Original Research Article

Isolation of Microalgae for Biomass and Lipid Enhancement through Plackett-Burmann Design

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A B S T R A C T

The algae are one among the biomass energy source which can be promising. The microalgae are the diverse organisms which can inhabit in different climatic conditions and adapt themselves and perpetuate forming their own niche. There is need to improve the local flora for biomass and lipid content instead of culturing exotic species. *Chlorella* the green microalgae, was isolated from fresh water and was characterized through molecular studies using 18s rRNA homology. The study has revealed that isolated species belong to Chlorophytes (green microalgae). The isolated species was further subjected to media optimization studies through Plackett-Burman which has revealed Urea being the highest positive effect of (7.6833) among seven variables ($NaNO_3$, $CaCl_2$, $MgSO_4$, K_2HPO_4, KH_2PO_4, Urea, 2-4,D) R^2 value of 93.16% and p value of 0.033(at 5 %) and K_2HPO_4 had a negative effect (-0.3833) for Biomass. $NaNO_3$ had highest positive effect and K_2HPO_4 had least effect on the lipid content and R^2 value of 61.14% and p value of 0.5788 (at 5 %) indicating the variables had less influence compared to Biomass. Further the variables which had highest positive effect on biomass and lipid will be selected for Response Surface methodology studies, aim will be to enhance both components in a single media, which will be the future scope of work.

Introduction

Biodiesel is currently receiving much attention due to its potential as a sustainable and environment friendly alternative fuel. Use of edible food crops for the production of biodiesel has raised the concerns of food insecurity. Therefore, algae as a feedstock for the production of biofuels has emerged at the fore front in the biofuel research (Chisti 2007, Chisti, 2008; Sivasubramanian, 2009, Michael *et al.,* 2010, Wayer *et al.,* 2010 John *et al.,* 2011). Microalgae are source of all nutrients

and are power houses and promising source of biofuel due to their simple cellular structure, higher growth rate and lipid content than conventional edible crops (Becker, 1994). Some algal species store 50- 60% of their dry cell weight as lipids which may also vary by adopting variety of environmental and nutritional stress conditions (Hu *et al.,* 2008; Breuer *et al.,* 2012)

Current biomass energy obtained from crop species like corn, sugarcane, soyabean, rape seed and others. The major problem is

competition with food and land resources. In order to combat with the problem, algae can be better alternate source for biomass energy as the algae grows in wide conditions right from fresh water to waste water. They have greater adaptability for different salinity and pH conditions. Algae can be used to treat the waste water and same biomass can be harvested for biomass. The obtained biomass can be used to extract lipid and can be extracted for biodiesel and leftover biomass can be fermented for ethanol production or else can be explored for other purposes like proteins for feed and micronutrients for other needs. But the level of toxicity has to be evaluated. The laboratory studies need to begin with collection, isolation and characterization and later on strain improvement. In the current study an attempt has been made to collect, isolate and characterize the isolated species. Among different isolates *Chlorella* sp. axenic culture was obtained, which was supported by partial sequencing of 18S rRNA homology study and phylogenetic analysis. The nucleotide partial sequence of isolate *Chlorella sorokiniana* PCPBEC was submitted to NCBI (accession number KY471550). The pure culture is submitted to NCIM, Pune. Fewer studies have been done on strain improvement of the local flora. As the species was isolated from fresh water local flora it was important to subject it for further investigation. When it was cultured on BBM it was clear that it can be grown in laboratory conditions but commercial applications would be limited. So it was important to subject it for further growth studies for better biomass and lipid enhancement. When it comes to the question of optimization it can be done through strain improvement by genetic Engineering studies, mutagenesis and by changing nutrition. In the current study Plackett-Burmann design is been adopted to know the effect of $NaNO_3$, $CaCl_2$, $MgSO_4$, K_2HPO_4, KH_2PO_4, Urea and 2-4,D. The advantage of Plackett-Burmann design, it is helpful in choosing all the variables at a time instead of studying them individually (Yang *et al.,* 2014, Mostafa *et al.,* 2016). Plackett-Burmann also helps in finding out the effectiveness of each variable and which further can be used for optimization parameters. Out of seven variables except K_2HPO_4 all had positive effect on biomass. $NaNO_3$, $CaCl_2$, $MgSO_4$, KH_2PO_4 and Urea had positive effect on lipid. It is critical to have balance between both biomass and lipid in same the media. An effort will be made to optimize both through Response Surface methodology using Box-Behnken design which will be future scope the work.

Materials and Methods

Collection of water samples

Different fresh water samples were collected from various water sources such as ponds (from Badami, Bagalkot district, Karnataka, India), domestic water and other resources from Bagalkot district for isolation of microalgae. Collection of fresh water sample was done in clean and dry sterilized water bottles. Water was collected in bottles, which was then sealed by cap and taken to the laboratory.

Isolation of microalgae using BBM

The collected fresh water samples were inoculated in Bold's Basal Media ($NaNO_3$25.0, $CaCl_2.2H_2O$ 2.5, $MgSO_4.7H_2O$ 7.5, $K_2HPO_4$7.5, $KH_2PO_4$17.5, NaCl 2.5 EDTA-50.0 KOH 31.0g, $FeSO_4.7H_2O$ 4.98 H_2SO_4 1.0 ml, $H_3BO_3$11.42 g L^{-1} Micronutrients- $ZnSO_4.7H_2O$ 8.82, $MnCl_2.4H_2O$ 1.44, MoO_3 0.71, $CuSO4.5H_2O$ 1.57, $Co(NO_3)2.6H_2O$0.49 gL^{-1}) for the growth of microalgae present in the water sample. The inoculated media was incubated at room temperature 25°±3C with pH of 6.8-7.0.

To approximately 900 mL of distilled water were added to all the components in the order specified. Total volume was brought to 1 L with distilled water. Then covered and autoclaved at 121°C for 15 min at 15 psi.

Pure culture of algae (Centrifuge Washing Technique)

The tubes containing the culture sample was centrifuged at 3000 rpm for 15 minutes. After centrifugation the supernatant was discarded and the cells were suspended in fresh sterile water in tube using vortex mixer to complete centrifuge-washing process. Centrifugation and washing was repeated for three times to expel most of the microorganisms present in the algal sample and the cells were then streaked on the agar plates.

Axenic culture by Streak Plate Technique

Washed microalgae were streaked on solidified Bold's Basal Medium in aseptic condition and kept for fifteen days for the growth. Repeated streak-platings was carried out to pick up single colony from earlier streaked plates and to make it free from bacteria. From last streaked plates, the single colonies were picked up by loop and allowed to grow in tubes and vials.

Axenic cultures were isolated by repeated sub-culturing. (Parvin *et al.,* 2007)

Characterization of isolated microalgae

The isolated microalgae were characterized by observing the cells under light microscope. To a clean and dry glass slide a drop of culture was added. This was covered with cover slip and was observed under 40 X objective. Later pictures were captured in camera attached trinocular microscope (OLYMPUS, India) under 100X. The size of the Chlorella species was measured using stage-ocular micrometer.

Study of growth pattern of *Chlorella* species

The isolated Chlorella sp. was grown in 500ml flask (Borosil) with 250ml Bold's Basal media with 16:8 hours light and dark cycles at room temperature 25 ± 2^0 C. The growth pattern of isolated *Chlorella* species was studied using optical density method. The OD was measured at 540 nm using spectrophotometer at an interval of 24 hours in triplicates for 30 days.

DNA extraction, PCR amplification and sequencing

DNA was isolated from axenic culture using genomic DNA isolation kit (Himedia, India). The purity of the DNA was checked by measuring 260/280 ratio and was quantified based on Agarose gel electrophoresis and bands were observed under gel documentation using gel doc (UVCI-1100-MSmajor Science). The extracted DNA was further subjected to PCR amplification using Oligonucleotide primers (18S F: 5'-GTAGTCATAKGCTNGTCTS-3'; 18S R 5' GARACCTDGTTAVGACTY-3') (Indhumati *et al.,* 2013, Franziska *et al.,* 2014).

PCR amplification conditions

PCR reaction was performed in a thermal cycler (MJ research PTC200). The reaction mixture was DNA 1μl (100ng), Forward Primer400ng,Reverse Primer 400ng,dNTPs (10mM each),4μl 10X Chrom Taq DNA Polymerase, Assay Buffer 10 μl,Chrom Taq DNA Polymerase Enzyme (3U/ μl)1 μl, Water X μl, Total reaction volume:100 μl

PCR Conditions: (25 cycles): Initial Denaturation: 96°C for 1min, Denaturation: 96°C for 10sec, Hybridization: 50 °C for 5 sec Elongation: 60 °C for 4 m (Manisha *et al.,* 2014, Franziska *et al.,* 2014). The amplified PCR was sequenced (Chromous Biotech Pvt., Ltd., Bengaluru, India).

Phylogenetic analysis

The 18S rRNA sequence of the strain was searched for homology in NCBI against BLAST. The partial 18S rRNA sequences of the isolated culture showing homology with known sequences of *Chlorella sp.* KMMCC FC-69 18S (Accession No. GQ122376.1) *Chlorella sorokiniana* strain UTEX 2805 18S (Accession No KR092112.1) existing in the database.

Plackett–Burman design

Seven variables were selected viz., NaNO$_3$, CaCl$_2$, MgSO$_4$, K$_2$HPO$_4$, KH$_2$PO$_4$, Urea, 2-4,D. Each variable had two levels: '-' low level and '+' high level, all were chosen in g L^{-1} and 2,4-D in mg L^{-1}. Higher concentration was selected as twice the higher concentration of BBM composition and lower concentration was half of the BBM composition. Urea and 2,4-D were chosen as an additional variables apart from the BBM composition. Urea a synthetic fertilizer, major source of nitrogen for the plant nutrition is a widely used in agriculture. The surface runoff water of agricultural land consists of urea which leads to Eutrophication problem in water bodies.

2,4-D at lower concentration is used as a growth hormone and at higher concentration used as a herbicide and is also one of the component of agricultural surface runoff water. Algae can be used for bioremediation of waste water bodies and also the biomass obtained from algal blooms can be used as a source of biofuels. The isolated species was subjected for the effect of urea and 2,4-D to reveal their effect on biomass and lipid content. Rest of the media composition was unchanged and was kept as dummy variables (Devendra and Pravin 2010, Reddy and Rao 2012, Manisha *et al.,* 2014, Yang *et al.,* 2014, Sheekh *et al.,* 2015). The layout of Plackett-Burmann is shown in table 1.

Biomass study

The biomass was determined based on the cell density using Neabauer haemocytometer for every interval 48 hours (Weena *et al.,* Larson and Aburg, 1997). The data was collected in triplicates.

Lipid estimation

Lipid was photo metrically estimated on seventh day of inoculation using colorimeter at 430 nm. 5ml algal sample from each run was centrifuged and the fresh pellet was used for quantification and was estimated in terms of mgL^{-1}of the media. The quantification is based on the fatty acids dissolved in chloroform. Saponified fatty acids were mixed in a copper reagent and chloroform was added and was measured colorimetrically by using sodium diethyl thiocarbamate which develops into yellow color and is proportional to the amount of lipid present in it. The palmitic acid was used for standard curve (make Himedia, 99% purity) (Duncombe, 1963; Wawrik and Harriman, 2010).

Results and Discussion

In most of the water samples green algae were found viz., *Chlorella sp.,* filamentous algae and diatoms. *Chlorella* sp. was isolated and further subjected for pure culture.

Isolation, characterization and growth of microalgae

Among different samples, Filamentous algae, Diatoms and Microalgae were found. *Chlorella* was chosen because of its wide adaptability over wide range of pH. Based on the microscopic observation, cells of *Chlorella* are round, small and normally individually dissociated (Ying *et al.,* 2009 and Rosen 1990). The size of the *Chlorella* sp., ranged from 6-12µm using stage-ocular micrometer.

Table.1 Seven variables with twelve runs of two level Plackett-Burmann design

Runs	$NaNO_3$	$CaCl_2$,	$MgSO_4$	K_2HPO_4	KH_2PO_4	Urea	2-4,D
	$g\ L^{-1}$ stock solution					$g\ L^{-1}$	$mg\ L^{-1}$
+	50	5	15	15	35	5	3
-	12.5	1.2	3.2	3.25	8.75	0.1	0.1
1	+	-	+	-	-	-	+
2	-	-	-	+	+	+	-
3	+	-	+	+	-	+	-
4	-	+	-	-	-	+	+
5	-	-	+	+	+	-	+
6	-	+	+	-	+	-	-
7	-	-	-	-	-	-	-
8	-	+	+	+	-	+	+
9	+	+	-	+	+	-	+
10	+	+	+	-	+	+	-
11	+	-	-	-	+	+	+
12	+	+	-	+	-	-	-

'+' level indicates high value and '-' level indicates low value.

Table.2 The Estimated effects and coefficients for biomass response of Plackett-Burman

Variable	Effect	Co-efficient	SE Coefficient	t-value	p-value
Constant		13.7917	0.5715	24.13	0.000
$NaNO_3$	0.2833	0.1417	0.5715	0.25	0.816
$CaCl_2$	2.8833	1.4417	0.5715	2.52	0.065*
$MgSO_4$	1.0833	0.5417	0.5715	0.95	0.397
K_2HPO_4	-0.3833	-0.1917	0.5715	-0.34	0.754
KH_2PO_4	0.0167	0.0083	0.5715	0.01	0.989
Urea	7.6833	3.8417	0.5715	6.72	0.003*
2-4,D	1.5500	0.7750	0.5715	1.36	0.247

SE, standard error; *Significant at 95% level ($p<0.05$)

Table.3 ANOVA of variance for Biomass response

Source	DF	Seq SS	Adj SS	Adj MS	F value	P value
Main Effects	7	213.45	213.45	30.493	7.78	0.033
Residual Error	4	15.68	15.68	3.919		
Total	11	229.13				

Table.4 The Estimated effects and coefficients for lipid response of Plackett-Burman

Variable	Effect	Co-efficient	SE Coefficient	t-value	p-value
Constant		71.750	6.158	11.65	0.000
NaNO$_3$	16.833	8.417	6.158	1.37	0.243
CaCl$_2$	-2.500	-1.250	6.158	-0.20	0.849
MgSO$_4$	14.500	7.250	6.158	1.18	0.304
K$_2$HPO$_4$	-10.167	-5.083	6.158	-0.83	0.455
KH$_2$PO$_4$	14.167	7.083	6.158	1.15	0.314
Urea	9.167	4.583	6.158	0.74	0.498
2-4,D	-8.167	-4.083	6.158	-0.66	0.544

SE, standard error; ($p<0.05$)

Table.5 ANOVA of variance for lipid response

Source	DF	Seq SS	Adj SS	Adj MS	F value	P value
Main Effects	7	2864	2864	409.1	0.90	0.578
Residual Error	4	1820	1820	455.1		
Total	11	4684				

Fig.1 Microscopic view of *Chlorella sorokiniana* PCPBEC under 100x compound microscope
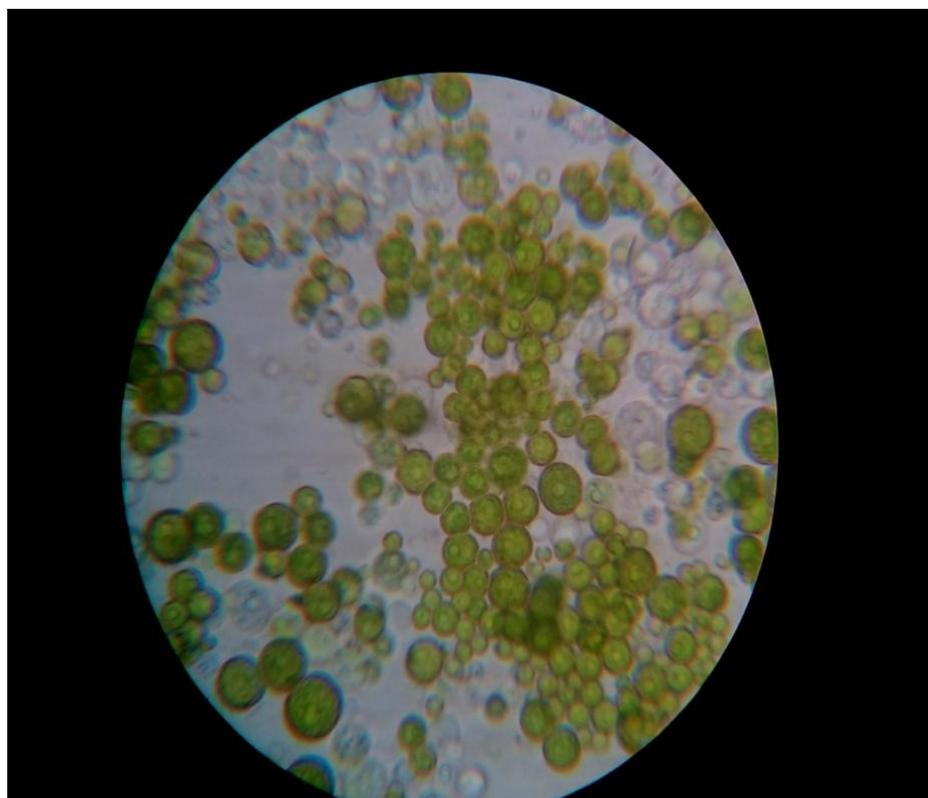
Fig.2 Extraction of genomic DNA from Isolated sample

PCPBEC

Fig.3 PCR amplification of 18s rDNA region from the isolate. The size of PCR amplified product is ~ 2kb (sequences)

2.5Kb

500bp
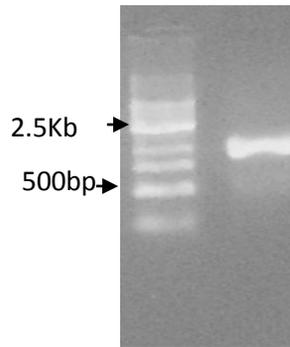
Fig.4 Results of blast analysis of *Chlorella sorokiniana* PCPBEC compared with other related species

Fig.5 Phylogenetic Analysis of *Chlorella sorkiniana* PCPBEC accession number KY471550 to predict the evolutionary relationship
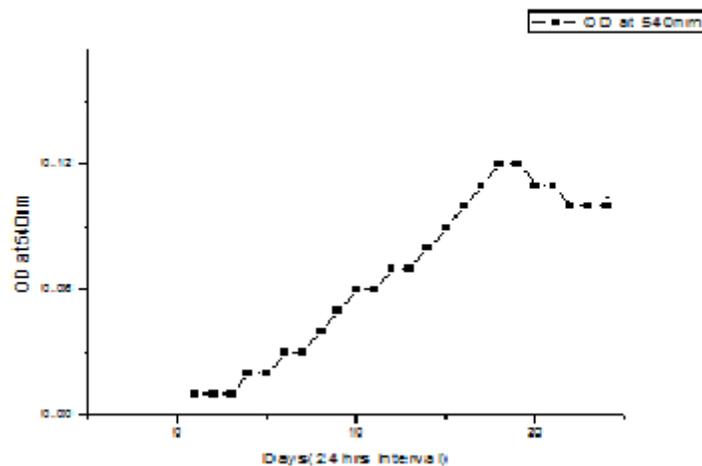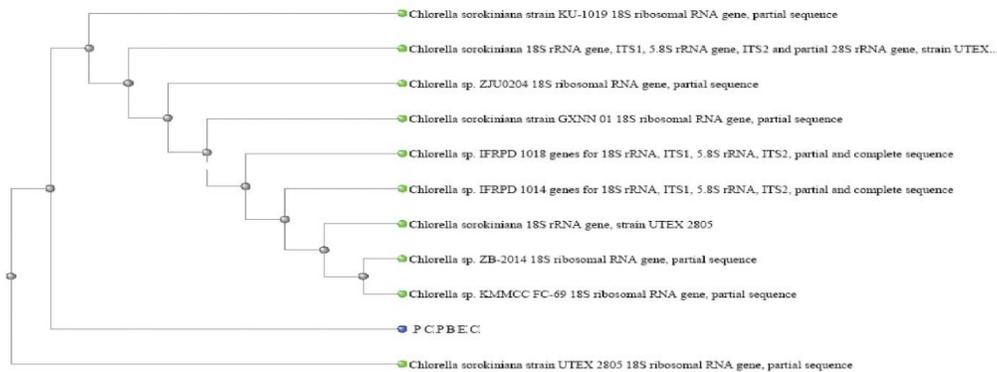
Graph 1. Growth pattern of Chlorella sp.

Chlorella sorokiniana observed under 100x compound microscope (Fig. 1).

Study of growth pattern of *Chlorella* species

The growth pattern of isolated *Chlorella* species was studied using optical density method. The OD was measured at 540 nm using colorimeter at an interval of 24 hrs.

The growth pattern is shown in the graph1. (Graph was plotted using Orion-8).

DNA extraction, PCR amplification and sequencing

The blast sequences of *Chlorella sorokiniana* PCPBEC:

CTGACTTTATACTGGTGAAACTGCGAA
TGGCTCATTAAATCAGTTATAGTTTAT
TTGATGGTACCTACTACTCGGATACCC
GTAGTAAATCTAGAGCTAATACGTGCG
TAAATCCCGACTTCTGGAAGGGACGTA
TTTATTAGATAAAAGGCCGACCGGGCT
CTGCCCGACTCGCGGTGAATCATGATA

ACTTCACGAATCGCATGGCCTTGCGCC
GGCGATGTTTCATTCAAATTTCTGCCC
TATCAACTTTCGATGGTAGGATAGAGG
CCTACCATGGTGGTAACGGGTGACGG
AGGATTAGGGTTCGATTCCGGAGAGG
GAGCCTGAGAAACGGCTACCACATCC
AAGGAAGGCAGCAGGCGCGCAAATTA
CCCAATCCTGACACAGGGAGGTAGTG
ACAATAAATAACAATACTGGGCCTTTT
CAGGTCTGGTAATTGGAATGAGTACAA
TCTAAACCCCTTAACGAGGATCAATTG
GAGGGCAAGTCTGGTGCCAGCAGCCG
CGGTAATTCCAGCTCCAATAGCGTATA
TTTAAGTTGCTGCAGTTAAAAAGCTCG
TAGTTGGATTTCGGGTGGGGCCTGCCG
GTCCGCCGTTTCGGTGTGCACTGGCAG
GGCCCACCTTGTTGCCGGGGACGGGCT
CCTGGGCTTCACTGTCCGGGACTCGGA
GTCGGCGCTGTTACTTTGAGTAAATTA
GAGTGTTCAAAGCAGGCCTACGCTCTG
AATACATTAGCATGGAATAACACGAT
AGGACTCTGGCCTATCCTGTTGGTCTG
TAGGACCGGAGTAATGATTAAGAGGG
ACAGTCGGGGGCATTCGTATTTCATTG
TCAGAGGTGAAATTCTTGGATTTATGA
AAGACGAACTACTGCGAAAGCATTTG
CCAAGGATGTTTTCATTAATCAAGAAC
GAAAGTTGGGGGCTCGAAGACGATTA
GATACCGCTGACTTTATACTGGTGAAA
CTGCGAATGGCTCATTAAATCAGTTAT
AGTTTATTTGATGGTACCTACTACTCG
GATACCCGTAGTAAATCTAGAGCTAAT
ACGTGCGTAAATCCCGACTTCTGGAAG
GGACGTATTTATTAGATAAAAGGCCGA
CCGGGCTCTGCCCGACTCGCGGTGAAT
CATGATAACTTCACGAATCGCATGGCC
TTGCGCCGGCGATGTTTCATTCAAATT
TCTGCCCTATCAACTTTCGATGGTAGG
ATAGAGGCCTACCATGGTGGTAACGG
GTGACGGAGGATTAGGGTTCGATTCCG
GAGAGGGAGCCTGAGAAACGGCTACC
ACATCCAAGGAAGGCAGCAGGCGCGC
AAATTACCCAATCCTGACACAGGGAG
GTAGTGACAATAAATAACAATACTGG
GCCTTTTCAGGTCTGGTAATTGGAATG
AGTACAATCTAAACCCCTTAACGAGGA

TCAATTGGAGGGCAAGTCTGGTGCCAG
CAGCCGCGGTAATTCCAGCTCCAATAG
CGTATATTTAAGTTGCTGCAGTTAAAA
AGCTCGTAGTTGGATTTCGGGTGGGGC
CTGCCGGTCCGCCGTTTCGGTGTGCAC
TGGCAGGGCCCACCTTGTTGCCGGGGA
CGGGCTCCTGGGCTTCACTGTCCGGGA
CTCGGAGTCGGCGCTGTTACTTTGAGT
AAATTAGAGTGTTCAAAGCAGGCCTAC
GCTCTGAATACATTAGCATGGAATAAC
ACGATAGGACTCTGGCCTATCCTGTTG
GTCTGTAGGACCGGAGTAATGATTAAG
AGGGACAGTCGGGGGCATTCGTATTTC
ATTGTCAGAGGTGAAATTCTTGGATTT
ATGAAAGACGAACTACTGCGAAAGCA
TTTGCCAAGGATGTTTTCATTAATCAA
GAACGAAAGTTGGGGGCTCGAAGACG
ATTAGATACCGTCCTAGTCTCAACCAT
AAACGATGCCGACTAGGGATCGGCGG
ATGTTTCTTCGATGACTCCGCCGGCAC
CTTATGAGAAATCAAAGTTTTTGGGTT
CCGGGGGGGAGTATGGTCGCAAGGCTG
AAACTTAAAGGAATTGACGGAAGGGC
ACCACCAGGCGTGGAGCCTGCGGCTTA
ATTTGACTCAACACGGGAAAACTTACC
AGGTCCAGACATAGTGAGGATTGACA
GATTGAGAGCTCTTTCTTGATTCTATG
GGTGGTGGTGCATGGCCGTTCTTAGTT
GGTGGGTTGCCTTGTCAGGTTGATTCC
GGTAACGAACGAGACCTCAGCCTGCT
AAATAGTCACGGTTGGCTCGCCAGCCG
GCGGACTTCTTAGAGGGACTATTGGCG
ACTAGCCAATGGAAGCATGAGGCAAT
AACAGGTCTGTGATGCCCTTAGATGTT
CTGGGCCGCACGCGCGCTACACTGATG
CATTCAACGAGCCTAGCCTTGGCCGAG
AGGCCCGGGTAATCTTTGAAACTGCAT
CGTGATGGGGATAGATTATTGCAATTA
TTAATCTTCAACGAGGAATGCCTAGTA
AGCGCAAGTCATCAGCTTGCGTTGATT
ACGTCCCTGCCCTTTGTACACACCGCC
CGTCGCTCCTACCGATTGGGTGTGCTG
GTGAAGTGTTCGGATTGGCGACCGGG
GGCGGTCTCCGCTCTCGGCCGCCGAGA
AGTCATAAAC

Phylogenetic analysis

Plackett- Burman design

The experimental design analysis was done using MINITAB-14 software. To test the significance of the hypothesis *p-value* was used. If the p-value was less than 5%, indicates the significance of the chosen variable and greater value indicates non-significance of the hypothesis. Among the seven variables chosen for biomass urea and $CaCl_2$ had positive effect with p-value 0.003 and 0.685, indicating highest effect on biomass. Rest of the variables had positive effect except K_2HPO_4. But urea had a highest effect among seven variables. In fact the urea had a greater effect than the $NaNO_3$ indicating the species can be further explored for Agricultural surface runoff water treatment. In contrast to this, for lipid studies the effect of these variables was different. The p-value was 0.578 indicating non significance. $CaCl_2$, K_2HPO_4 and 2-4D, had negative effect. Interestingly $NaNO_3$ had highest positive effect followed by $MgSO_4$, KH_2PO_4 and Urea. Many reports suggest that increased nitrogen concentration reduces lipid production but that was not observed in this study. In fact Urea had a positive effect on both biomass and lipid. This clearly indicates that further urea need to be properly evaluated for standardizing both biomass and lipid production. The results are shown in Table 2, 3, 4 and 5 (Fig. 1–5).

The current study aimed at isolating the local flora and screening it for biomass and lipid enhancement studies. Effort was made to culture and improve the strain for its for commercial applications. Morphological and molecular characterization was done through gene sequencing, which revealed the species to be microalgae of genera *Chlorella sorokiniana* PCPBEC (accession number KY471550) and database is been submitted to NCBI. Further it was subjected to Biomass

and lipid enhancement studies through Plackett-Burmann, additionally to BBM media. $NaNO_3$ $CaCl_2$, $MgSO_4$, K_2HPO_4, KH_2PO_4, Urea, and 2-4,D (Urea and 2,4-D were added deliberately, which are pollutants found in agriculture surface runoff water). Biomass had a better response for all the variables except K_2HPO_4. Variables $CaCl_2$, K_2HPO_4, 2-4,D had a negative effect on lipid enhancement. Overall K_2HPO_4 had a negative effect on both studies. So Urea had resulted positive effects on both studies which need to be further optimized. The study has a scope for further media optimization. The isolate can be used for waste water treatment studies.

Acknowledgments

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