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Green renewable energy production from *Myxosarcina sp*: media optimization and assessment of biodiesel fuel properties

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Renewable energy resources like biomass from plant and algae have gained more interest for biodiesel production as an energy source to reduce the consumption of fossil fuels and elevated global warming. In this study, *Myxosarcina* sp., an unicellular cyanobacterium was evaluated for higher biomass and lipid production by the supplementation of sugar industrial waste (SIW), sodium chloride (NaCl) and media optimization with response surface methodology (RSM) for biodiesel production. The outcome of the findings showed that biomass and lipid productivities of 28.5 ± 2.4 (1.2 fold) and 3.4 ± 0.2 (1.3 fold) mg/L/day were observed in the BG-11 media supplemented with SIW than control (24 ± 1 and 2.6 ± 0.1 mg/L/day). However hyper lipid content 20.6 ± 1.8 % (1.5 fold) was achieved by RSM optimized media including NaCl at 0.8 M, SIW at 2 mL/L, sodium nitrate ($NaNO_3$) at 2.5 g/L and magnesium sulphate ($MgSO_4$) at 0.075 g/L than control (13.6 ± 1.4 %). Fatty acid characterization by GC-MS anlysis revealed that Myxosarcina sp. yielded 39 ± 5.3 % of saturated fatty acids (SFA) and 61 ± 5.3 % of mono unsaturated fatty acid (MUFA) and its biodiesel fuel properties evaluated using empirical equations showed that almost all the properties calculated were in concurrence with the national and international biodiesel standards EN 14214 (Europe), ASTM D6751-02 (US) and IS 15607 (India). Thus, Myxosarcina sp. can be utilized as the environmental friendly biodiesel feedstock for high quality biodiesel production in the current scenario for the escalating energy demand.

Introduction

Hydrocarbon fuels from green biomass have received more attention towards diminishing petro fuels for the production of renewable energy for the continuous energy requirement and mitigation of global warming. In recent years, due to increase in the population and industrial development across the world, fuel crisis has become one of the biggest bottleneck to the global economy and human survival. On the other hand, carbon dioxide emissions have increased throughout the globe especially from the top emitting countries like USA, Japan, China and India due to rapid industrialization.[1] In addition, as an outcome of urbanization the quantum of liquid and solid wastes accumulation to the environment has increased immensely.[2] In order to overcome the aforementioned problems, extensive research is being explored for a versatile feedstock for biofuel that could fix the atmospheric CO_2 and utilizes the industrial wastes as a substrate. Also many researchers addressing that fossil fuels satisfies only 85% of today's total global energy demand and their main drawback is that they would be depleted in the near future.[3] Biodiesel production came into practice even before 50 years using animal fat, plant oil and waste cooking oil with soybean biodiesel widely produced and commercialized in United States.[4] However almost all the feedstocks used for biodiesel production has its own downside like vegetable oil feedstock in competing with a human consumption and less availability of animal fat and used cooking oil.[5] In the recent decades, algal oil (oilgae) achieved much interest among entrepreneurs and scientists as a feedstock for biodiesel production by its added advantages like atmospheric nitrogen and carbon fixation, cheap nutrient requirements for growth, waste water utilization, high productivity (per-acre), utilization of non-arable land and value added co-products along with the biofuel.[6] Cyanobacteria have the capability to uptake inorganic nitrogen and phosphorous in the wastewater, and have been identified to be applicable in bioremediation of the wastewater.[7] Furthermore most of the cyanobacteria exhibits nitrogen fixation capability which is an added advantage that

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eukaryotic algae.[8] Significance of utilizing cyanobacteria for biodiesel production over microalgae includes, thin cyanobacterial cell walls with less complexity than microalgae[9] that accomplish the lipid extraction process simpler. Furthermore the photosynthetic ability of cyanabacteria are greater than algae since cyanobacteria can convert up to 10% of solar energy into biomass that augments higher biomass production whereas only 5% conversion occurs in algae.[6] Subsequently large scale cultivation of cyanobacteria for biodiesel production can be performed effectively than microalgae. In contrast to other microalgae, a cyanobacteria (either filamentous or unicellular algae) contains diacylglycerol dispersed throughout thylakoid membrane of the cytoplasm whereas an eukaryotic microalga accumulates triacylglycerol as storage bodies.[10] The major advantage of cyanobacteria is that, can also be cultivated in the photobioreactors (PBRs) either indoor or outdoor level for higher biomass production and it can be of three major types, flat panel PBR, vertical column PBR and tubular PBR.[11] Algae can also be cultivated in open raceway ponds but major hindrances with the processes are contamination, evaporation of the medium, decreased sunlight diffusion, huge cultivation land requirement, expensive biomass recovery and thus appropriately designed outdoor PBRs under controlled conditions are highly preferable for outdoor cultivation of algae.[12,13] Before cultivating the biomass in large scale in either indoor or outdoor the media formulation would play a crucial role. Hence optimizing media for enhanced production of biomass and lipid productivity by response surface methodology (RSM) has greater benefits such as less time consuming, evaluating the combinatorial effects of independent variables on the responses than the conventional methodologies.[14]

Biodiesel synthesis comprises of two essential steps, 1) lipid extraction from the feedstock 2) transesterification of lipids to fatty acid alkyl ester (FAAE).[15] Chloroform and methanol in different ratios is being widely used for lipid extraction whereas other methods like direct transesterification, supercritical fluid extraction using green solvents, microwave and sonication assisted methods can also be employed.[16,17] Extracted lipids can be transesterified into fatty acid methyl esters (FAME) in the presence of alcohol *i.e.* methanol along with catalyst *i.e.* acid or alkali or lipase.[18,4]

Though, biodiesel can be produced from various feedstock, it is mandatory that fuel from any sources should comprise the critical fuel parameters such as saponification value (SV), iodine value (IV), cetane number (CN), degree of unsaturation (DU), long chain saturation factor (LCSF), cold filter plugging point (CFPP), cloud point (CP), pour point (PP), kinematic viscosity (ν), density (ρ) and higher heating value (HHV), which can be calculated using the empirical equations by assessing the fatty acid composition.[19]

Since informations pertaining to lipid enhancement for biodiesel production in cyanobacteria are sparse, this study was designed to improve the biomass and lipid production in *Myxosarcina* sp. using RSM and to evaluate critical biodiesel fuel properties by analyzing the fatty acid profile. To the best of our knowledge, this is the first extensive report stating the media optimization methodology enhanced the lipid production in *Myxosarcina* sp. and it could be used as a biodiesel feedstock for biodiesel production.

Materials and Methods

Batch cultivation of *Myxosarcina* sp.

Myxosarcina sp. used in this study was purchased from National Facility for Marine Cyanobacteria (NFMC), Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. *Myxosarcina* sp. was cultivated and maintained in BG-11 medium.[20] All the media components were purchased from Himedia, Mumbai, India. To achieve higher biomass production, *Myxosarcina* sp. was grown autotrophically in an indoor bench top photobioreactor (BORG, India) of working volume 3 litres. Aeration was provided during day time by sterile air (0.2 µm pore size) using sparger at a flow rate of 5 lpm. Baffles were fixed inside and the impeller was rotated at a speed of 150 rpm to provide equal mixing of media. The pH of the growth medium was maintained (at pH 6.8±0.2) manually by collecting 1ml of culture and measuring pH at regular intervals. Photobioreactor culture vessel was irradiated with 12 white lamps providing 2000 lux at an alternate photoperiod (12:12 h light/dark cycle). Cultures were grown for 35 days at 28°C and then they were used for the further experiments.

Improvement in biomass and lipid production by SIW supplementation and saline stress

SIW (waste water discharged after sugarcane processing) was collected from Dharani Sugars & Chemicals Ltd, Vasudevanallur, Tamil Nadu, India. Different concentrations of SIW (0.625, 1.25, 2.5 ml/L) and NaCl (0.5, 1, 1.5 M) for salinity stress were added to BG-11 media inoculated with *Myxosarcina* sp. (8 mg dry cell weight) to improve the lipid production. Cultures were grown at 25°C under illumination of 1500 lux with photoperiod as described above. The various properties of SIW was characterized and reported in our previous study.[21]

Determination of biomass and lipid production

Myxosarcina sp. cells were harvested during exponential phase and they were dried at 45°C until the constant dry cell weight (DCW) was obtained. Dried cells were preweighed (Shimadzu, Germany) and the DCW was calculated gravimetrically. They were then subjected to lipid extraction according to Folch et al., (1957).[22] Briefly, cells were ground in a mortar and pestle, added with chloroform:methanol (2:1) until the cell debris becomes colorless. Contents were centrifuged at 8500 rpm for 12 min and the lower organic layer was transferred to separating funnel. 1% NaCl was added to the lower organic layer and the contents were shaken intensely to aid phase separation. The lower organic layer containing lipid was collected, evaporated to dryness and were quantified gravimetrically. The difference in the initial and final DCW of the biomass has been employed to estimate the biomass productivity, lipid productivity and lipid content using equations 1 to 3.[23]

Biomass productivity, mg/L/day (BP) = $(A_2-A_1) / (t_2-t_1)$ (1)
Lipid content, % (LC) = DLW / DCW (2)
Lipid productivity, mg/L/day (LP) = BP × LC (3)
Whereas DLW and DCW denotes dry lipid weight and dry cell weight respectively, A_1 and A_2 denotes DCW of initial and final biomass concentration respectively, (t_1-t_2) represents the time interval. All the experiments were performed in triplicates and the results were expressed as mean ± standard deviation.

Optimization of media for enhanced lipid production by RSM

The statistical experimental design through RSM is being widely used by researchers for enhanced production of metabolites.[24,25] Design Expert® Software Version 9 was used to optimize the media conditions for biomass, lipid productivity and lipid content by a central composite rotatory design (five-level–two-factor) using NaCl (A), SIW (B), $NaNO_3$ (C) and $MgSO_4$ (D) as independent variables. The variables NaCl and SIW were supplemented to the media whereas $NaNO_3$ and $MgSO_4$ were the components of BG-11 media. Central composite rotatory design (CCRD) contains a total of thirty experimental runs at five coded levels (-α, -1, 0, +1, +α) with six replications at the centre values and the whole experiment was performed in triplicates. The levels of composition of each factor for the CCRD are shown in Table 1. The responses biomass productivity in mg/L/day (Y1), lipid productivity in mg/L/day (Y2) and lipid content in % (Y3) respectively were calculated using equations. 1 – 3 as mentioned before. Quadratic equation was used for statistical analysis is given below.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum\sum \beta_{ij} X_i X_j + \varepsilon$$

where Y is the response, β_0 the intercept term, β_i the linear effect, β_{ii} the squared effect, and β_{ij} is the interaction effect, X_i, X_j are the factors independent variables and ε is the error.

Fatty acid profiling of *Myxosarcina* sp. by GC-MS analysis

To analyze the fatty acids obtained from *Myxosarcina* sp. the extracted lipids were transesterified into fatty acid methyl esters through acid catalysis using modified method.[26] Briefly, lipids were dissolved in 1.5 mL of methanol, 50 µl of 35% conc. HCl (final concentration 0.39 M) and were further boiled at 100°C for 1 h and 30 min. The contents were then cooled at room temperature and to that 1 ml of hexane was added. They were then vortexed for 2 min and the hexane layer was collected and transferred to GC vial (Cyber lab). FAME were analysed by injecting 1 µl of sample into GC (Agilent Technologies 6890, N Series, USA) and their individual *m/z* values were identified by EI-MS (Electron impact ionization - mass spectrometry), JEOL GC MATE-II, JEOL Ltd, Tokyo, Japan) equipped with HP-5 MS column, photon multiplier tube detector and quadruple double focusing mass analyzer. Each FAME was identified by comparing with Supelco 37 component FAME mix (Sigma - Aldrich, USA) as standard. Besides each peaks were also compared with NIIST (National Institute for Standards and Technology) database at SAIF (Sophisticated Analytical Instrument Facility), Indian Institute of Technology – Madras (IITM), Chennai, Tamil Nadu, India (www.saif.iitm.ac.in). The relative percentage of each fatty acid was calculated by area normalization method.

Assessment of biodiesel fuel properties

Important fuel properties of biodiesel were evaluated for *Myxosarcina* sp. from its fatty acid profile using empirical equations (4) to (14)[19,21] and it includes SV, IV, CN, DU, LCSF, CFPP, CP, PP, *v*, ρ and HHV.

$$SV = \sum (560 \times F)/M \quad (4)$$

$$IV = \sum (254 \times F \times D)/M \quad (5)$$

$$CN = (46.3 + [5458/SV]) - (0.225 \times IV) \quad (6)$$

where *F* is the percentage of each type of fatty acid, *M* is the molecular mass of the corresponding fatty acid, and *D* is the number of double bonds.

DU = MUFA wt.% + (2 × PUFA wt.%) (7)
Where MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids.

LCSF = (0.1×C16)+(0.5×C18+(1×C20)+(1.5×C22)+(2×C24) (8)
CFPP = (3.1417 × LCSF) – 16.477 (9)
Where C16, C18, C20, C22, C24 are percentage of corresponding fatty acids.

CP = (0.526 × C16) – 4.992 (10)
PP = (0.571 × C16) – 12.240 (11)
Where C16 denotes C16:0 content (wt.%) in fatty acid profile

$$\ln(v_i) = -12.503 + 2.496 \times \ln(M_i) - 0.178 \times N \quad (12)$$

$$\rho_i = 0.8463 + 4.9/Mi + 0.0118 \times N \quad (13)$$

$$HHV = 46.19 - 1794/M_i - 0.21 \times N \quad (14)$$

where, v_i is the kinematic viscosity at 40 °C in $mm^2\ s^{-1}$; ρ_i is the density at 20°C in g/cm^3 and HHV_i is the higher heating value in MJ/kg of i^{th} fatty acid, M_i is the molecular mass of i^{th} fatty acid and N denotes number of double bonds.

Results and Discussion

Effects of SIW and salinity stress in biomass and lipid contents of *Myxosarcina* sp.

Productivities of biomass and lipid are the key parameters to figure out a best strain for biodiesel production. Lipid contents in microalgae can be enhanced by different strategies like nitrogen starvation, salinity stress and nutrient supplementation.[21] Whereas in the case of cyanobacteria, little information is available regarding lipid enhancement methods for biodiesel production. Hence, in this study an attempt was made to enhance the biomass and lipid by providing *Myxosarcina* sp. with SIW as a substrate and imposing this strain to salinity stress. Batch cultivation of *Myxosarcina* sp. in photobioreactor (3 litres) was carried out (Supplementary Fig.1.) for raising the biomass and that was harvested after 35 days and maintained to carry out the further experiments like media optimization by RSM. In this study, during exponential growth phase *Myxosarcina* sp. under saline stress (0.5 M) showed similar lipid content of 11 ± 0.6 % (Fig. 1.) with that of the control 10.9 ± 1.8 % whereas decreased biomass and lipid productivities in the different concentrations of NaCl provided in the BG-11 media was observed. *Lyngbya* sp. and *Synechococcus* sp. was enforced to saline stress and found increased lipid content of 8 % (1.2 fold) and 9.8 % (1.1 fold) in 1 M concentration, and found that upon increasing the concentration of salinity had shown inhibitory effect in

growth.[27] Similarly, green microalgae *Coelastrella* sp. (37%) and *Micractinium* sp. (23%) under 2-3 % saline stress yielded higher lipid contents respectively.[21] On the other hand, microalgae under salinity stress could influence the biomass and lipid productivity at lower concentration NaCl (1 g) and that may inhibit the growth of microalgae where the concentration is increased.[28] The results are indicating that algae under saline stress may be an important tool for the production of biomass, lipid and that may also negatively affect the growth of algae at higher concentration. In addition the stimulation of lipid production by salinity stress would aid to increase the viscosity of plasma membrane and turgor pressure of the plant cell or algae and thereby preventing the efflux of water from the cell for its adaptation.[29] In addition, salinity stress could cause the synthesis of osmoregulants rather than the other cellular constituents and which may increase the tolerance level to stress by aiding the various functions like carbon and nitrogen storage and stabilization of sub cellular structure in plants and microalgae.[30,31]

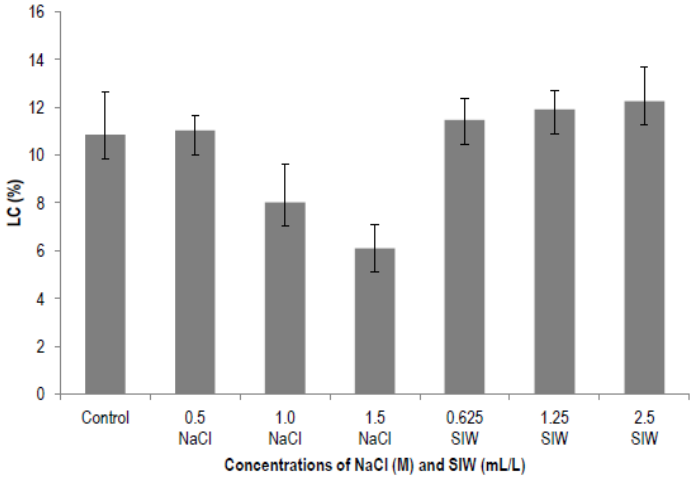

Fig. 1. Lipid content (LC) in % of *Myxosarcina* sp. control, under saline stress and SIW supplementation. All the experiments were performed in triplicates and the data represent mean of the triplicates. Standard deviations are shown in error bars.

However 1.2 fold (28.5 ± 2.4 mg/L/day) biomass productivity and 1.3 fold (3.4 ± 0.2 mg/L /day) lipid productivity increases were found in BG-11 medium supplemented with 2.5 mL/L SIW supplementation (Fig. 2 and Fig. 3). Moreover lipid content was found to be marginally increased (12.2 ± 1.5 %) than control (10.9 ± 1.8 %) when 2.5 mL/L of SIW was added (Fig. 1). Interestingly, CO_2 from gaseous effluent of cement industry at lower concentrations can be used to grow microalgae and higher dust nature of cement industry had not affected the algal growth.[32] Supplementation of 0.625 mL/L sugar industrial waste and 2% NaCl resulting in higher lipid productivities of 10.8 (1.2 fold) and 13.9 (1.6 fold) mg/L/day respectively in *Coelastrella* sp. and also reported that the presence of organic nitrogen in the effluent would influence the lipid productivity.[21] Biomass and lipid productivities of five different cyanobacteria in normal growth medium were found to be existing in the range of 3.7 to 52.7 mg/L/day and 0.8 to 14.2 mg/L/day respectively.[33]

Hence from these experiments, it can be concluded that SIW supplementation enhanced biomass and lipid production in *Myxosarcina* sp. and to the best of our knowledge, this would be the first report that *Myxosarcina* sp. utilizes SIW for its growth and lipid production.

Response surface methodology

RSM for the production of secondary metabolites is always

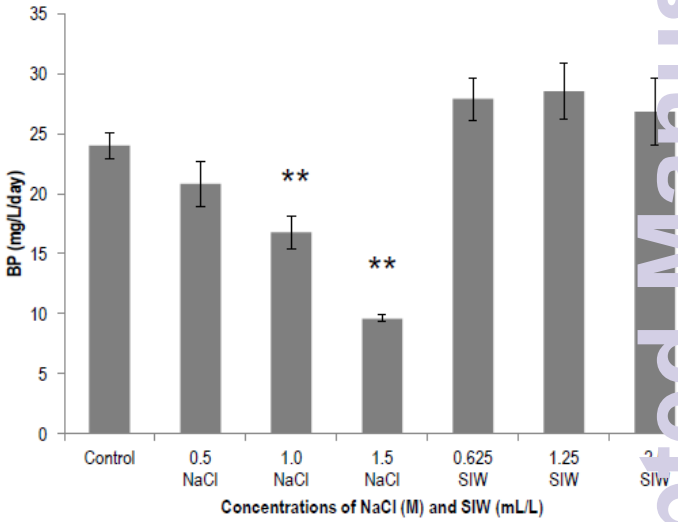

Fig. 2. Biomass productivity (BP) in mg/L/day of *Myxosarcina* sp. control, under saline stress and SIW supplementation. Asterisk denotes the significance value ** $p < 0.01$. All the experiments were performed in triplicates and the data represent mean of the triplicates. Standard deviations are shown in error bars.

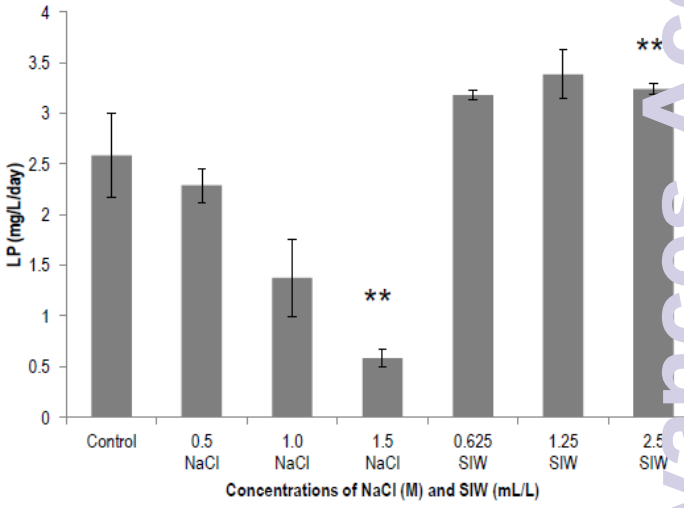

Fig. 3. Lipid productivity (LP) in mg/L/day of *Myxosarcina* sp. control, under saline stress and SIW supplementation. Asterisk denotes the significance value ** $p < 0.01$. All the experiments were performed in triplicates and the data represent mean of the triplicates. Standard deviations are shown in error bars.

been a part of the media optimization strategies and the combined effect of different nutrient variables in this design would yield maximum production. Since salinity stress and SIW supplementation resulted in only marginal increase in the biomass and lipid production, the combinatorial effect of the aforementioned two factors along with the BG-11 media components *i.e.* $NaNO_3$ and $MgSO_4$ for optimum biomass and

lipid production was designed through a CCRD. The CCRD consisted a total of 30 experimental runs comprising 6 trials for replication at the central points, 8 trials for axial points and 16 trials for factorial design along with responses of each factor were shown in Table 2. The interaction between four factors on lipid productivity response was shown in three-dimensional response surface plot (Fig. 4). The data from RSM studies exhibited that among the 30 runs, 25th run showed highest lipid productivity and content (2.3 ± 0.5 mg/L/day and 20.6 ± 1.8 %) respectively.

Table. 1. Factors and their level of composition for the CCRD

Independent variables	Symbols	Coded levels				
		-α	-1	0	+1	+α
NaCl (M)	A	0.4	0.6	0.8	1	1.2
SIW (mL/L)	B	1	1.5	2	2.5	3
$NaNO_3$ (g/L)	C	0.5	1	1.5	2	2.5
$MgSO_4$ (g/L)	D	0.045	0.06	0.075	0.09	0.105

It is obvious that the lipid productivity and content were positively regulated by the media components at the concentration of 0.8 M NaCl, 2 mL/L SIW, 2.5 g/L of $NaNO_3$ and 0.075 of g/L of $MgSO_4$. The indication of these findings would reveal that higher concentration of nitrogen and SIW along with slight saline stress improve the lipid production in *Myxosarcina* sp. Nitrogen ($NaNO_3$) starvation in *Oscillatoria willei* BDU 130511) resulted in many metabolic changes among which decreased fatty acid and lipid content, photosynthetic impairment and reduced pigment production which implies that nitrogen is needed for cyanobacterial growth and lipid production.[34] Moreover, the luxurious growth of cyanobacteria in industrial effluents that contains tolerable pH and nutrients with low oxygen content was also noted.[8] Sugar industrial effluent comprises of high content of organic nitrogen and a minor amount of carbohydrates as reported in our earlier study.[21] Furthermore, cyanobacteria can readily uptake and utilizes organic compounds containing reduced nitrogen and inorganic nutrients NH_4^+, NO_3^-, and NO_2^- have also been reported.[35,36] Significant increase of 3.2 fold in biomass yield was observed in *Oscillatoria annae* by employing RSM.[17] In another study, plant growth promoter calliterpenone was used for enhanced biomass and lipid production in *Synechocystis* PCC 6803 by RSM and reported that 20 µL/100 mL (0.01 mM) of calliterpenone enhanced biomass (346.95 %), lipids (134.46 %) and carbohydrates (187.2 %) respectively.[37] Similarly, RSM was employed to enhance biomass and lipid production in *Synechococcus* sp. HS01 and reported that maximum lipid productivity of 56.5 mg/L/day was achieved (2.82 folds higher than control) in the media containing 0.09% NaCl, 1.12 g/L of $NaNO_3$ and 1% ostrich oil.[38] The experimental results analysed by RSM yielded CCRD design for lipid productivity response can be defined by a quadratic (Equation.15.)

Lipid productivity = +1.65 – (0.16A) + (0.032B) + (0.15C) + (0.3D) – (0.13AB) + (0.059AC) + (0.07AD) – (0.12BC) + (0.16BD) – (0.083CD) – (0.013A^2) – (0.21B^2) + (0.24C^2) – (0.17D^2) (15.)

Hence by modifying the concentrations of NaCl, SIW, $NaNO_3$ and $MgSO_4$ using above equation.15, maximum lipid productivity would be obtained. Analysis of variance (ANOVA) by f-test showed that model developed for lipid productivity response was significant whereas the other two responses biomass productivity and lipid content were insignificant with "Prob>F" values 0.00226, 0.131445 and 0.052093 respectively (Table. 3). Model for the lipid productivity response with *F*-value 4.84 admits that the model is significant and there is only 0.22% chance for the large *F*- value occurrence due to noise. Regression analysis revealed that *R*-squared value of 0.818613 which means 81.86% variability in the lipid productivity response and insignificant lack of Fit F-value of 2.57 implies that the model fits with the experimental data. The measure of signal to noise ratio by adequate precision was found to be 10.075% for lipid productivity response denotes that the model can be adopted to navigate the design space. In this context, A, D, B^2, C^2 model terms for the lipid productivity response were found to be significant. Obviously it is also evident that this RSM study for lipid productivity, the optimum values of each factor lies adjacent to the central values except $NaNO_3$ where the optimum value was 2.5 g/L. The validation of RSM - CCRD optimized media was also performed to verify the model and found that they are in parallel with the optimized media. The findings of validation RSM-CCRD optimized medium showed that biomass, lipid productivities and lipid contents (15.82 ± 0.7 mg/L/day, 2.64 ± 0.1 mg/L/day and 16.72 ± 1.1 %) than the normal BG-11 medium (12.06 ± 0.8 mg/L/day, 1.63 ± 0.1 mg/L/day and 13.6 ± 1.4 %).

Fatty acid profiling by GC-MS

Unsaturation of fatty acid plays an important role in performance of biodiesel. In this study, the relative percentage of individual fatty acid of *Myxosarcina* sp. was estimated using fatty acid profiling by GC-MS is shown in Fig.5. The observation of resulted fatty acids of *Myxosarcina* sp. revealed that 39 5.3% of SFA, 61 ± 5.3% of MUFA along with undetectable or unquantifiable level of PUFA, which clearly evidenced that the absence of PUFA in *Myxosarcina* sp. paving a way for better biodiesel stability. Similar results were also observed in *Myxosarcina* PCC 7312 showed 26.2% of SFA, 64.5% of MUFA along with the absence of PUFA [39] that substantiates the fatty acid composition of *Myxosarcina* sp. in our study. Moreover, the higher percentage (>76%*)* of SFA, MUFA and absence of PUFA would determine the suitability of good biodiesel in in *Scenedesmus abundans*.[40] Similarly, the findings of fatty acid composition from *Myxosarcina* sp had shown absence or undetectable level PUFA may contribute an appropriate nature of biodiesel. On the other hand, twelve heterocystous nitrogen fixing, filamentous cyanobacteria of different species of *Anabena* sp., *Nodularia* sp. and *Nostoc* sp. consisting the lipids in the range of 8-13% and the fatty acid composition revealed higher levels of polyunsaturated fatty acids and saturated fatty acids than monounsaturated fatty acids.[41] Correspondingly the fatty acid composition of six freshwater cyanobacteria had revealed that 33-62% of unsaturated and 32-49% of saturated fatty acids and palmitic acid as the dominant fatty acid (24-40%) was also noticed.[42]

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Table.2. Observed and predicted responses of biomass productivity (BP) in mg/L/day, lipid productivity (LP) in mg/L/day and lipid content (LC) in % along with the experimental layout of RSM – CCRD. Factor A – NaCl; B –SIW; C – $NaNO_3$; D – $MgSO_4$

Runs	Factors				Observed responses			Predicted responses		
	A (M)	B (mL/L)	C (g/L)	D (g/L)	BP (mg/L/day)	LP (mg/L/day)	LC (%)	BP (mg/L/day)	LP (mg/L/day)	LC (%)
1	0.8	2	1.5	0.075	13.07 ± 1.2	1.54 ± 0.2	12.1 ± 3.0	13.42 ± 0.5	1.65 ± 0.1	13.11 ± 1.2
2	0.6	1.5	2	0.09	13.16 ± 2.0	2.18 ± 0.6	16.53 ± 4.3	12.11 ± 2.0	1.69 ± 0.2	13.61 ± 2.3
3	1	2.5	2	0.09	12.33 ± 0.5	1.69 ± 0.2	13.77 ± 1.7	14.2 ± 2.0	1.77 ± 0.2	12.64 ± 2.1
4	0.8	3	1.5	0.075	11.56 ± 0.4	1.07 ± 0.2	9.19 ± 1.8	12.98 ± 1.2	0.88 ± 0.3	7.01 ± 2.3
5	1	1.5	2	0.09	18.96 ± 5.6	2.10 ± 0	11.1 ± 3.5	15.5 ± 2.0	1.89 ± 0.2	13.12 ± 2.3
6	0.8	2	1.5	0.075	13.33 ± 0.4	2.01 ± 0.2	15.1 ± 1.6	13.42 ± 0.5	1.65 ± 0.1	13.11 ± 1.2
7	0.8	2	1.5	0.075	12.78 ± 0.1	2.02 ± 0.5	15.82 ± 1.4	13.42 ± 0.5	1.65 ± 0.1	13.11 ± 1.2
8	1	2.5	2	0.06	10.49 ± 1.4	0.49 ± 0.1	4.63 ± 0.2	8.74 ± 2.0	0.89 ± 0.2	8.82 ± 2.3
9	0.4	2	1.5	0.075	14.9 ± 0.8	1.36 ± 0.3	9.16 ± 2.0	15.49 ± 1.2	1.91 ± 0.3	13.15 ± 2.3
10	0.8	2	1.5	0.075	15.76 ± 1	1.87 ± 0.4	11.92 ± 2.5	13.42 ± 0.5	1.65 ± 0.1	13.11 ± 1.2
11	0.8	2	1.5	0.105	15.05 ± 1.2	1.27 ± 0.3	8.45 ± 1.7	15.51 ± 1.2	1.57 ± 0.3	10.28 ± 2.3
12	0.6	2.5	1	0.06	11.51 ± 0.3	1.24 ± 0.5	10.94 ± 4.9	15.7 ± 1.2	1.37 ± 0.3	9.06 ± 2.3
13	0.6	1.5	2	0.06	15.10 ± 0.7	1.69 ± 0.1	11.22 ± 0.8	15.07 ± 1.2	1.71 ± 0.3	11.02 ± 2.3
14	0.8	2	1.5	0.045	10.50 ± 0.4	0.59 ± 0.2	5.74 ± 2.2	11.33 ± 1.2	0.38 ± 0.3	3.59 ± 2.3
15	0.6	2.5	2	0.06	15.05 ± 1.2	1.69 ± 0.5	11.05 ± 2.2	12.99 ± 1.2	1.47 ± 0.3	9.97 ± 2.3
16	1.2	2	1.5	0.075	11.38 ± 0.3	1.75 ± 0.7	15.51 ± 6.2	11.34 ± 1.2	1.28 ± 0.3	11.18 ± 2.3
17	1	1.5	2	0.06	11.73 ± 0.5	1.42 ± 0.2	12.19 ± 2.4	13.04 ± 1.2	1.63 ± 0.3	11.69 ± 2.3
18	1	1.5	1	0.06	9.72 ± 0.9	0.83 ± 0.4	8.85 ± 4.7	9.98 ± 1.2	0.81 ± 0.3	8.53 ± 2.3
19	0.6	1.5	1	0.09	11.53 ± 0.4	1.73 ± 0.4	15.1 ± 3.5	14.01 ± 1.2	1.44 ± 0.3	11.06 ± 2.3
20	0.8	1	1.5	0.075	10.68 ± 1.3	0.49 ± 0.1	4.76 ± 1.3	13.86 ± 1.2	0.75 ± 0.3	6.61 ± 2.3
21	1	2.5	1	0.06	10.25 ± 0.3	0.46 ± 0.1	4.48 ± 0.9	8.17 ± 1.2	0.55 ± 0.3	7.59 ± 2.3
22	0.8	2	1.5	0.075	12.61 ± 0.6	1.63 ± 0.4	12.8 ± 2.2	13.42 ± 0.5	1.65 ± 0.1	13.11 ± 1.2
23	1	1.5	1	0.09	15.19 ± 0.8	1.36 ± 0.2	8.96 ± 0.7	14.11 ± 1.2	1.40 ± 0.3	10.24 ± 2.3
24	0.6	2.5	1	0.09	13.07 ± 1.2	1.54 ± 0.2	12.1 ± 3.0	17.43 ± 1.2	2.31 ± 0.3	14.33 ± 2.3
25	0.8	2	2.5	0.075	11.1 ± 1.8	2.3 ± 0.5	20.6 ± 1.8	12.75 ± 1.2	2.88 ± 0.3	20.11 ± 2.3
26	0.6	2.5	2	0.09	12.56 ± 1.3	1.82 ± 0	14.49 ± 1.0	13.04 ± 1.2	2.08 ± 0.3	14.95 ± 2.3
27	0.6	1.5	1	0.06	20.3 ± 1.2	1.39 ± 0.1	6.85 ± 0.2	15.29 ± 1.2	1.13 ± 0.3	8.18 ± 2.3
28	0.8	2	1.5	0.075	13.14 ± 0.9	1.44 ± 0.1	10.91 ± 0.3	13.42 ± 0.5	1.65 ± 0.1	13.11 ± 1.2
29	1	2.5	1	0.09	14.55 ± 0.8	1.65 ± 0.7	11.35 ± 4.0	15.31 ± 1.2	1.77 ± 0.3	11.69 ± 2.3
30	0.8	2	0.5	0.075	12.36 ± 0.7	2.01 ± 0.1	16.41 ± 1.6	14.08 ± 1.2	2.29 ± 0.3	16.32 ± 2.3

Characterization of biodiesel fuel properties

In order to have favourable biodiesel fuel properties like oxidative stability, the proportions of SFA and MUFA should be higher than PUFA.[42] In this study, eleven different biodiesel fuel properties were evaluated using empirical equations and were listed in Table.4. The results showed that almost all the parameters were in concurrence with the biodiesel standards EN 14214 (Europe), ASTM D6751-02 (US) and IS 15607 (India) except kinematic viscosity and HHV with marginal increase in density than the standard levels. SV which denotes the number of milligrams of KOH required to saponify 1 g of oil was found to be 210 ± 3 mg KOH g^{-1} of oil for biodiesel from

Table.3. Statistical analysis of CCRD by analysis of variance (df denotes degree of freedom and p - value Prob>F less than 0.05 denotes that the model is significant; NA- not applicable)

Source	Lipid productivity (mg/L/day)				
	Sum of squares	df	Mean square	F - value	P - value Prob>F
Model	8.36	14	0.60	4.84	0.00223
A-NaCl	0.58	1	0.58	4.70	0.05
B-SIW	0.02	1	0.02	0.20	0.66
C-$NaNO_3$	0.52	1	0.52	4.19	0.06
D-$MgSO_4$	2.12	1	2.12	17.21	0.00
AB	0.25	1	0.25	2.05	0.17
AC	0.06	1	0.06	0.46	0.51
AD	0.08	1	0.08	0.64	0.44
BC	0.23	1	0.23	1.88	0.19
BD	0.40	1	0.40	3.21	0.09
CD	0.11	1	0.11	0.90	0.36
A2	0.00	1	0.00	0.04	0.85
B2	1.18	1	1.18	9.53	0.01
C2	1.52	1	1.52	12.31	0.00
D2	0.78	1	0.78	6.29	0.02
Residual	1.85	15	0.12	NA	NA
Lack of fit	1.55	10	0.16	2.57	0.15

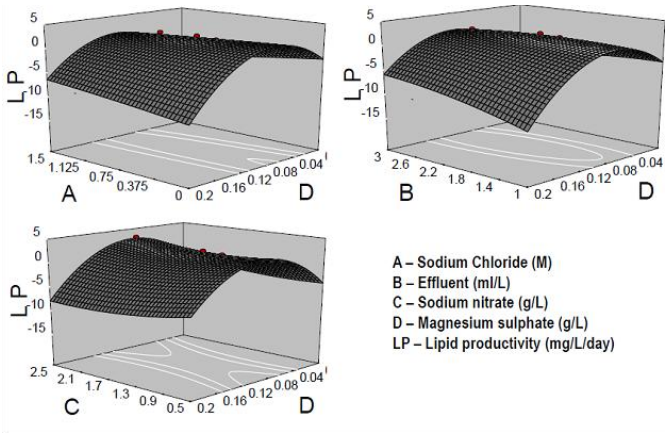

Fig.4. Three dimensional surface plots showing interaction of various factors with the response lipid productivity (mg/L/day) of *Myxosarcina* sp. grown in the CCRD optimized media.

Myxosarcina sp. which is almost similar (188 – 210 mg KOH g^{-1} of oil) in the cases of SV of several microalgae reported earlier.[43] CN of biodiesel denotes the time interval between fuel injection and ignition. CN increases with the increase of SFA content in the FAME whereas kinematic viscosity, density and CFPP are influenced by unsaturated fatty acid and thus it is important to maintain appropriate balance between saturated and unsaturated fatty acids which necessitates high quality of biodiesel.[44] CN for biodiesel from *Myxosarcina* sp. was found to be 60 ± 0.2 that surpass the ASTM D6751-02 and EN 14214 specifications for biodiesel which is minimum of 47 and 51 respectively. FAME with high content of saturated esters will have preferable CN with low viscosity whereas polyunsaturated esters results in poor CN and oxidative stability. Monounsaturated esters have considerable level of viscosity and low temperature properties.[45] IV determines the unsaturated fatty acid levels and thus higher the unsaturatio higher the IV. Our study states that, IV of biodiesel from *Myxosarcina* sp. 56 ± 5 g I_2 $100g^{-1}$ of oil is lower than EN 14214 specification for biodiesel that is 120 g I_2 $100g^{-1}$ of oil s maximum limit. Similar to that IV of fatty acids were analysed and reported for *Microcystis*

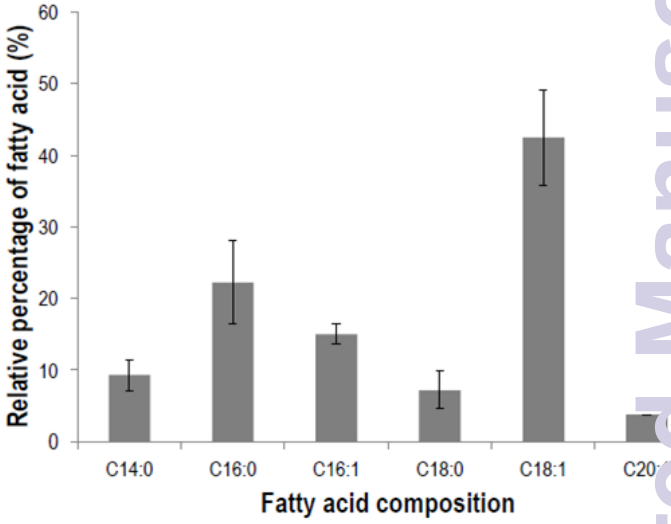

Fig.5. Relative percentage of fatty acids (*Myxosarcina* sp.) calculated by area normalization method. Experiment was performed in duplicates and the data represents the mean of the duplicates. Standard deviations are shown in error bars.

aeruginosa NPCD-1,[34] an unicellular cyanobacterium as 57 g I_2 100 g^{-1} and IV of FAME was reported for *Oscillatoria* sp,[43] a filamentous cyanobacterium as 67 g I_2 100 g^{-1} that substantiates our study revealing the lower IV among the cyanobacteria, which were reported earlier. DU of biodies from *Myxosarcina* sp. from the fatty acid profile was found as 61 ± 5.3 wt% and this lower DU value was mainly due to the absence of PUFA. Biodiesel flow performances at the cold countries are highly determined by the parameter CFPP which is being allowed a maximum of 19°C as a limit.[46] Presence of higher degree of longer carbon chains or saturated carbon chains in the biodiesel results in the crystallization and clogging of fuel lines. Thus, lower the value of CFPP, lower the clogging and better the engine performance.[47] CFPP and LCSF of biodiesel from *Myxosarcina* sp. was found to be 2 ± 2 °C and 6 ± 1 °C respectively thus meeting the standard specifications. CP and PP denotes the temperatures when they are cooled at which the cloud of wax crystals first appear and the range allotted by ASTM D6751 are -3 to 12 °C and -15 to 16 °C respectively.[48] CP and PP of biodiesel from *Myxosarcina* sp. was found to be 7 ± 3 °C and 0.5 ± 3 °C respectively affirm that this strain has good CP and PP. The CP and PP of eleven cyanobacterial strains showed that values existed in the range of -4.54 to 9.71 °C and -11.76 to 3.71 °C respectively[19] and th corroborates with our study. To the best of our knowledge this is the first report stating biodiesel fuel properties like SV IV DU, CFPP, LCSF, CP, and PP of *Myxosarcina* sp. concurrence with the national and international standards

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Table.4. Biodiesel fuel properties of *Myxosarcina* sp. evaluated from the fatty acid profile. Experiment was performed in duplicates and the data represent the mean of duplicates.

SV (mg KOH g^{-1} of oil)	IV (g I_2 $100g^{-1}$ of oil)	CN	DU (Wt %)	LCSF (°C)	CFPP (°C)	CP (°C)	PP (°C)	*v* (mm^2 sec^{-1})	ρ (g m^{-3})	HHV (MJ kg^{-1})	SFA	MUFA	PUFA
210 ± 3	56 ± 5	60 ± 0.2	61± 5	6 ± 1	2 ± 2	7 ± 3	0.5 ± 3	-1.5 ± 0	1.1 ± 0	35 ± 0	39 ± 5.3	61 ± 5	0

Kinematic viscosity of biodiesel from *Myxosarcina* sp. was found to be -1.5 ± 0 mm^2 s^{-1} which is lower than the ASTM D6751-02 (1.9 – 6.0 mm^2 sec^{-1}), EN14214 (3.5 – 5.0 mm^2 s^{-1}) and IS 15607 (2.5 – 6.0 mm^2 s^{-1}) specifications respectively. High viscosity of fuel results in poor engine performance with higher exhaust smoke and emissions.[49] Density of the biodiesel from *Myxosarcina* sp. was 1.1 ± 0 g m^{-3} which is slightly higher than all the standard specifications (0.86 to 0.9 g m^{-3}). HHV was not specified in any of the biodiesel standards, however they were found to be 35 ± 0 MJ kg^{-1} for biodiesel from *Myxosarcina* sp. which is slightly lower than the standard set range for biodiesel (39.8–40.4 MJ kg^{-1}) as mentioned earlier.[48] The mass balance equation for biodiesel production from *Myxosarcina* sp. would be derived from the FAME content and glycerol obtained through transesterification of lipid extracted from biomass cultivated in larger scale near future. Hence, the biodiesel as a renewable green energy obtained from *Myxosarcina* sp. have met almost all the required fuel properties which were also in concurrence with the national and international standards. Furthermore, race way ponds or outdoor photobioreactors can be employed for mass cultivation of *Myxosarcina* sp. in order to implement real-time application of the biodiesel in future. Thus this cyanobacteria would be one of the suitable feedstock among cyanobacteria could satisfy the recent energy demand.

Conclusion

In this study, *Myxosarcina* sp. has been evaluated for biodiesel production and RSM-CCRD was employed to enhance the biomass and lipid content. This work highlights that enhanced biomass and lipid productivities were observed in BG-11 medium supplemented with 1.25 mL/L SIW whereas higher lipid content was observed in RSM optimized media supplemented with SIW as a cheap nutrient source and $NaNO_3$ with slight saline stress (0.8 M). The fatty acid composition of this strain showed mainly of SFA and MUFA which are very much amenable for biodiesel production. Almost all the biodiesel fuel properties assessed were also found in concurrence with the national and international standards. Thus, *Myxosarcina* sp. can be used as an alternate versatile biodiesel feedstock that can mitigate atmospheric CO_2 and utilizes wastewater simultaneously.

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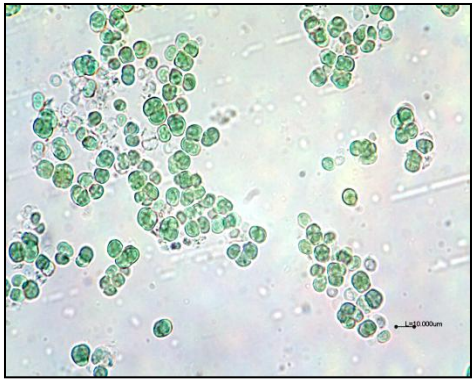
Biomass production of *Myxosarcina* sp. in photobioreactor



***Myxosarcina* sp.**

Salinity stress & sugar industrial waste (SIW) supplementation

RSM - CCRD (NaCl, SIW, $NaNO_3$ and $MgSO_4$)

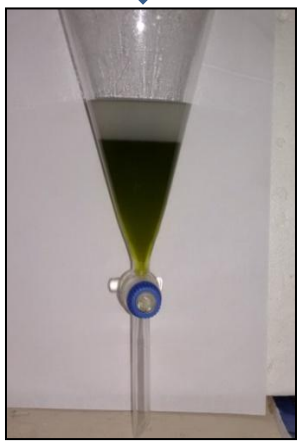
Characterization of Biodiesel Parameters

SV= ∑ (560*F)/MW

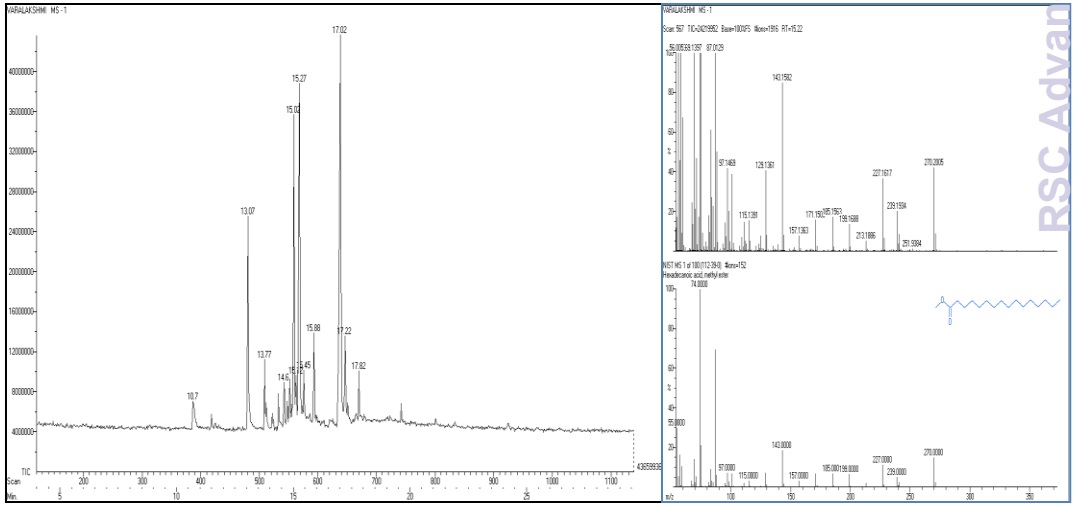
IV = ∑ (254 *F*D)/MW

CN= (46.3+5458/SV)-(0.225*1V)

DU= (MUFA,wt%) +(2*PUFA,wt%)



Lipid extraction and quantification



GC-MS analysis