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EVALUATION OF BLUE GREEN ALGAE GROWN UNDER DIFFERENT pH CONDITION FOR BIOFUEL PRODUCTION

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ABSTRACT

Blue green algae was isolated from fresh water bodies of Allahabad region, Yamuna River, India to produce biodiesel. Based on rate of growth in cultural media and morphological characters and biomass production, 8 colonizing blue green algae isolates were selected for the further biofuel production work. To further narrow down the number of isolates, the carbonic anhydrase enzyme assay was used as biochemical marker for identifying high CO_2 fixation or hydrocarbon accumulation in the isolated colonizing blue green algae. Based on carbonic anhydrase enzyme assay of selected algal isolates, three potential high CA activity colonizing blue green algae (BIOLBG-6, BIOLBG-7 and BIOLBG-8) were selected. The algal growth was investigated in liquid media of BG-11 for 21 days and the growth was calculated in gram biomass per litre using different pH (5 to 9) condition by taking absorbance using Spectrophotometer at a wavelength of 680nm..The best growth of biomass was seen in pH 8 for blue green algae. For 0.15483 ml/g D.W. Biomass blue green algae 3 ml of methyl ester (biodiesel) was formed.

KEYWORDS:Blue green algae, Spectrophotometer, methyl ester.

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INTRODUCTION

Energy is the most fundamental and essential input for economic development of the country. During the industrial revolution in 19th century, coal was the important source of energy. Next to coal, petroleum and diesel are the main sources.The use of fossil fuels is unsustainable due to the depletion of resources and accumulation of green house gasses in the environments that have already exceeded the threshold level of 450 ppm of carbon dioxide [1].Biofuel is eco-friendly, alternative fuel prepared from domestic renewable resources i.e. vegetable oils and animal fats. These natural oils and fats are made up mainly of triglycerides. These triglycerides when reacted chemically with lower alcohol in presence of a catalyst results in fatty acid esters. These esters show striking similarity to petroleum derived diesel and called "Bio-diesel". Biodiesel is produced currently from plant and animal oils, but not from microalgae and this is likely to change, as several companies are attempting to commercialize micro algal biodiesel. Biodiesel is a proven fuel and technology or producing and using biodiesel has been known for than 50 years. In United States, bio-diesel is produced mainly from soyabeans and other sources of commercial biodiesel include canola oil, animal fat, palm oil, corn oil, waste cooking oil[2]. Algal biofuels are seen as one of the most promising solutions of global energy crisis and climate change in the years to come. Major advantages of algae are potentially high yield and no competition with food crops for arable land and fresh water resource[3]. To achieve environmental and economic sustainability, the renewable energy sources like solar, wind, tidal and use of biomass is essential. Many governmental organizations and corporations have taken serious interest on production of liquid biofuels which are derived from biomass. The existing alternatives such as ethanol and hydrogen production have less impact, because of cost competitiveness with petroleum. The biodiesel derived from biomass is advantageous because the fuel can be used in existing diesel engines and can be used with or without blending with petroleum and diesel in engines[4].Microalgae can be used as an alternative fuel energy source because it contains lipids, which can be extracted, processed, and converted into transportation fuels using available technology. Biodiesel production using microalgae offers the following advantages: (1) microalgae have a rapid growth rate and can be grown without plantation on the land: (2) microalgae cultivation consumes less water than land crops: (3) microalgae have the same process with higher plants to reduce the greenhouse effect with capture of carbon dioxide in photosynthesis reaction using energy from light, so that it is environmental friendly[5]. Microalgae are photosynthetic microorganism which converts sunlight, water and Co_2 to sugars, from which macromolecules such as lipids and triacylglycerides (TAGs) can be obtained. The TAGs are the promising and sustainable feedstock for biodiesel production. Many micro algae has the ability to produce substantial amounts (20-50%) of triacylglycerols (TAGs) as a storage lipid under photo-oxidative stress or other adverse environmental conditions. However, the expression of genes involved in fatty acid synthesis is poorly understood in micro algae. Algae are the major health indicator of oceans in which 71% of the earth surface is covered by these species. Algae are the original source of fossil carbon found in the crude oil and in natural gas. Microalgae, which cover almost 75% algae species, contribute approximately 40% of the oxygen in the atmosphere[6]. The pH value for optimum growth of algae ranges between 7-12. Every algal species has a different optimum salinity range. It found a salinity range of 10 to 34 ppt for growth of clones of Emiliania huxleyi.[7]

MATERIALS AND METHODS

(i) Collection of sample

Fresh water algae sample was collected from river Yamuna (saraswati ghat), pond (yeshu darbar) or fresh water bodies (college campus). Culturing of blue green algae was carried out on

agar solified (2%) BG-11 media[8] on sterilized petriplates with chromic acid and, the culture was grown for one week at 22 ^{0}C in culture room. Sample was collected by pressing a sterile test tube into an algal mass of steam water or can be collected by sterilized pipette for specific sample collection or either by collecting crude mass with a spatula and placing in a petridish, Bottle or conical flasks. During the time when the sample was collected, temperature and pH of fresh sample were recorded.

(ii) Isolation of blue green algae

The sample was centrifuged in 3 ml DW at 5000rpm for 3 mins and Supernatant was discarded and pellet was used and Dilution was carried out and pellet was further purified by serial dilution technique till 10^{-1} and 10^{-2} dilution. After autoclave pour 25 ml of mediad with 1-1.5% agar then Leave a side for solidification and moisture formation was avoided.After solididification, inoculums of about 1 mL were added by spread method and then plates in a growth chamber were incubated at 25+1^{0}C temperature with 1.2+0.2 klux light intensity and 16:8 light dark cycles for 3 days. After all these process when carried out growing colonies of microalgae were observed.

(iii) Maintaining blue green algae culture by sub-culturing

Sterilization of all transfer pipettes was carried out before beginning the culture transfers. All the glass wares were washed thoroughly, rinsed several times and were soak in hot water for a final rinse. 100 ml flask was used and filled approximately with 40 ml with freshly prepared and sterilized media; a small amount (1ml) of inoculums from a stock culture was added. Media was sterilized to culture on agar by using sterile technique; alga from stock culture was transferred in the petridish containing solidified agar media and was spread with the help of spreader and then, they were kept at an optimum condition.

(iv) Identification of blue green algae

After the isolation of blue green algae the colonies from the culture blue green algae were taken out with the help of blade and were centrifuged with DW at 5000 rpm for 3 mins. Now a drop of aliquot was kept on glass slide and cover slip was placed over .Slide was observed under microscope (40X).The identification of the algal cultures was done by observing under the compound microscope up to genera level.The morphological characters considered for identification of blue green algae were cell structure, chlorophyll present, spines present on the cell.The algal samples were collected and analyzed were identified as *spirogyra, chlorella, claymydomonas, and cynobacteria.*Based on rate of growth in cultural media and morphological characters and biomass production, 8 colonizing blue green algae isolates were selected for the further biofuel production work. To further narrow down the number of isolates, the carbonic anhydrase enzyme assay was used as biochemical marker for identifying high CO_2 fixation or hydrocarbon accumulation in the isolated colonizing blue green algae. Based on carbonic anhydrase enzyme assay (Enzymatic Assay of carbonic anhydrase (EC 4.2.1.1) Tris-Sulfate Buffer) of selected algal isolates, three potential high CA activity colonizing micro algae were selected for further biofuel production work.

(v) Evaluation of blue green algae growth and biomass production using different pH stress

Freshly prepared media with different pH was autoclaved and transferred into 24 sterile test tubes (5 ml in each test tube). In each test tubes 1 ml of algal solution was inoculated (after serial dilution) and kept under optimum condition for sample up to 21 days. Each pH experiment was run in triplicate. If the pH differed by more than 0.2 from the target pH, it was adjusted by addition of small amounts NaOH or HCl. The pH was measured by pH meter clonizing blue green algae were selected for further biofuel production work.

(vi)Growth determination of blue green algae

The algal growth was investigated in liquid media of BG-11 for 21 days and the growth was calculated in gram biomass per liter using blue green algae growth formulae and evaluated for

growth enhancement. Absorbance was determined by Spectrophotometer (Systronics-167) at a wavelength of 680nm.The growth was estimated by taking absorbance using Spectrophotometer .The growth was calculated using formula to [9].

Growth (gm/l) = 0.939 X A_{680} +0.011 (r2=0.994).

(vii) Biodiesel production

The methyl esters (biodiesel) production was carried out according to following methods:

1 .Harvesting

The algal culture of different concentration was filtered with the help of filter paper then weighed separately. Then the filtrate was dried in Hot Air Oven at 60-80^{0}C for 2 hrs. And It was carried out 1:20(w/v) dry biomass and petroleum ether (60-80^{0}C)[10]. The mixture of dry biomass and petroleum ether was taken in reflex condenser and add 25 ml of N/2 alcoholic KOH was added and it was then reflex it for half an hour or till oil from at the end.

2.Extraction of TAG or fatty acid by partition chromatography

The end product was mixed with diethyl ether in ratio (1:50) separating funnel with 1 ml of 1% KOH is added which acts as sponifiny agent .the mixture is separated in two distint layers.the upper layer of golden brown colour is triacylglyceride (TAG).Transfer this oil crude into separating funnel and add diethyl ether in ratio 1:50 then shake vigorously and then leave it undisturbed for few mins.A layer of fatty acid will be formed at lower part of the separating funnel .Separate these layer into a test tubes covered with aluminium foil and seal it with a cork.

3. Transestreification of Triacylgylcerides (TAG) to produce methyl esters

TAG was collected using pippet and mixed in it it three times 80% methanol and catalyst potassium hydroxide (1%KOH) after then took it into a beaker and placed it hot air oven at 65^{0}C for 90^{0}C min. Methyl ester and glycerol was obtained in 3:1 ratio.

4. Biodiesel recovery

Biodiesel was purified by using partion chromatography.Methyl ester and glycerol mixture was placed in separating funnel along with distilled water. Since glycerol was soluble in distilled water hence two separate layers was observed.The upper layer was bio-diesel and lower was glycerol. Extracted oil (TAGs) we used in making bio-diesel consists of triglycerides in which three fatty acid molecules are esterifies with a molecule of glycerol. In making biodiesel, triglycerides are reacted with methanol in a reaction known as transestrification or alcoholysis. Transestrification produces methyl esters of fatty acids that are biodiesel and glycerol. The reaction occours stepwise trigylcerides are first converted to diglycerides, then to monoglycerides and finally to glycerol.Transestrification requires 3 mol of alcohol for each mole of triglyceride to produce 1 mol of glycerol and 3 moles of methy esters. The reaction is equilibrium. Industrial processes use 6 mol of methanol for each mol of trigyceride.

RESULTS AND DISCUSSION

The investigations were carried out to isolate and growth prospecting of fresh water blue green algae for biofuel production. The following results were obtained during the study.

1. Isolation and identification

The blue green algae were isolated based on morphological characteristics on BG-11 medium.The plates were kept at 22^{0}C at light intensity of 1600 W. The growth was appeared after 48 hrs and the proper colonial blue green algae were isolated at 72 hrs for the growth studies and TAG production. During the investigation the following blue green algae was isolates and identified on BG 11 media which was collected from Allahabad region ,was identified under electric and compound microscope.

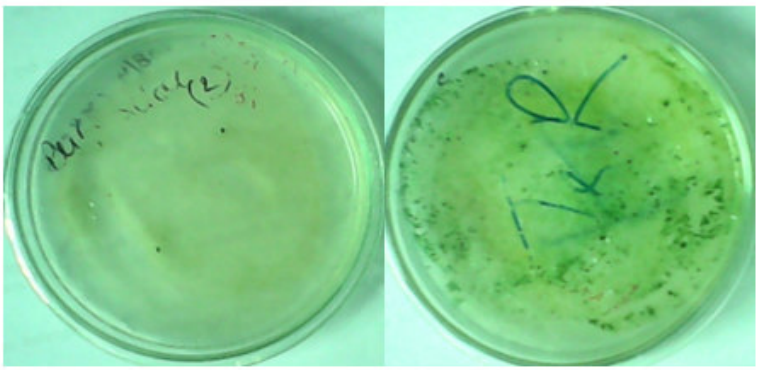

Figure 4.1
Isolates colonies of blue green algae in BG-11 media

2.Growth evaluation of selected algal isolates

Based on carbonic anhydrase enzyme assay of selected algal isolates, three potential high carbonic anhydrase activity colonizing blue green algae (BIOLBG-6, BIOLBG-7, and BIOLBG-8) were selected for further biofuel production work. The algal growth was investigated in liquid media of BG-11 for 21 days and the growth was calculated in gram biomass per litre using blue green algae growth formulae and evaluated for growth enhancement.

Table 1
BIOLBG-6 growth evaluation under different pH.

pH	Biomass of blue green algae g/L						
	Day 3	Day 6	Day 9	Day 12	days 15	days 18	days 21
5	0.02	0.08	0.10	0.10	0.11	0.12	0.13
6	0.02	0.02	0.04	0.06	0.08	0.14	0.20
7	0.10	0.12	0.15	0.18	0.19	0.28	0.33
8	0.04	0.08	0.14	0.19	0.20	0.21	0.22
9	0.05	0.14	0.21	0.21	0.24	0.31	0.36
F-test					S		
S.Ed					0.041		
CD(0.05)					0.082		

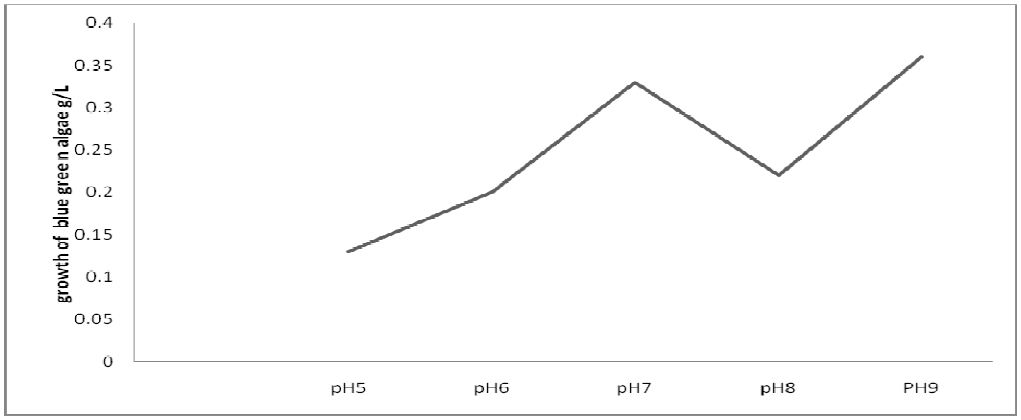

Figure 4.2
BIOLBG-6 growth evaluation under different pH on 21 days

Table 2
BIOLBG-7 Growth evaluation under different pH

pH	Biomass of blue green algae g/L						
	Day 3	Day 6	Day 9	Day 12	days 15	days 18	days 21
5	0.06	0.09	0.10	0.14	0.15	0.18	0.19
6	0.03	0.11	0.11	0.12	0.13	0.14	0.14
7	0.08	0.09	0.09	0.14	0.14	0.15	0.28
8	0.15	0.15	0.16	0.17	0.18	0.21	0.26
9	0.13	0.15	0.18	0.19	0.19	0.21	0.22
F-test					S		
S.Ed					0.023		
CD(0.05)					0.048		

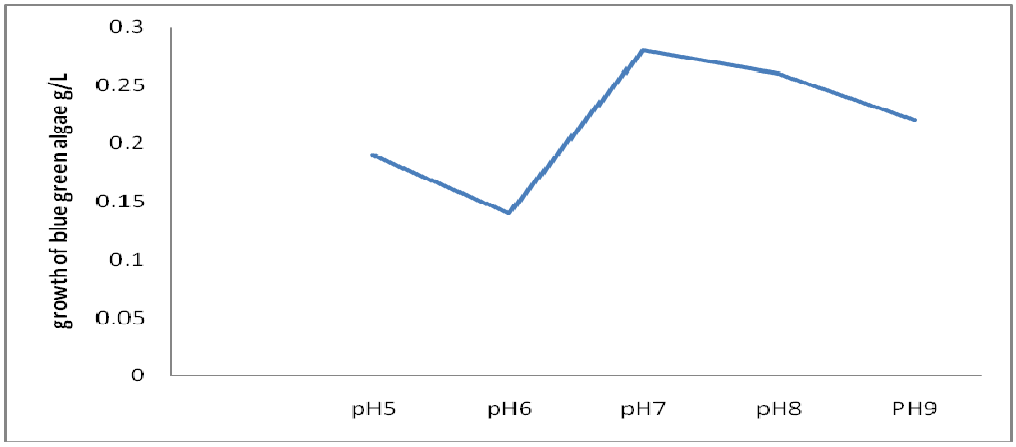

Figure 4.3
Growth evaluation under different pH on 21 days

Table 3
BIOLBG-8 Growth evaluation under different pH

pH	Biomass of blue green algae g/L						
	Day 3	Day 6	Day 9	Day 12	Day15	Day 18	Day 21
5	0.02	0.07	0.09	0.09	0.11	0.12	0.15
6	0.01	0.04	0.07	0.09	0.12	0.09	0.24
7	0.02	0.01	0.12	0.13	0.13	0.14	0.14
8	0.09	0.09	0.14	0.18	0.19	0.20	0.21
9	0.14	0.15	0.18	0.18	0.19	0.21	0.22
F-test					S		
S.Ed.					0.02927		
					0.055		

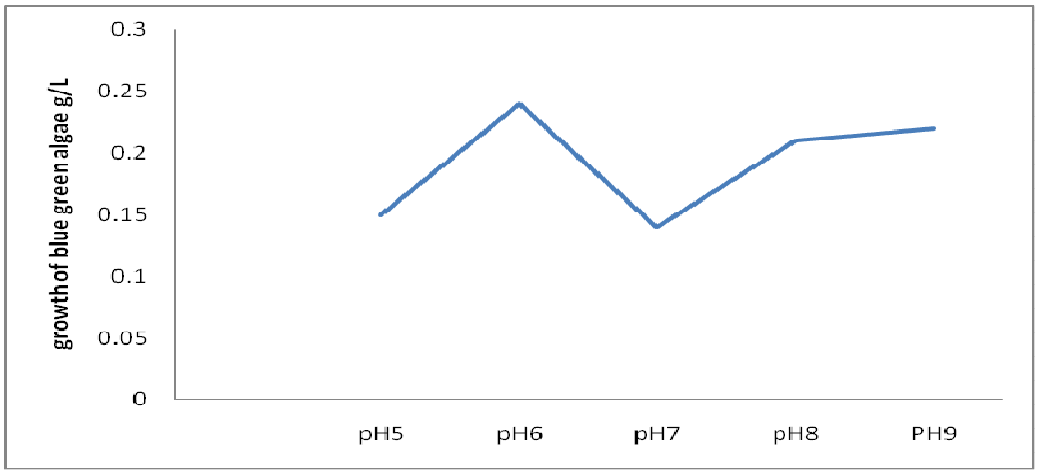

Figure 4.6
Growth evaluation under different pH on 21 days

Among all the pH enrichment algal samplas growth of BIOLBG-6 was found maximum in pH 9 where at 21th day yield was 0.36mg/ml and minimum was found in 0.05 mg/ml.Statical analysis shows significant difference under different pH stress.

3. TAG estimation and Biodiesel potential

In our experiment, extracted TAG's by reflex digestion ,petroleum ether was added in 1:20 ratio ,in dry biomass and the mixture was digested in evaporation condenser .The gel type end product obtained .To it diethyl ether was added in 1:50 ratio and TAG's was separated by partial choromotography.

Table 4
Triglyceride content (ml/g d.w.) from blue green algae and MEFA production

S.No	pH	Sample code	TAG (Ml/g D.W.)	MEFA ml
1	5	BIOLBG-6	0.01569	nill
		BIOLBG-7	0.015	nill
		BIOLBG-8	0.0153	nill
2	6	BIOLBG-6	0.0139	2
		BIOLBG-7	0.142	0.5
		BIOLBG-8	0.07073	0.5
3	7	BIOLBG-6	0.18107	2
		BIOLBG-7	0.1248	2
		BIOLBG-8	0.0752	1
4	8	BIOLBG-6	0.15483	3
		BIOLBG-7	0.13367	2
		BIOLBG-8	0.101	1.5
5	9	BIOLBG-6	0.16733	2
		BIOLBG-7	0.13695	1
		BIOLBG-8	0.181	0.5

DISSCUSSION

In the past, pH has not been considered to be an important chemical parameter influencing biotic processes in marine environments. However, a number of studies have shown that pH and, in some cases, inorganic carbon may be important in regulating the growth rate and distribution of marine algae[11].Directly or indirectly pH seems likely to be an important factor in determining why oligotrophic species do not grow in hard-water lakes. Effects of pH on growth do not, however, explain why growth of eutrophic algae does not supersede that of oligotrophic algae in soft waters. This problem is discussed by[12]. There are several ways in which high pH might exclude oligotrophic algae from eutrophic waters: (1) an intrinsic effect of pH on enzymes, in the cell wall or membrane, responsible for uptake of one or more essential nutrients; (2) inability of oligotrophic species to absorb trace elements present in low concentration at high pH; (3) a toxic effect of relatively high total dissolved ion content associated with high pH; (4) coprecipitation of phosphate with calcium, magnesium, and carbonate at high carbonate levels; (5) a direct toxic effect of carbonate or of hydroxide ions, levels of which increase with increasing pH; (6) differential availability of different inorganic carbon compounds for photosynthesis.Investigated the tolerance pH in some species of microalgae .The species showed growth pH 8.1and above. Doubling rate has observed as 0.64 l/g at pH 8.1 where as maximum 0.67 l/g at pH 9.05. This alga will show good growth pattern if pH maintained from 8.01 to 9.05, however decrease in growth rate had been observed above pH 9.20. Maximum growth rate was observed at pH 8.43. Overall moderate growth pattern observed at pH neutral to basic. No growth has observed at pH above 9.35. The alga is more comfortable with basic pH as compare to acidic[13].The effect of pH of the growth of the green algae and *Cyanobacteria* was studied using AF6 and BG11 media in different pH level viz 4,6,7,8 and 9 .[14] *C*. pyrenoidosa was cultivated in BG-11, Bold's Basal, Bristol and Fogg's medium and growth was evaluated as cell count in all the media. BG-11 medium showed remarkably high growth rates (14.2 x 104 cells / ml / d) as compared to BBM (2.68 x 104 cells / ml / d), Bristol (5.2 x 104 cells / ml / d) and Fogg's (3.6 x 104 cells / ml / d) medium and was therefore used as an optimum growth medium for further studies[15].

CONCLUSION

Fresh water bodies were undertaken for investigating the occurance and identification of blue green algae which are rich in hydrocarbon and related as a renewable source of energy and a potential model for carbon dioxide fixation, fresh water blue green algae was isolated on BG-11 nutrient media the growth was enhanced by using different pH .the colonies were isolated and after centrifugation with distilled water they were inoculated into BG-11 media .the culture were maintained by sub culturing and biomass was obtained by spectro (1ml inoculums was used for each concentration in 10 ml BG-11 media) at 680nm for 21 days.On the basis of results obtained it can be concluded that the best growth of biomass (0.15483 ml/g D.W.) was observed at pH 8 in BIOLBG-6 group of fresh water microalgae, in this group conversion of biomass to TAGs and its further conversion to Biodiesel was found maximum thus, we can use this group of blue green algae for industrial production of methyl ester (Bio-diesel).

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