution with sterile syringe and kept for curing at 4°C for 1 h. Gellations of beads were done by addition of fresh CaCl₂ solution for 24 h with gentle agitation. Finally, these beads were washed with distilled water to remove excess calcium ions and unbound proteins. Beads were preserved in 0.9% sodium chloride solution in refrigerator¹³ until used.

For transesterification, extracted oil (3 g) and methanol (1:4) were taken in a conical flask. Immobilized lipase (2 g) was added to this solution and incubated at 40°C with a constant shaking at 250 rpm for 12 h, and then allowed to settle for 12 h. Then content was centrifuged to separate biodiesel layer from glycerin layer. Yield (%) was calculated as weight of alkyl esters obtained per unit weight of oil.

Response Surface Methodology (RSM) - Central Composite Rotatable Design (CCRD)

RSM was applied to optimize media for biomass and lipid production by using CCRD. Experimental design and statistical data analysis were done by using Design Expert (version 8.0.1) on windows platform. CCRD was applied to determine optimum level of variables. Effect of 4 different variables (pH, MgSO₄, NaNO₃, molasses) on lipid level was studied (Table 1) at 5 experimental levels (-α, -1, 0, +1, +α) for α=2ⁿ, where, n is number of variables and 0 corresponds to central point. Lipid content was analyzed by using second order quadratic equation and data were fitted into the equation by multiple regression procedure. Model equation for lipid content is

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{55} X_5^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{15} X_1 X_5 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{25} X_2 X_5 + \beta_{34} X_3 X_4 + \beta_{35} X_3 X_5 + \beta_{45} X_4 X_5 \quad \dots(1)$$

where, Y-predicted response, X₁, X₂....X₅ are levels of the factors and β₁, β₂.... β₅ are linear coefficients, β₁₁, β₂₂....β₅₅ are quadratic coefficients and β₁₂, β₁₃.... β₄₅ are interactive coefficients estimates with β₀ having a role of a scaling constant. Analysis of variance (ANOVA), regression analysis, was done and contour plots were drawn by using Design Expert (version 8.0.1) on windows platform.

Quality of Biodiesel

Quality of biodiesel was checked by visual examination and checking its neutral pH. For wash test, unwashed biodiesel (150 ml) was allowed to settle for 12 h. Glycerin was taken in a glass jar (½ l) or PET bottle and water (150 ml) was added at RT. This mixture was mixed vigorously for 20-30 s. For biodiesel separation from water, contents were allowed to settle for 30 min. For methanol test, in a measuring glass, biodiesel (25 ml) was dissolved in methanol (225 ml), stirred for 2 min, and then left to settle for 30 min. Biodiesel was fully solubilized in methanol, and formed a clear bright phase. For reprocessing test, finished, settled and separated biodiesel (100 ml) was taken and processed again using methanol (20 ml) and NaOH (0.35 g) or the equivalent of KOH. This reaction mixture was left to settle for 30 min and checked for any more glycerin by-product drops out.

Results and Discussion

Biomass Growth and Lipid Extraction

Cyanobacterial biomass (CB) yield was examined by growing *O. annae* in NPK media supplemented with and without molasses. CB yield was exponential up to 4 weeks of incubation, and the medium with molasses (optimum yield on dry wt basis, 3.86 g/l) showed better

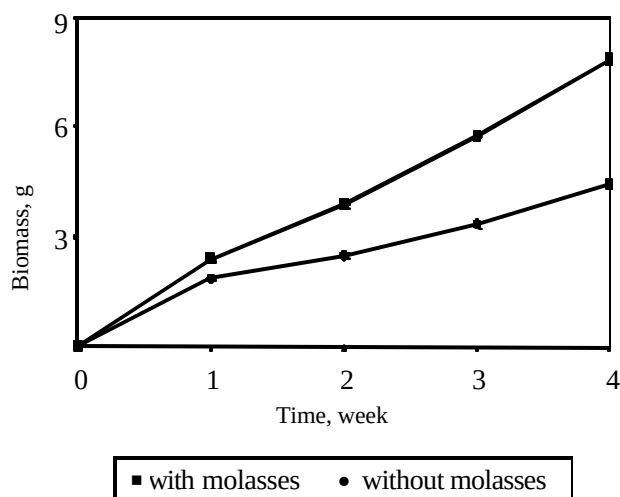


Fig. 1—Comparison of biomass production of *O. annae* with and without molasses in NPK medium

results (Fig. 1) in terms of biomass growth in comparison with the medium with no molasses (optimum yield on dry wt basis, 2.45 g/l). Similarly, influence of molasses in the biomass growth has been shown in microalgae, *Spirulina platensis*¹⁴. Molasses has been used extensively as carbon source for commercial production of several microorganisms. Besides carbohydrates¹⁵, it also contains nitrogenous substances, and water soluble vitamins (thiamin, riboflavin, pyridoxine, folic acid, biotin, pantothenic acid, and trace elements). Thus, induced biomass production by molasses can be used as a low-cost supplement for mass cultivation of cyanobacteria. Among three different methods, followed to extract total lipids from CB (1 g), ultrasonication mediated extraction facilitated higher lipid recovery (50.9%) than by solvent alone (36.2%) and homogenization followed by solvent extraction (40.4%).

Characterization of Lipid by HPLC

Lipid extracted from CB was subjected to HPLC analysis using methanol/water system as mobile phase. Sample volume required for injection was 0.2 ml under standard operating conditions. Olive oil standard had various retention times (1.17, 1.803, 2.067, 2.280, 2.403 min). Similarly, extracted cyanobacterial lipid samples also showed three similar peaks at 1.19, 2.07 and 2.287 min, which clearly indicates the presence of identical fatty acid fractions in standard and cyanobacterial extracts (Fig. 2).

Transesterification Process

Alkali mediated Transesterification (AMT)

Methanol is preferred for transesterification because it is less expensive than ethanol¹⁶. Required quantity of catalyst (lye) was determined by means of titration using isopropyl alcohol and found to be 3.5 g of catalyst for every one liter of fresh oil. Titration value showed 1.2 ml and the volume of oil transesterified was 50 ml. So, $(3.5 \times 0.05 + 1.2 \times 0.05)$ 0.235 g of lye required to transesterify 50 ml of cyanobacterial extract. Conversion of olive oil to biodiesel and *O. annae* lipids to biodiesel was determined. AMT products from standard olive oil and cyanobacterial oil were compared. Maximum biodiesel obtained from 20 ml standard olive oil was 96%, while maximum of 86% biodiesel conversion was obtained with 20 ml of cyanobacterial oil (Table 2).

Lipase mediated Transesterification (LMT)

Enzymatic assay gave lipase activity as 445 U/l. Immobilized lipase was used for transesterification of olive oil and cyanobacterial oil. LMT products from standard olive oil and cyanobacterial oil were compared. From lipase mediated standard olive oil, biodiesel

Table 2—Alkali-mediated and lipase-mediated transesterification of olive oil and cyanobacterial oil

Sl No	Amount of oil ml		Alkali mediated transesterification						Lipase mediated transesterification					
			Sodium methoxide ml	Biodiesel ml		Conversion %		Lipase g	Biodiesel ml		Conversion %		Olive oil	Cyano oil
	Olive oil	Cyano oil		Olive oil	Cyano oil	Olive oil	Cyano oil		Olive oil	Cyano oil	Olive oil	Cyano oil		
1	10	10	2.5	9.3 ± 0.02	8.45 ± 0.033	93	84.5	5	8.10 ± 0.02	7.85 ± 0.039	81	78.5		
2	15	15	4	13.8 ± 0.03	12.8 ± 0.028	92	85.6	8	11.6 ± 0.03	11.05 ± 0.035	77	73.6		
3	20	20	5	18.8 ± 0.04	17.2 ± 0.035	96	86	10	16.1 ± 0.02	15.3 ± 0.034	80.5	76.5		

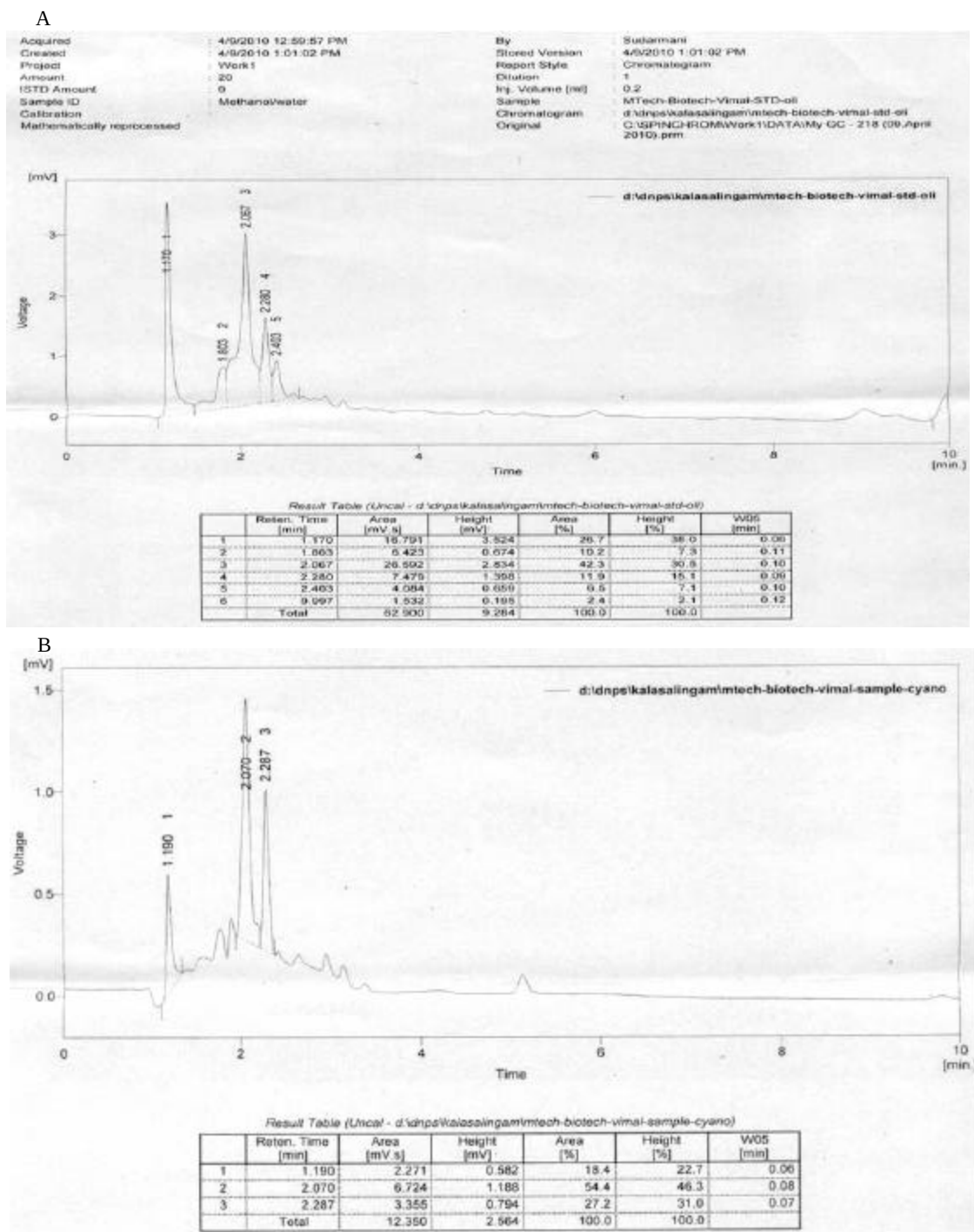


Fig. 2—Quantification of lipids in: A) standard olive oil: and B) cyanobacterial oil

conversion was 80.6% but for cyanobacterial oil, biodiesel conversion was 76.5% (Table 2). Maximum biodiesel was produced in AMT than LMT. Low amount of biodiesel from LMT is because activity of lipase would be lost in the presence of too much methanol and lipase expressed relatively poor stability¹⁶. Moreover, lipases

are not currently feasible because of the relatively high cost of catalyst¹⁷.

RSM – CCRD Design

Using RSM, a significant improvement in biomass can be obtained in a shorter time than the traditional ‘one-

Table 3—Design of experimental runs and results of the central composite design

Run	Std	Factor 1 A:pH	Factor 2 B:MgSO ₄ ·7H ₂ O	Factor 3 C:NaNO ₃	Factor 4 D:Molasses	Response 1 Biomass, g	Response 2 Lipids, g/g
1	10	8.00	0.05	1.25	4.00	3.24	0.61
2	28	7.00	0.08	1.50	3.00	3.75	0.55
3	20	7.00	0.13	1.50	3.00	2.91	0.55
4	8	8.00	0.10	1.75	2.00	2.55	0.55
5	6	8.00	0.05	1.75	2.00	3.02	0.59
6	21	7.00	0.08	1.00	3.00	3.52	0.58
7	30	7.00	0.08	1.50	3.00	3.84	0.70
8	29	7.00	0.08	1.50	3.00	3.80	0.69
9	18	9.00	0.08	1.50	3.00	2.40	0.50
10	1	6.00	0.05	1.25	2.00	2.63	0.56
11	14	8.00	0.05	1.75	4.00	3.25	0.60
12	26	7.00	0.08	1.50	3.00	3.65	0.74
13	23	7.00	0.08	1.50	1.00	2.12	0.42
14	9	6.00	0.05	1.25	4.00	2.85	0.49
15	2	8.00	0.05	1.25	2.00	2.55	0.53
16	5	6.00	0.05	1.75	2.00	2.08	0.54
17	22	7.00	0.08	2.00	3.00	3.98	0.70
18	12	8.00	0.10	1.25	4.00	3.01	0.65
19	7	6.00	0.10	1.75	2.00	2.45	0.55
20	13	6.00	0.05	1.75	4.00	3.22	0.58
21	19	7.00	0.03	1.50	3.00	2.98	0.51
22	25	7.00	0.08	1.50	3.00	3.64	0.64
23	24	7.00	0.08	1.50	5.00	3.80	0.67
24	3	6.00	0.10	1.25	2.00	2.45	0.49
25	27	7.00	0.08	1.50	3.00	4.12	0.68
26	15	6.00	0.10	1.75	4.00	3.09	0.66
27	16	8.00	0.10	1.75	4.00	2.51	0.56
28	11	6.00	0.10	1.25	4.00	2.85	0.49
29	17	5.00	0.08	1.50	3.00	2.01	0.46
30	4	8.00	0.10	1.25	2.00	3.00	0.58

Table 4—ANOVA for response surface reduced quadratic model analysis of variance table [Partial sum of squares - type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.13	8	0.016	6.17	0.0004	significant
A-pH	6.017E-003	1	6.017E-003	2.29	0.1450	
B- MgSO ₄ · 7H ₂ O	5.042E-004	1	5.042E-004	0.19	0.6658	
C-NaNO ₃	9.204E-003	1	9.204E-003	3.50	0.0752	
D-Molasses	0.023	1	0.023	8.92	0.0070	
AC	8.556E-003	1	8.556E-003	3.26	0.0854	
A ²	0.054	1	0.054	20.49	0.0002	
B ²	0.026	1	0.026	10.06	0.0046	
D ²	0.020	1	0.020	7.75	0.0111	
Residual	0.055	21	2.626E-003			
Lack of Fit	0.034	16	2.108E-003	0.49	0.8716	not significant
Pure error	0.021	5	4.284E-003			
Cor Total	0.18	29				

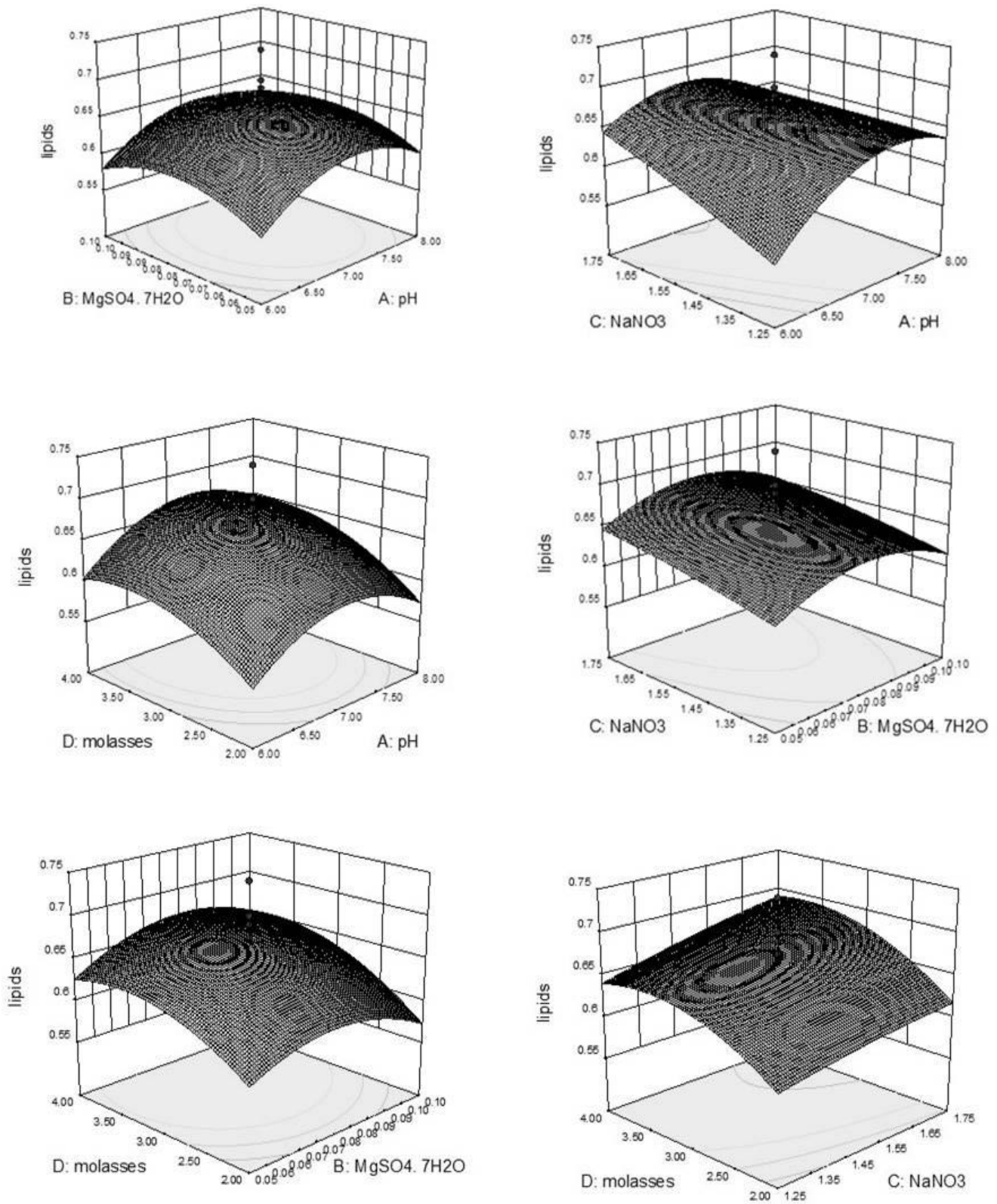


Fig. 3—Response surface for lipid yield from ar:*O. annae* biomass [Surface curves showing effects of variables (pH, NaNO_3 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and molasses) and their interactions in lipid yield from *O. annae* biomass]

factor-at-a-time' technique that requires a considerable amount of work and time¹⁸. Experimental results of biomass and lipid yield by a complete four-factor-two-level factorial experiment design with six replications of central point and eight axial points are shown (Table 3). Responses of CCRD design were fitted in Eq. (1) to get Y (total lipid yield) = $+ 0.66 + 0.016 * A + 4.583E - 003 * B + 0.020 * C + 0.031 * D - 0.023 * A * C - 0.044 * A^2 - 0.031 * B^2 - 0.027 * D^2$, where A is pH, B is $MgSO_4 \cdot 7H_2O$, C is $NaNO_3$ and D is molasses.

Fit of the model was checked by coefficient of determination R^2 (0.7016), indicating that 70.2% of variability in response could be explained by the model. Statistical significance of the model equation was evaluated by F-test for ANOVA. F-value (6.17) implies the model is significant. P -value ($P < 0.0004$) indicated significance of the model. Values of Prob $> F$ (< 0.05) indicated that model terms were significant. In this case, four model terms D , A^2 , B^2 , D^2 were significant (Table 4). Further, coefficient of variation (CV) indicates the degree of precision. Lower CV means a higher reliability of the experiment. Lower value of CV (8.83%) demonstrated the performed experiments were reliable. Lack of Fit F-value (0.49) implies that it was not significant relative to pure error. There was a 87.16% chance that a Lack of Fit F-value could occur due to the noise. Pred R-Squared (0.3373) is not as close to Adj R-Squared (0.5880) as one might normally expect. This may indicate a large block effect or a possible problem with the model and/or data. Things to consider are model reduction, response transformation, outliers, etc. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 8.771 indicates an adequate signal and therefore the model was significant for this process.

Three-dimensional RSM curves (Fig. 3) were plotted on the basis of the model equation, to explain interaction among variables and to determine optimum concentration of each level of each variable for maximum yield of total lipid content from the biomass of *O. annae*. Each figure demonstrates the effect of two factors keeping other factors fixed at zero level. Optimum parameters were found as follows: $NaNO_3$, 1.50 g; $MgSO_4 \cdot 7H_2O$, 0.08 g; molasses, 3 ml; and pH, 7.0. With the optimized medium and cultural conditions, biomass of *O. annae* was enhanced to 5.3 g⁻¹ dry wt/g of inoculum when compared with the biomass obtained with initial medium (1.6 g⁻¹ dry wt/g of inoculum). Similarly, influence of pH, temperature, and urea in the biomass growth of *Porphyridium*

*purpureum*¹⁸ and *Arthrospira platensis*¹⁹ has been optimized using RSM.

Quality Testing

Biodiesel was subjected to wash test and impurities that found in washed liquid was very less and biodiesel phase also separated in < 20 min. Biodiesel was completely dissolved in methanol and left no undissolved materials at the bottom and it give a single phase of solution. Under reprocessing test, by mixing biodiesel with methanol, no any glycerin was produced and solution was found very clear.

Conclusions

Cyanobacteria are an economical choice for biodiesel because of its easy availability and low cost. Production of *O. annae* biomass has improved with the addition of molasses to growth media. Oil extraction from *O. annae* by ultrasonication showed better extraction than homogenization. AMT showed efficient conversion of triglycerides into biodiesel (86%) than LMT (76.5%). Media optimization for maximum production of biodiesel was carried out using RSM and CCRD, method which resulted in 3.2-fold increase in biomass yield compared to initial level. Recovery of total lipids from biomass obtained with the medium before and after optimization was 0.50 g/g of biomass and 0.74 g/g of biomass, respectively. Quality testing of biodiesel also reveals the effectiveness of conversion of methyl esters and purity of fatty acid methyl esters. In this way, *O. annae* would be a novel efficient strain that produces eco-friendly, economically viable, environmentally safe, alternate fuel for this 21st century.

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