RESEARCH PAPER | Biotechnology | Volume : 4 | Issue : 9 | September 2014 | ISSN - 2249-555X

Studies on Cultivation of Lipid Accumulating *BOTRYOCOCCUS BRAUNII* from North Gujarat Inland Waters for Generation of 3rd Generation Biofuels

KEYWORDS: Renewable, Microalgae, Lipids, Biofuel, Photoperiod.

Kommu Sasikanth
Department of Biotechnology, Kadi Sarva Vishwavidyalaya,II Floor, KBIPER Building, Kadi Pharmacy Campus, Sector – 23, Gandhinagar.

Tyagi Jyotsna
Department of Biotechnology, Kadi Sarva Vishwavidyalaya,II Floor, KBIPER Building, Kadi Pharmacy Campus, Sector – 23, Gandhinagar.

Padhiar Anjali
Department of Biotechnology, Kadi Sarva Vishwavidyalaya,II Floor, KBIPER Building, Kadi Pharmacy Campus, Sector – 23, Gandhinagar.

Sharma M.C
Department of Biotechnology, Kadi Sarva Vishwavidyalaya,II Floor, KBIPER Building, Kadi Pharmacy Campus, Sector – 23, Gandhinagar.

ABSTRACT *Microalgae are a source of biofuels to address ever growing fuel demands and environmental problems associated with fossil fuels. KSV1, a free floating, green, unicellular, fresh water microalga, isolated from North Gujarat inland waters and provisionally identified as Botryococcus braunii has shown optimal growth and lipid synthesis in BG11medium, at 20°C, pH of 7.0 to 8.0 with 16:8 hrs Light (5.0 ± 0.2 k lux): Dark period cycle under agitation at 120 rpm. This alga exhibits tolerance to wide range of pH (4.0-10.0) and agitation (0-180 RPM) but is sensitive to light: dark period cycles and temperature. Lipid content of 15-19% of the dry weight makes this strain of B. braunii, a potential candidate for 3rd generation biofuels.*

INTRODUCTION:

The renewable biofuels are ideal substitutes for Fossil fuels that are currently fulfilling significant proportion of energy requirements, despite severe environmental problems due to emissions and their non-renewable nature (Mata et al., 2010). Microalgae have been focus of attention for their potential as resource of Third-Generation biofuels (Beer et al., 2009; Scott et al., 2010; Wij els and Barbosa, 2010; Picazo-Espinosa et al., 2011; Alam et al., 2012; Sharma et al., 2012; Lee and Lavoie, 2013; Li-Beisson and Peltier, 2013; Cheng and He, 2014), though all the algae may not be suitable for biodiesel production(Chisti, 2008; Manzanera, 2011; Picazo-Espinosa et al., 2011).

Microalgae are the organisms of choice today, being fast growing phototrophs (Li et al., 2008), with Photosynthesis at 50 times higher rates than terrestrial plants (Suali & Sarbatly,2012), thereby yielding 10 to 100 times higher lipids per unit land area than the terrestrial plants (Dayananda et al., 2007; Li et al., 2008) and higher biomass production per unit area and higher hydrocarbon / lipid / carotenoid content per unit dry weight (Pirt, 1986; Sawayama et al., 1994; Sheehan et al., 1998; Banerjee et al., 2002; Metzger & Largeau, 2005; Chisti, 2007; Dayananda et al., 2007a;Patil et al., 2008; Sathya & Srisudha, 2013). Many research reports and articles describe advantages of using microalgae for biodiesel production in comparison to other available feed stocks (Sheehan et al., 1998; Tsukahara & Sawayama, 2005; Chisti, 2007; Hossain et al., 2008; Hu et al., 2008; Li et al., 2008a; Li et al., 2008b; Rosenberg et al., 2008; Schenk et al., 2008; Rodolfi et al., 2009). Microalgae can be feedstock for several different types of renewable fuels such as biodiesel, methane, hydrogen and ethanol (Mata et al., 2010). Algae biodiesel contains no sulphur and performs as well as petroleum diesel, while reducing emissions of particulate matter, CO, hydrocarbons and SOx. However emissions of NOx may be higher in some engine types (Malcata, 2011). Bio-fixation of CO2 by algae (Hirano et al., 1997) also reduces the GHG emissions significantly (Maršálková et al., 2010).

Microalgal oils have potential to completely replace petroleum as a source of hydrocarbon feedstock for the petro chemical industry (Bajhaiya et al., 2010) and the algal strains with high oil content are of great interest in search for sustainable feedstock for the production of biodiesel (Chisti,2007; Bajhaiya et al.,2010; Spolaore et al. 2010). In view of their ability to grow under harsher conditions, and their reduced needs for nutrients, Alga are easy to cultivate, can be grown in areas unsuitable for crops, do not compete for arable land use, grow with little or no care, completing an entire growth cycle every few days, getting adjusted to local environment, and can utilize wastewater streams (including municipal effluents) unsuitable for human consumption as the culture medium (Hannon et al., 2010). According to Aslan & Kapdan (2006), Picazo-Espinosa et al., (2011) and Zhou et al., 2011 the production of microalgal biodiesel could be combined with the treatment of waste water for removal of nitrogenous and phosphorus pollutants.

Various algal forms have the capacity to produce and accumulate energy-rich oil content in the biomass (Rodolfi et al., 2009). Botryococcus spp. have been reported to contain up to 50% of their dry mass as long- chain hydrocarbons (Kojima & Zhang, 1999). According to Mata et al., (2010), the oil content in microalgae can reach 75% by weight of dry biomass (e.g. Botryococcus braunii). The most frequented algae Botryococcus, Chlorella, Crypthecodinium, Cylindrotheca, Dunaliella, Isochrysis, Nannochloris, Nannochloropsis, Neochloris, Nitzschia, Phaeodactylum, Porphyridium, Schizochytrium and Tetraselmis store oil levels between 20 and 50% and even higher productivities can be achieved through optimization. There are several factors influencing algal growth including temperature, nutrients, CO_2, pH, salinity, agitation as well as a few other biotic and abiotic

Volume : 4 | Issue : 9 | September 2014 | ISSN - 2249-555X

factors. Thomas et al., (1984) concluded that cultivation of Botryococcus, Isochrysis and Dunaliella species under nitrogen deficient conditions /stress increase the lipid contents in these microalgae. Macedo and Alegre (2001) observed that decrease of nitrogen content and temperature resulted in approximately 3 times increase in Spirulina lipids. Lipid inducing stress conditions for microalgae described by Sharma et al. (2012) and Rukminasari (2013) include nutrient limitation, temperature (low, high) shift, change in salinity & pH and light / dark cycles during the production period as determinants of the lipid content.

In an attempt to explore the utilization of Microalgae native to Gujarat's rural water-bodies as biofuel feedstock, a free floating microalga from Panchot village in Mahesana of North Gujarat was isolated, purified and cultivated in laboratory to optimize conditions for growth and lipid yields in this study. Pheneticidentification has been attempted.

MATERIALS & METHODS:

Sample collection, isolation, purification and Phenetic identification:

Water samples from surface of a large pond (Fig. 1a) at Panchot (Latitude 23.63° E and Longitude 72.34° N), in District Mahesana (Gujarat) were collected during winter. Microscopic examination and plating of serial dilutions on Himedia's Rose Bengal (RB) Chloramphenicol Agar and BG11 agar (Hong et al., 2012) containing Bavistin (1,250 µg/ml) were carried out for bacteria and fungi contamination free isolations. Uniform green microalgal isolates as single colony were selected (hereafter termed as KSV1) and maintained on BG11 medium slants at 20 + 1°C.

Optimization of Media and Culture Parameters:

For the growth studies and optimization, the culture from slants was inoculated in 50 mL of BG-11 medium in 250 mL flask and incubated on Orbital shaker at 120 RPM and 20°C+ 1°C under 5.0 ± 0.2 k lux light intensity with 16:8 hrs of light: dark cycles. 10 mL of growth obtained after 7 days was used to inoculate 100 mL of algal growth media including Bold's

Table 1. Composition of Various Culture Media** utilized for the growth of KSV 1**

Component	Modified Bold's Basal (g/L)	Modified	Modified	Modified	Modified
$NaNO_3$	0.250	0.250	1.500	------	0.250
$CaCl_2.2H_2O$	0.025	0.025	0.036	0.0362	0.025
$MgSO_4.7H_2O$	0.075	0.075	0.007	0.0044	0.075
K_2HPO_4	0.075	0.075	0.040	0.0572	0.075
KH_2PO_4	0.175	0.175	-------	-------	0.175
NaCl	0.025	0.025	-------	-------	0.025
H_3BO_3	0.028	-------	-------	-------	-------
Citric Acid	-------	-------	0.006	0.0008	-------
Amm. Ferric Citrate	-------	-------	0.006	-------	-------
$Na_2EDTA.2H_2O$	-------	-------	0.001	-------	-------
Na_2CO_3	-------	-------	0.020	-------	-------
KNO_3	-------	-------	-------	0.0002	-------
Ferric Citrate	-------	-------	-------	0.0016	-------
Peptone	-------	-------	-------	-------	0.010
Others	1mL of BB Trace Mix*	-------	1 ml of BG-11 Trace Mix#	1 mL of Chu13 Trace Mix•	-------
	1mL of EDTA stock**	-------	-------	1 drop of 0.072 N H_2SO_4	-------
	1mL of Fe solution***	-------	-------		-------

*BB Trace Metal Mix ($ZnSO_4·7H_2O$ - 8.82 g/L, $MnCl_2·4H_2O$ -1.44 g/L, MoO_3 - 0.71 g/L, $CuSO_4·5H_2O$ - 1.57 g/L, $Co(NO_3)_2·6H_2O$ - 0.49 g/L); ** EDTA stock ($EDTANa_2$ - 50.0 g/L, KOH - 31.0 g/L); ***Fe solution ($FeSO_4·7H_2O$ - 4.98 g/L, Conc. H_2SO_4 - 1.0 ml); #BG-11 Trace Metals Mix (H_3BO_3- 2.86 g/L, $MnCl_2·4H_2O$ - 1.81 g/L, $ZnSO_4·7H_2O$ - 0.22 g/L, $Na_2MoO_4·2H_2O$ - 0.39 g/L, $CuSO_4·5H_2O$ - 0.079 g/L, $Co(NO_3)_2·6H_2O$ - 49.4 mg/L); •Chu13 Trace metal Mix ($CoCl_2$ - 0.02 g/L, H_3BO_3 - 5.72 g/L, $MnCl_2·4H_2O$ - 3.62 g/L, $ZnSO_4·7H_2O$ - 0.44 g/L, $CuSO_4·5H_2O$ - 0.16 g/L, Na_2MoO_4 - 0.08 g/L).**** Autoclaved at 121°C for 20'

Basal, Bristol, BG11, Chu13 and Proteose media detailed in Table 1 (composition details based on information from http://www.ccap.ac.uk/media/pdfrecipes.htm collected on 29.05.2012) in 250 mL flasks to determine the optimal media for biomass yield. The flasks were incubated as above for 14 days, whereafter the growth was centrifuged and the pellet washed with distilled water 2-3 times and oven dried. The dry algal mass obtained in various media was weighed. All the experiments were carried out in triplicates.

For studying the impact of Temperature on growth and Lipid biosynthesis, the cultures were grown at 15°C, 20°C, 25°C, 30°C and 35°C in BG-11 medium flasks. For the study of the effect of pH on growth, it was grown similarly on BG-11 with pH values ranging from 4.0 – 10.0. To study the effect of agitation on the growth of KSV1, the cultures were grown in BG11 medium at 20 + 1°C on orbital shaker maintained at agitation speeds: 0, 60, 120 and 180 RPM. To check the impact of length of Photoperiod, KSV1 was grown in BG11 medium maintained at 20 + 1°C under static conditions, with photo periods of 4:20, 8:16, 16:8 and 20:4 hrs of light: dark periods. The % dry biomass was calculated and lipids in the biomass of KSV1 from various treatments were isolated by modified method of Ryckebosch et al. (2011), using Chloroform: Methanol: Water (10:5:1) mixture initially and then mixture of Chloroform: Methanol (1:1) finally. Cell debris, if any, in lipid fraction was filtered off through Whatman No.1 filter paper with solvent washings. Solvent evaporation resulted in gravimetric determination of Lipids. All the experiments were carried out in triplicate.

Phenetic Identification of Microalgal Isolate:

The Microalgal isolate was sent to an Algal Speciality Research Organization: Soley Institute, Istanbul, Turkey for Phenotypic identification and Lipid content Analysis.

RESULTS & DISCUSSION:

Isolation & Microscopic Examination:

Fig. 1a presents the natural occurrence of the predominant free floating KSV1, Fig. 1b depicts the pure culture of this alga in Petri plates and Fig. 1c shows it's 400 x magnified microscopic view.

Different algal members viz., Chlamydomonas, Scenedesmus, diatoms were found in the fresh inland waters of Panchot along with KSV1. Through careful

Volume : 4 | Issue : 9 | September 2014 | ISSN - 2249-555X

microscopic examination and serial dilutions (Fig. 1b), KSV1 was isolated as green, unicellular, oval microalgal cells, often clustered together (Fig. 1c).

Fig. 1a. KSV1 in water body

Fig. 1b. KSV1 on Chu-13 Plate

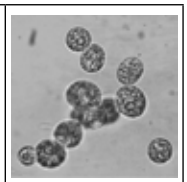


Fig. 1c. 400 X image of KSV1

Optimization of Media and Culture Parameters:
The detailed data on the impact of various media and parameters on the growth and Lipid content of KSV1 are presented in Tables 1 & 2 and Figs. 2, 3 & 4.

Table 1: Effect of different Media on Biomass and Lipid Content of KSV1

Medium	Yields of Biomass and Lipids		
	Dry weight (g /100 ml)	Total Lipid(g)/100 ml	% Lipid content
Bold's Basal	0.057 ± 0.001	0.011 ± 0.0002	19.290 ± 0.10
Bristol	0.071 ± 0.003	0.014± 0.0007	19.718± 0.26
BG-11	0.098 ± 0.002	0.016 ± 0.0005	16.326± 0.38
Chu13	0.090 ± 0.005	0.011 ± 0.0007	12.222 ± 0.22
Proteose	0.084 ± 0.004	0.011 ± 0.0006	13.095± 0.44

The growth of KSV1 was best in BG11 medium, followed by that in Chu13, Proteose, Bristol and Bold's Basal medium respectively(Table1). KSV1 preferred BG11 to other media as also preferred by several members of Chlorophyceae (Ren et al., 2013; Zhou et al., 2011) and Botryococcus braunii (Ranga Rao et al., 2010; Tran et al., 2010; Dayananda et al., 2007).

KSV1 exhibited slow growth between pH range of 4.0 - 5.5 and high growth at pH range 6.0 – 8.0 indicating its preference of near neutral pH (Table 2). The increase of pH above 8.0 resulted in gradual retardation of growth. Reports indicate that very high pH values of the medium (>8) do not support the growth of algae due to reduced availability of CO_2 to algae(Chen and Durbin, 1994; Pruder& Bolton, 1979) and the same trend has been observed in these studies.

Table2: Effect of Cultivation Parameters on Biomass and Lipid content of KSV1

Parameter		Dry weight (g /100 ml)	Total Lipid (g)/100 ml	% Lipid content
Temperature (°C)	15	0.053 ± 0.001	0.008 + 0.0006	15.09± 0.91
	20	0.094 ± 0.001	0.015 + 0.0006	15.96 ± 0.48
	25	0.070 ± 0.002	0.011 + 0.0006	15.71 ± 0.62
	30	0.015 ±0.001	0.002 + 0.0000	13.33± 0.48
pH	4.0	0.079 ± 0.002	0.011 ± 0.0006	13.92 ± 0.37
	4.5	0.082 ± 0.001	0.012 ± 0.0006	14.63± 0.66
	5.0	0.083 ± 0.001	0.012 ± 0.0006	14.46± 0.56
	5.5	0.083 ± 0.002	0.012 ± 0.0000	14.46± 0.27
	6.0	0.090 ± 0.001	0.013 ± 0.0006	14.44± 0.52
	6.5	0.092 ± 0.001	0.014 ± 0.0006	15.22± 0.50
	7.0	0.093 ± 0.003	0.015 ± 0.0006	16.13± 0.38
	7.5	0.092 ± 0.002	0.015 ± 0.0006	16.30± 0.46
	8.0	0.091 ± 0.002	0.015 ± 0.0006	16.48± 0.95
	8.5	0.079 ± 0.003	0.012 ± 0.0000	15.19± 0.50
	9.0	0.077 ± 0.003	0.012 ± 0.0010	15.58± 0.80
	9.5	0.075 ± 0.003	0.011 ± 0.0010	14.67± 0.85
	10.0	0.076 ± 0.003	0.011 ± 0.0010	14.47± 0.84
Light : Dark	4:20	0.032 ± 0.002	0.004 ± 0.0000	12.50± 0.58
	8:16	0.044 ± 0.001	0.006 ± 0.0000	13.64± 0.36
	12:8	0.066 ± 0.002	0.009 ± 0.0006	13.64 ± 0.54
	16:8	0.092 ±0.002	0.014 ± 0.0006	15.22 ± 0.39
	20:4	0.091 ± 0.001	0.013±0.0000	14.29 ± 0.50
	0	0.063 ± 0.002	0.009 ± 0.0006	14.29± 0.62
	60	0.075 ± 0.002	0.011 ± 0.0006	14.67± 0.40
	120	0.086 ± 0.002	0.012 ± 0.0006	15.12± 0.43
	180	0.085 ± 0.001	0.012 ± 0.0006	14.11± 0.64

Results indicate the growth optima of KSV1 at 20°C, declining growth at 30°Cand KSV1 did not revive once grown at 35°C and beyond. According to Chisti (2007), most algal members may not survive high temperatures or get damaged when exposed to temperatures higher than 30°C. The occurrence and profuse growth of KSV1 and other algae during September to January in Gujarat and their reduced occurrence during summers can be assigned to the temperature impact. Significant reduction of growth of this isolate was observed when grown at 15°C.

Lipid content of KSV1 varied from 12 - 20 % (Tables 1 & 2; Figs. 2 & 3)under the impact of the various parameters. The highest lipid quantity was produced in BG-11 medium, at 20° C, between pH values of 7.0 to 8.0 and at 120 RPM agitation. Temperatures around 20 - 25° C have been reported to be optimal for lipid productivity in Chlorella sp. and Asteracys and temperatures above this result in decline of the lipid production by the algae (Suali & Sarbatly, 2012). The media composition and fermentation parameters are reported to influence the total lipid concentration in Scenedesmus sp. (Ren et al., 2013) and the same proved true for KSV1 as well, stresses like low temperature, pH between 7.0 to 8.0 and light : dark cycles having enhanced the lipid yields identical to the observations of Sharma et al. (2012) and Rukminasari (2013) on nutrient limitation, temperature shift, change in salinity & pH and light / dark cycles during the production period determining the lipid content .

Volume : 4 | Issue : 9 | September 2014 | ISSN - 2249-555X

Soley Institute, Istanbul, identified KSV1 as Botryococcus braunii on the basis of Phenetic (morphological and cultural)characteristicsand reported 19%Lipid content with Bold's Basal medium, wherein our results showed 19.7% lipid. However, the biomass in our studies in Bold's Basal medium was less than that obtained by Soley Institute, perhaps because they had used CO_2 feeding which was not employed here. Further studies to confirm Genetic identification through 18 S rRNA gene sequencing of KSV1 are being initiated.

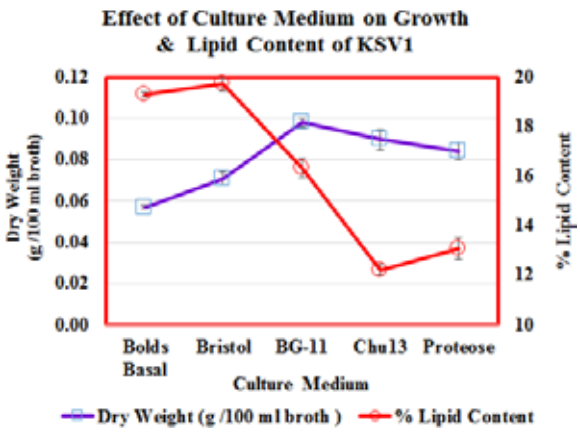
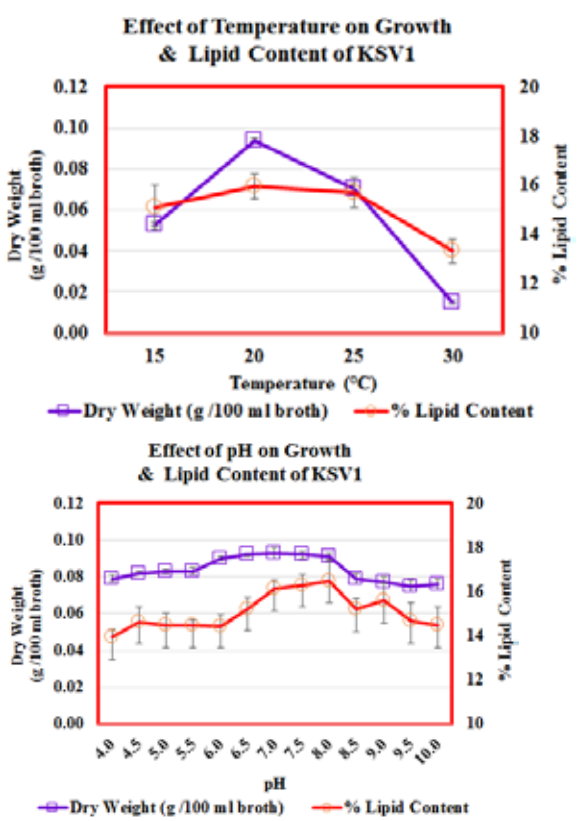
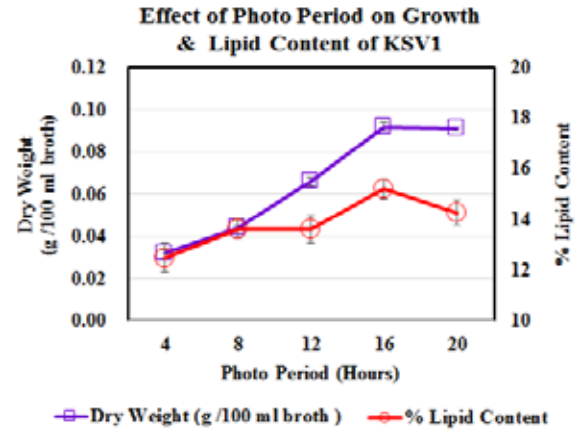


Fig. 2 Effect of Culture Media on Biomass and Lipid Content of KSV1

Utility of Antibacterials and Antifungals for obtaining Axenic Algal cultures:

The study has established the utility of Rose Bengal Medium with Chloramphenicol and Bavistin (1,250 µg/ml) in BG-11 medium for obtaining axenic culture of Botryococcus braunii





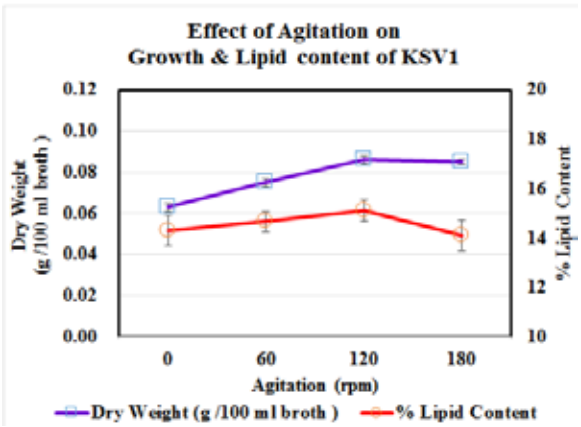


Fig. 3 Effect of Cultivation Parameters on Biomass and Lipid content of KSV1

(KSV1), avoiding the bacterial and fungal contaminants during isolation of algae from mixed populations from inland waters. This may prove useful for other algal forms as well. Droop (1967) and Guillard (1973) have reported the use of antibiotics mix including Penicillin, Streptomycin, Chloramphenicol as antibacterials and Actidione, Nystatin& Amphotericin B as antifungals for axenic cultures of Algae, though reporting toxicity of chloramphenicol to some algal forms. However, Guillard (2005) and Sena et al. (2011) have opined that no realistic recommendation of any particular procedure for contamination control can be made. In case of KSV1, the combined/ sequential use of BG-11 medium with Bavistin and Rose Bengal Medium with chloramphenicol proved useful and can be tried for obtaining axenic cultures of other algal forms.

CONCLUSION:

A free floating alga from mixed populations in inland water ponds in Gujarat, was purified through the use of Rose Bengal Medium with Chloramphenicol and Bavistin in BG-11 medium and provisionally identified as Botryococcus braunii. It shows optimal growth and lipid accumulation in BG11 medium at 20°C within a pH range of 7.0 to 8.0 provided with 16:8 hrs Light (5.0 ± 0.2 k lux): Dark period cycle under agitation at120 rpm. Lipid content under optimal conditions ranged between 15-19% of the algal dry weight.

ACKNOWLEDGEMENTS:

The authors are thankful to the Management of Kadi Sarva Vishwavidyalaya, Gandhinagar, for facilitating this research and to Soley Institute, Istanbul, Turkey, for the Identification and Analysis of KSV1.

Volume : 4 | Issue : 9 | September 2014 | ISSN - 2249-555X

REFERENCE

1. Alam, F., Date, A., Rasjidin, R., Mobin, S., Moria, H. and Baqui, A. (2012) Biofuel from algae- Is it a viable alternative? Procedia Engineering, 49, 221 – 227. | 2. Banerjee, A., Sharma, R., Chisti, Y. and Banerjee, U.C. (2002)Botryococcus braunii: A Renewable Source of Hydrocarbons and Other Chemicals. Crit. Rev. Biotechnol. 22, 245-279. | 3. Bajhaiya, A. K., Mandotra, S. K., Suseela, M.R., Toppo, K. and Ranade, S. (2010) Algal Biodiesel: The next generation biofuel for India. Asian J. Exp. Biol. Sci., 1, 728-739. | 4. Beer, L. L., Boyd, E. S., Peters, J. W. and Posewitz, M. C. (2009) Engineering algae for biohydrogen and biofuel production. Curr. Opin. Biotechnol.20, 264–271. | 5. Chen, C. Y. and Durbin. E. G. (1994) Effects of pH on the growth and carbon uptake of marine phytoplankton. Mar. Ecol. Prog. Ser. 109: 83-94. | 6. Chisti, Y. (2007) Biodiesel from Microalgae. Biotechnol. Adv. 25,294-306. | 7. Chisti, Y. (2008) Biodiesel from microalgae beats bioethanol. Trends in Biotechnology.26, 126-131. | 8. Dayananda, C., Sarada, R., Kumar, V. and Ravishankar, G. A. (2007) Isolation and characterization of hydrocarbon producing green alga Botryococcus braunii from Indian freshwater bodies. Electronic Journal of Biotechnology, 10, 78 -91. | 9. Dayananda, C., Sarada, R., Usha Rani, M., Shamala, T. R. and Ravishankar, G. A. (2007a) Autotrophic cultivation of Botryococcus braunii for the production of hydrocarbons and exopolysaccharides in various media. Biomass and Bioenergy, 31, 87-93. | 10. Droop, M. (1967) A procedure for routine purification of algal cultures with antibiotics. Br. Phycol. Bull. 3, 295-297. | 11. Evans, G.M. and Furlong, J.C. (2003) J.C. Environmental Biotechnology: Theory and Applications. John Wiley & Sons Ltd, West Sussex, England. | 12. Guillard, R. R. L. (1973) Methods for microflagellates and nanoplankton. In Handbook of Phycological Methods: Culture Methods and Growth Measurements ed. Stein, J.R., Cambridge University Press, Cambridge, 69- 85. | 13. Guillard, R. R. L. (2005) Purification Methods for Microalgae. In Algal Culturing Techniques, ed.Andersen. R. A., 117-132, Elsevier Academic Press. | 14. Hirano, A., Ueda, R., Hirayama, S. and Ogushi.Y. (1997) CO2 fixation and ethanol production with microalgal photosynthesis and intracellular anaerobic fermentation. Energy, 22, 137–142. | 15. Hong, J. W., Kim, S. A., Chang, J., Yi, J., Jeong, J., Kim, S., Kim, S. H. and Yoon, H. S. (2012) Isolation and description of a Korean microalga, Asterarcys quadricellulare KNUA020, and analysis of its biotechnological potential. Algae, 27, 197-203 | 16. Hossain, B.M.S., Salleh, A., Boyce, A.N., Chowdhury, P. and Naqiuddin, M. (2008) Biodiesel fuel production from algae as renewable energy. American Journal of Biochemistry and Biotechnology, 4, 250–254. | 17. Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M. and Seibert, M. (2008) Microalgal triacylglycerols as feed stocks for biofuels production: perspectives and advances. The Plant Journal, 54, 621–639. | 18. http://www.ccap.ac.uk/media/documents/BB_000.pdf (29.05.2012) | 19. Kojima, E. and Zhang, K. (1999) Growth and hydrocarbon production of microalga Botryococcus brauniiin bubble column photobioreactors. Journal of Biosciences Bioengineering, 87, 811–815. | 20. Lee, R.A. and Lavoie, J.M. (2013). From first- to third-generation biofuels: Challenges of producing a commodity from a biomass of increasing complexity. Animal Frontiers, 3, 115-140. | 21. Li-Beisson, Y. and Peltier, G. (2013). Third-generation biofuels: current and future research on microalgal lipid biotechnology. OCL, 20, available at http://www.ocl-journal.org/articles/ocl/full_html/2013/06/ocl130012/ocl130012.html | 22. Li, Y., Wang, B., Wu, N. and Lan, C.Q. (2008) Effects of nitrogen sources on cell growth and lipid production of Neochloris oleoabundans. Applied Microbiology and Bio- technology, 81, 629–636. | 23. Li, Y., Horsman, M. Wu, N., Lan, C.Q. and Dubois-Calero, N. (2008) Biofuels from microalgae. Biotechnology Progress, 24, 815–820. | 24. Malcata, F.X. (2011) Microalgae and biofuels: a promising partnership? Trends Biotechnol, 29, 542–549. | 25. Maršálková, B., Širmerová, M., Ku ec, M., Bránÿik, T., Bránÿiková, I., Melzoch, K. andZachleder, V. (2010) Microalgae Chlorella sp. As an alternative source of fermentable sugars. Chem. Eng. Trans, 21, 1279–1284. | 26. Mata, T. M., Martins, A. A. and Caetano, N.S. (2010) Microalgae for biodiesel production and other applications: A review. Renewable and Sustainable Energy Reviews, 14, 217–232. | 27. Macedo, R.V.T. andAlegre, R.M. (2001). Influencia do Teor de Nitrogenio no Cultivo de Spirulina Maxima emDois Ni veis de Temperatura – Parte II. Produc¸a ode LipidiosCiencTecnol Aliment Campinas, 21, 183–186. | 28. Metzger, P and Largeau,C. (2005) Mini review. Botryococcus braunii: A rich source for hydrocarbons and related ether lipids. Applied Microbiology and Biotechnology, 66, 486-496. | 29. Patil, V., Tran, K. Q., and Giselrød, H. R. Towards Sustainable Production of Biofuels from Microalgae. Int. J. Mol. Sci. 2008, 9, 1188-1195. | 30. Picazo-Espinosa, R., Gonza´lez-Lo´pez, J. and Manzanera, M. (2011). Bioresources for Third Generation Biofuels, Biofuel's Engineering Process Technology, Dr. Marco Aurelio Dos Santos Bernardes (Ed.), ISBN: 978-953-307-480-1, InTech, Available at :http://www.intechopen.com/books/biofuel-sengineering-process-technology/bioresources-for-third-generation-biofuels | 31. Pirt, S.J (1986). The Thermodynamic Efficiency (Quantum Demand) and Dynamics of Photosynthetic Growth. New Phytol. 102, 3-37. | 32. Pruder, G. D. and Bolton, E. T. (1979). The role of CO2 enrichment of aerating gas in the growth of an estuarine diatom. Aquaculture 17, 1-15. | 33. Raja, R., Shanmugam, H., Ganesan, V. and Carvalho, I.S. (2014) Biomass from Microalgae: An Overview. Oceanography, 2, 118, available at http://esciencecentral.org/journals/biomass-from-microalgae-an-overview-2332-2632-2-118.php?aid=23654 | 34. Ranga Rao, A., Dayananda, C., Sarada, R., Kumar, V., Shamala, T. R. andRavishankar, G. A. (2007) Effect of salinity on growth of green alga Botryococcus braunii and its constituents. Bioresource Technology, 98, 560- 564. | 35. Ranga Rao, A., Sarada, R. and Ravishankar, G. A. (2010) Enhancement of Carotenoids in Green Alga Botryococcus braunii in Various Autotrophic Media under Stress Conditions. International Journal of Biomedical and Pharmaceutical Sciences, 4, 87-92, Global Science Books. | 36. Ren, H. Y., Liu, B. F., Ma, C., Zhao, L. and Ren, N. Q. (2013) A new lipid-rich microalga Scenedesmus sp. Strain R-16 isolated using Nile red staining: effects of carbon and nitrogen sources and initial pH on the biomass and lipid production. Biotechnology for Biofuels 6, 143, available at http://www.biotechnologyforbiofuels.com/content/6/1/143 | 37. Rodolfi, L., Zittelli, G. C., Bassi, N., Padovani, G., Biondi, N. and Bonini, G.(2009) Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photo bioreactor. Biotechnology and Bioengineering, 102,100–112. | 38. Rosenberg, J. N., Oyler, G. A, Wilkinson, L. and Betenbaugh, M. J. (2008) A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution. Current Opinion in Biotechnology, 19, 430–436. | 39. Rukminasari, N. (2013) Effect of Temperature and Nutrient Limitation on the Growth and Lipid Content of Three Selected Microalgae (Dunaliella tertiolecta, Nannochloropsis sp. and Scenedesmus sp.) for Biodiesel Production. International Journal of Marine Science, 3, 135-144. | 40. Ryckebosch, E., Muylaert, K. and Foubert, I. (2012) Optimization of an analytical procedure for extraction of lipids from microalgae. Journal of the American Oil Chemists' Society, 89, 189–198. | 41. Sathya, S. and Srisudha, S. (2013) Fatty Acid and Hydrocarbon Composition in Asteracys quadricellulare (Behre). Indian journal of applied research, 3, available at http://www.biotechnologyforbiofuels.com/content/6/1/143 | 42. Sawayama, S., Inoue, S. and Yokoyama, S. (1994) Continuous culture of hydrocarbon rich microalga Botryococcus braunii in secondarily treated sewage. App. Microbiology and Biotechnology, 41, 729 -731. | 43. Schenk, P. M., Hall, S. R. T., Stephens, E., Marx, U. C., Mussgnug, J. H. S. and Posten, C. (2008). Second generation biofuels: high-efficiency microalgae for biodiesel production. Bioenergy Research, 1, 20–43. | 44. Scott, S. A., Davey, M.P. and Dennis, J.S.(2010) Biodiesel from algae: Challenges and Prospects. Curr. Opin. Biotechnol., 21, 277–286. | 45. Sena, L., Rojas, D., Montiel, E., González, H., Moret, J. and Naranjo, L. (2011) A strategy to obtain axenic cultures of Arthrospira spp. cyanobacteria. World J. Microbiol. Biotechnol., 27, 1045–1053. | 46. Sharma, K. K., Schuhmann, H. and Schenk, P. M. (2012). High Lipid Induction in Microalgae for Biodiesel Production. Energies, 5, 1532-1553. | 47. Sheehan, J., Dunahay, T., Benemann, J. and Roessler, P. (1998) A look back at the U.S. Department of Energy's Aquatic species Programme – Biodiesel from Algae. N.R.E.L. U.S.A. | 48. Spolaore, P., Joannis-Cassan, C., Duran, E. and Isambert, A. (2006) Commercial applications of microalgae. J. Biosci. Bioeng.,101, 87-96. | 49. Suali, E. and Sarbatly, R. (2012) Renewable and Sustainable Energy Reviews, 16, 4316– 4342. | 50. Thomas, W. H., Tornabene, T. G. and Weissman, J. (1984) Screening for lipid yielding microalgae: Activities for 1983. SERI/STR-231-2207. | 51. Tran, H., Kwon, J., Kim,Z., Oh, Y., and Lee. C (2010). Statistical Optimization of Culture Media for Growth and Lipid Production of Botryococcus braunii LB572. Biotechnology and Bioprocess Engineering, 15, 277-284. | 52. Tsukahara, K. and Sawayama, S. (2005) Liquid fuel production using microalgae. Journal of the Japan Petroleum Institute, 48, 251–259. | 53. Wij els, R.H. and Barbosa, M.J. (2010) An Outlook on Microalgal Biofuels. Science,329, 796–799. | 54. Zhou, W., Li, Y., Min, M., Hu, B., Chen, P. and Ruan, R. (2011). Local bioprospecting for high lipid producing microalgal strains to be grown on concentrated municipal wastewater for biofuel production. Bioresour. Technol., 102, 6909 – 6919. |