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Research Article

Microalgae Cultivation in Different pH, Temperature and Media for Lipid Production

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ABSTRACT

Lipids produced by microalgal biomass can be grouped into nonpolar lipids and polar lipids, which can be easily converted into biofuels. Microalgal samples were collected from three different ponds of Bangalore and cultured in the laboratory to find the effect of different pH, temperature and media on the production of biomass and lipids. Among these, pH-9, temperature -25C and Beneck's media was most suitable for production of biomass (35.80 g/L) and lipids from the isolated microalgae Chlorella sp. compare to Chladospora sp. (13.33 g/L). Chlorella sp. Showed 0.32 (OD) at pH-9, 0.43 (OD) at temperature-25C and 2.94 (OD) in Beneck's media. Our result revealed that nutrient supply along with measured variables affects the production of biomass and lipids in different microalgae.

INTRODUCTION

Algae are the best suitable feedstock for biofuel production because of their low cost, high growth and high biomass production rates (Thurm, 1997). Microalgae are the diverse group of eukaryotic microorganisms which will grow in both fresh and marine environments. Nowadays microalgae are used to produce biofuels; the biofuels are mainly classified into first, second and third generation biofuels (Naik et al., 2010). Biofuels can be produced by using biomass as a sustainable biological resource and to produce a good quality and pilot scale of biofuel. Selection of a suitable biomass is required (Abdeshahian et al., 2010). There are certain elements which are most important for the growth of microalgae, for example N, P, and K elements are added in the form of salts (Michael and Navid, 2013). The selection of a best medium is very much essential for growing microalgae and to obtain a biomass, different nutrient components influence the growth of algae. Before going to modern techniques involved in biofuel production from microalgal lipid, it is important to optimize the microalgal growth under different physiological conditions to obtain greater biomass (Kaushik, 1987). For the growth of any microalgae, obviously defined media and other parameters like salinity and illumination is required for the growth of algae (Stein, 1973). Lipids produced by microalgae mainly contain neutral lipids, polar lipids, wax esters, sterols hydrocarbons tocopherols, carotenoids, terpenes, quinines and pyrrole derivatives such as the chlorophylls. Lipids produced by microalgae can be grouped into polar and nonpolar lipids. Polar lipids have a high content of polyunsaturated fatty acids, which are essential for animals and humans. Nonpolar lipids are in the form of triacylglycerides (TAGs) made up of saturated fatty acids and some unsaturated fatty acids which can be transesterified to produce biodiesel (Rat and Priyadarshani, 2012). Under favourable conditions microalgae produce primarly polar lipids. However under unfavourable growth conditions microalgae accumulate neutral lipids in the cytoplasm. To obtain a large amount of biomass component, major requirement is to select the best medium. The selection of the medium depends on the several factors which include the chemical composition of the medium to obtain the maximum growth of microalgae. Hence, the main goal of the present study is to evaluate the effect of different pH, temperature, and media on the growth and lipid content of isolated microalgae.

MATERIALS & METHODS

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Microalgae culture

The algal water samples were collected from three different ponds in the city of Bangalore (India) as follows: 1. BBMP park pond- Rajarajeshwarinagar (RR Nagar); 2. BBMP park pond- Jayanagar; 3. BBMP Park pond- Bhuvaneshwarinagar. The collected samples were washed with distilled water for 2 - 3 times to remove the dust and mud particles. The cultures were transferred to plastic bottles for further studies.

Physico-chemical parameters of algal water samples

Water samples were also collected from the same sites along with algae in a sterile bottle and analyzed according to (Manjare et al., 2010) for temperature, pH, total alkalinity, Ca^{2+}, Mg^{2+}, dissolved oxygen, chemical oxygen demand, sodium and phosphorous, which favors the growth of algae.

Screening of algae isolates

To obtain pure culture, microalgae samples were spread on the plates containing standard Blue Green 11 (BG-11) medium and the plates were incubated for 10-15 days with 16:8 hours light/dark photoperiod. After incubation the cultures were subjected for continuous subculture till the pure culture is obtained (Asulabh et al., 2012).

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Identification

The isolated microalgae were identified microscopically according to algae identification field guided by (Huynh and Serediak, 2006).

Preparation of media and inoculation

Standard BG-11 media was prepared (Kuhl, 1964) and autoclaved at 121C for 15 min. The effect of pH, temperature, and media on the growth of isolated algae cultures and their lipid production was carried out using the following method. All the experiments were carried out in triplicate.

About pH

The pure cultures of microalgae were cultivated in 250 mL Erlenmeyer flasks containing 100 mL of standard Blue Green-11 media; the pH was set to 8.0, 9.0, and 10.0, respectively. The flasks were incubated for 10-15 days at 30°C with 16:8 hours of photoperiod (Asulabh et al., 2012).

Temperature

The pure cultures were inoculated into the standard BG-11 media, suspended in 250 mL Erlenmeyer flasks for optimizing the growth at different temperature. After inoculation the flasks were incubated in different temperature 30°C, 35°C, and 40°C for 10 - 15 days with 16 : 8 hours of photoperiod (Nurul Salma Adenan et al., 2013).

Preparation of Growth Media

The effect of media on the isolated cultures was studied. The microalgal cultures were cultivated in three different modified media like Zarrouk's media, Rao's media (Pandey et al., 2010) and Beneck's media (Aneja, 2003). The three media were prepared according to the composition given in Table1. Thus prepared 100 mL of medium was suspended into different 250 mL Erlenmeyer flasks and autoclaved at 121C for 15 min. After cooling, the culture was inoculated and incubated at room temperature for 10-15 days (Shilpkar et al., 2010).

Table 1. Media composition per litre

Sl. No.	Modified Zarrouk's media	Modified Rao's media	Modified Beneck's media
1.	$NaHCO_3$- 16.8g	Sodium bicarbonate-18g	KH_2PO_4-8.75g
2.	K_2HPO_4– 0.5g	Di-potassium hydrogen phosphate-1.5g	Calcium chloride-1ml
3.	$NaNO_3$-2.5g	Sodium nitrate-2.50g	Magnesium sulphate-3.75g
4.	Potassium sulphate-1g	Potassium sulphate-0.60g	Sodium nitrite-12.5g
5.	NaCl-1g	Sodium chloride-0.40g	K_2HPO_4– 0.5g
6.	$MgSO_4$-0.2g	Magnesium sulphate-0.04g	NaCl-1g
7.	Calcium chloride- Stock 4%-1ml	Calcium chloride- Stock 4%- 1ml	$MgSO_4$-0.2g
8.	$FeSO_4$ solution (1%)- 0.1ml	Fe-Ethylene diamine tetra acetate-0.08g	EDTA- 10g
9.	EDTA- 0.08g	A5 Solution- 1ml	H_2SO_4- 1ml
10.	Boric acid-1.8g		
11.	$Mncl_2$-1.8g		

Growth studies

After incubation, microalgal growth of each experiment was evaluated by measuring the optical density at 540 nm against media as a blank. The procedure was repeated for 10 days at regular time intervals of 0, 24, 48, 72, 96, 120, and 144 hours (Dong Yan et al., 2011).

Biomass determination

For each experiment, the cells were harvested by centrifugation at 3000 rpm for 10 min and washed with distilled water. The initial values were recorded and the biomass was dried in a hot air oven at 50C overnight. The obtained dry biomass was weighed and the difference between initial and final readings was expressed in terms of grams per litre (Lee and Zhu, 1997).

Analytical methods

The dried biomass was ground with a two solvent

system of chloroform: methanol (2:1, v/v) and hexane separately. The samples were then transferred to 50 mL falcon tubes and centrifuged at 3000 rpm for 10 min. The supernatant was collected and washed with 1% sodium chloride for three times by vigorous shaking. The layers formed were separated and the layer containing lipids with the solvent was evaporated in a rotary evaporator at 70°C under vacuum. After evaporation the sample was dissolved in two solvents, chloroform and hexane, and subjected to thin layer chromatography (Chiara Samori, 2013).

Thin layer chromatography

In order to check the effect of different pH, temperature and media on the lipid content, it is necessary to separate the lipids by thin layer chromatography (TLC). The solvents containing lipids were spotted on a silica TLC plate with a control containing C12, C14, C16, C18 carbon atoms (Joseph Sherma, 2000). Following the drying process, the plate was developed in mobile phase hexane and chloroform, 9:1 (v/v). The plate was removed from the mobile phase tank and dried. The dried plates were placed in an iodine chamber and visualized following which the Rf value was calculated.

RESULTS & DISCUSSION

The physico-chemical parameters of water sample collected from RR Nagar pond revealed the highest values, when compared to Bhuvaneshwarinagar and Jayanagara pond waters. The parameters observed in the RR Nagar pond water favoured the growth of algae when all these are supplied in a suitable media (Table 2).

Table 2. Physico-chemical parameters of different pond water samples

Parameters	BBMP park pond RR Nagara	BBMP park pond Jayanagar	BBMP park pond Bhuvaneshwarinagar
pH	8.7±0.0	8.9± 0.0	9.0±0.0
Temp.	28^0± 0.0C	30^0±0.0C	28.2^0±0.0C
Total alkalinity	134±0.0 mg/l	120 ±0.0mg/l	121±0.0mg/l
Ca $^{2+}$,	72±0.0 mg/l	55 ±0.0mg/l	30±0.0 mg/l
Mg $^{2+}$	5.2±0.0ppm	3.91±0.0ppm	4±0.0ppm
DO	5.5±0.0mg/l	5.2 ±0.0mg/l	4.0 ±0.0 mg/l
COD	35 ±0.0mg/l	26 ±0.0mg/l	10.2±0.0 mg/l
Na	2.7±0.0ppm	5.1± 0.0ppm	6.0±0.0ppm
P	0.15 ±0.0 mg/l	0.1± 0.0 mg/l	0.8±0.0mg/l

Identification

The algae obtained from different ponds after pure culture were identified as *Chlorella sp*. and *Chladospora sp.*

Growth studies

In growth studies, the Chlorella sp. showed more growth in pH-9 (OD-0.32) and less growth in pH-10 (OD-0.08). On the other hand, Chladospora sp. showed more growth in pH-8 (OD-0.27) and less growth in pH-10 (OD-0.03). Further, it was found that the growth was less in 24 hours when compared to the growth in 144 hours (r^2= 1) (Table 3 and Fig. 1). With respect to temperature Chlorella sp. And Chladospora sp. showed more growth at 25°C (OD- 0.43 and 0.36). No growth was seen at 30C and 35C (r2= 0.997) (Table 4 and Fig. 2). In the case of the media Chlorella sp. showed the highest growth in Beneck's media (OD-2.94), minimum growth in Zarrouk's medium (OD-0.53) and optimum growth in Rao's media (OD-0.56). On the other hand, Chladospora sp. showed maximum growth in Rao's media (OD-0.73), minimum growth in Zarrouk's media (OD-0.22) and optimum growth in Beneck's media (OD - 0.29) (r2= 1). The details are presented in Table 5 and Figure 3.

Table 3. Growth pattern of algae in different pH

Duration (hrs)	Absorbance (OD)					
	pH-8		pH-9		pH-10	
	Chlorella sp.	*Chladospora sp.*	*Chlorella sp.*	*Chladospora sp.*	*Chlorella sp.*	*Chladospora sp.*
24	0.13	0.09	0.17	0.12	0.04	0.00
48	0.16	0.11	0.22	0.16	0.05	0.02
72	0.21	0.15	0.29	0.17	0.07	0.02
96	0.26	0.19	0.30	0.20	0.07	0.03
120	0.29	0.22	0.31	0.22	0.08	0.03
144	0.30	0.27	0.32	0.24	0.08	0.03

Table 4. Growth pattern of algae in different time intervals

Duration (hrs)	Absorbance (OD)					
	Temp-25^{0}C		Temp-30^{0}C		Temp-35^{0}C	
	Chlorella sp.	*Chladospora sp.*	*Chlorella sp.*	*Chladospora sp.*	*Chlorella sp.*	*Chladospora sp.*
24	0.18	0.12	-	-	-	-
48	0.22	0.17	-	-	-	-
72	0.27	0.21	-	-	-	-
96	0.32	0.26	-	-	-	-
120	0.37	0.30	-	-	-	-
144	0.43	0.36	-	-	-	-

Table 5. Growth pattern of algae in different media

Duration (hrs)	Absorbance (OD)					
	Zarouk's media		Rao's media		Beneck's media	
	Chlorella sp.	*Chladospora sp.*	*Chlorella sp.*	*Chladospora sp.*	*Chlorella sp.*	*Chladospora sp.*
24	0.12	0.09	0.23	0.23	0.61	0.18
48	0.18	0.11	0.28	0.31	0.83	0.21
72	0.28	0.14	0.36	0.48	1.46	0.24
96	0.34	0.17	0.44	0.51	1.5	0.25
120	0.41	0.20	0.49	0.55	2.5	0.27
144	0.53	0.22	0.56	0.73	2.94	0.29

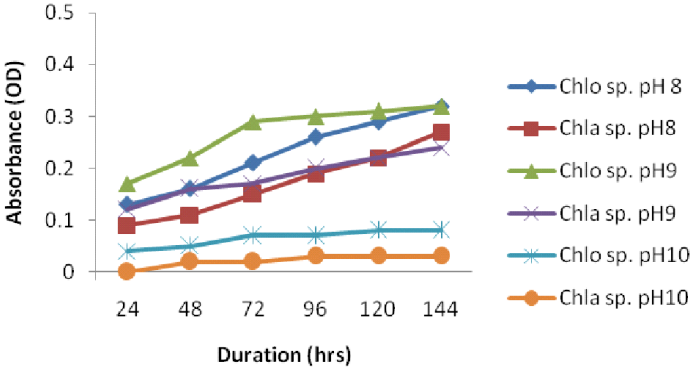

Figure 1. Growth curve of algae in different pH

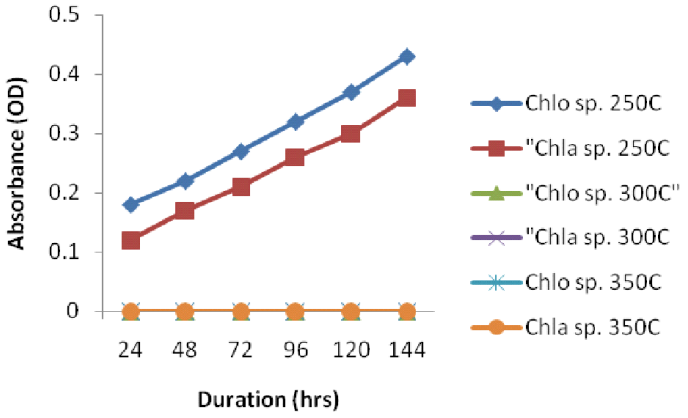

Figure 2. Growth curve of algae in different temperature

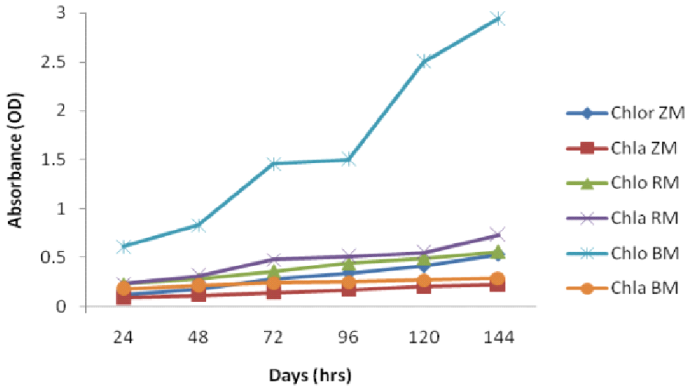

Figure 3. Growth curve of algae in different media

Biomass estimation

The wet biomass of the *Chlorella sp.* and *Chladospora sp.* was found to be 35.8 g/L and 13.33g/L, respectively. The dry biomass of these cultures was found to be 4.45g/L and 12.0g/L, respectively.

Thin layer chromatography

By Nile red staining *Chlorella sp*. showed lipid droplets inside the cell, whereas *Chladospora sp.* did not show the lipid droplets (data not shown). Hence TLC was performed only for *Chlorella sp.* Results from the TLC experiment shows that the solvent front (X) was 7.75 cm and the solute front (Z) was 5.5 cm. The Rf for the non-polar compound is therefore X/Z = 7.75 cm / 5.5 cm = 1.40. Fraction 1 contains standard C14 (myristic acid), C16 (palmitic acid) and C18 (stearic acid) carbon compounds. In this experiment, the C16 and C18 compounds were not clearly separated. Fractions 2 and 3 showed that the non-polar compound has eluted from the column. Fractions 4 show three mixture of lipids, which is from the microalgae grown in Beneck's media. Fractions 5-7 showed the separation of nonpolar lipids obtained from 8, 9 and 10 pH conditions. Moreover, it can be seen that higher carbon lipids are slightly eluted from the column and the bands are very light. Fraction 8 contains very less concentration of lipid which is obtained from cells grown at 25C (Fig. 4).

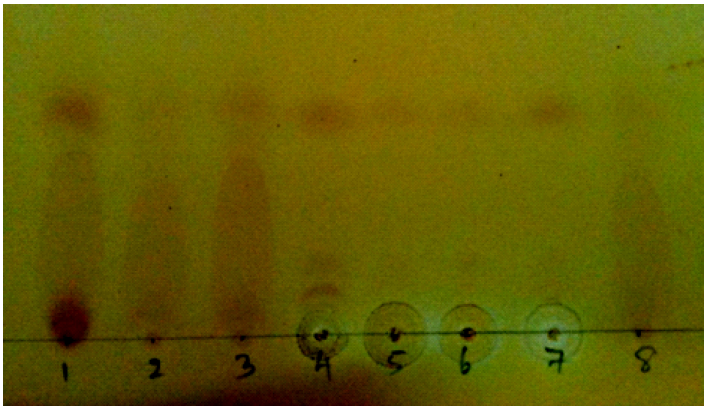

Figure 4. *Chlorella sp.* showing separation of non polar-lipids on TLC plate. Standard lipids (C14, C16, C18), 2. Lipids obtained from Zeroucks medium, 3. Lipids from Rao's medium, 4. Lipids from Beneck's media, 5. PH-8, 6. pH -9, 7. pH-10 and 8. Temperature - 25°C

CONCLUSION

The present study is the first report on growth of *Chladospora sp.* under different conditions of pH, temperature, and media, which significantly affects the cell growth. Edward (1994) has examined that some marine algae were able to grow at pH 8.8. The present study corroborates that algae can be grown above pH 8.8. The *Chlorella sp.* had a higher growth and biomass at 25C. These observations concur with the Bartosh (2007) study where the author grew *Chlorella vulgaris* under 5-25°C and obtained more growth at 15°C. Nutrients in the media play a major role in the cultivation of *Chlorella sp.* and *Chladospora sp.* This is also reported by Gopinath (2011) that certain nutrients in appropriate quantities are required for the growth of algae. Hence, the present study reveals the effect of different physical and chemical conditions on the growth of *Chlorella sp.* and *Chladospora sp.* Their growth can be improved further and there is a need to search for their low cost cultivation by using waste sources (agriculture and domestic wastes) to provide good sources of biomass to meet the primary requirements of alternative fuel production in future.

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