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NOTE

Biochemical composition of green alga *Chlorella minutissima* in mixotrophic cultures under the effect of different carbon sources

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Mixotrophic growth of *Chlorella minutissima* with carbon supplements such as glucose, glycerol, succinate, molasses and press mud resulted in maximum biomass accumulation in glucose supplemented culture. Lipid content was maximum with molasses followed by press mud, fatty acid compositions of which also were best suited for biodiesel production.

[**Key words:** *Chlorella minutissima*; Mixotrophic; Molasses; Lipid; Nuclear magnetic resonance; Gas chromatography]

The continuous rise in oil prices has pressurized the world to look for alternate sources of energy. Oil was also derived earlier from oil seed plants such as *Jatropha curcas* (1), palm (2), soybean (3) etc., for biodiesel production however oil rich microalgae are one step ahead as they utilize sunlight more efficiently and thus have higher photosynthetic efficiency than land plants (4). Mixotrophic growth of algae is considered better and more sustainable than the heterotrophic mode as it utilizes solar energy for lipid production and helps in biosequestration of CO_2 (5). Effect of glucose (6) and acetate (7) on *Chlorella protothecoides* and *Nannochloropsis* sp. (acetate) (8) have been studied for their effect on algal growth. In a study on *Chlorella zofingiensis*, glucose was observed to be the best carbon source for biodiesel production (9). *Phaeodactylum tricornutum*, when grown mixotrophically using glycerol, acetate and glucose resulted in higher biomass productivity and specific growth rate (10). *Botryococcus braunii* showed that mixotrophic growth with glucose yielded maximum growth rates in the absence of light (11). Fatty acid content in *Chlorella* sp. was also found to increase with glucose and sodium thiosulphate as supplements (12). Molasses supplementation increased the biomass productivity of *Spirulina platensis* (13). In the present study, *Chlorella minutissima* is grown mixotrophically using carbon sources, i.e., glucose, glycerol and succinate, molasses and press mud. Molasses/press mud, glycerol and succinate are the waste products of sugar industry, biodiesel industry and food/pharmaceutical industry, respectively. The analysis of lipids using proton nuclear magnetic resonance (NMR) and gas chromatography was carried to examine changes in the quality of lipids under autotrophic and mixotrophic conditions.

Chlorella minutissima was purchased from Indian Agricultural Research Institute (India) and maintained in BG-11 medium. The cells were grown in 500-ml Erlenmeyer flasks with manual shaking twice a day. Carbon (100 mM) of each of glycerol, glucose, succinate (sodium succinate), molasses and press mud was used in carbon supplemented cultures (CSC). Molasses and press mud were procured from All India Distiller's Association (India). Press mud was added to growth medium as aqueous extract. Control culture was not supplied with any carbon source. All estimations were performed on three independent replicates and are shown as the average of the triplicates. All chemicals used were bought from Northern Laboratory Implements (New Delhi, India) and were manufactured either by Fischer Scientific or Merck. The cultures were grown at a temperature of 28°C under a light intensity of ~2700 lux in pH range of 7.5–8. The inoculum amount was fixed to 10% from 10 days old culture. The ultimate analysis of *C. minutissima* biomass and oil was carried out in VarioEL cube CHNSO analyser from Elementar Analysensysteme GmbH (Hanau, Germany). The percentage of oxygen is calculated by difference. The growth rate was determined by extracting chlorophyll in methanol using the hot extraction method (14). The absorbance was recorded at 650 nm and 665 nm. Carotenoids were extracted using 85% acetone and the absorbance was recorded at 480 nm (15). Total soluble sugars were estimated colourimetrically using phenol sulphuric acid method at wavelength of 488 nm by comparing readings against glucose standard curve (16). Total soluble proteins were estimated by modified Folin's method (17). The absorbance was recorded at 650 nm and compared against bovine serum albumin (BSA) standard curve. The biomass from a fixed amount of culture was dried and weighed to estimate the biomass production. Lipids were extracted from algal biomass pretreated with perchloric acid (0.2N) using chloroform-methanol (2:1) solvent. Total lipids were estimated using dichromate solution prepared in sulphuric acid for oxidation of all types of lipids (18). The assay is based on the disappearance of absorbance at 350 nm as the dichromate is reduced. A palmitic acid standard is plotted using the same method and the concentration of the unknown lipid sample is

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calculated against palmitic acid standard curve. The oil extracted from different algal cultures was also characterized by NMR spectral studies using the instrument Bruker Spectrospin 300 NMR spectrometer. The solvent used for carrying out the NMR studies was $CDCl_3$ containing TMS as internal reference. The spectrum was recorded between 0 and 10ppm for ^1H NMR studies. For gas chromatography analysis, the extracted lipids were transesterified by refluxing lipid sample in 2% sulphuric acid solution prepared in methanol for 5–6 h followed by fatty acid methyl esters (FAME) extraction using ethyl acetate. FAME were analysed by GC (Shimadzu GC-2010) equipped with FID using SP™ – 2560 capillary column (100m × 0.25 mm 1D 0.20 μm film). The temperature programming was adjusted to 140°C–240°C at a rate of 4°C/min. The FAME(s) were identified by comparing their fragmentation pattern with suitable standard (Sigma).

Ultimate analysis of *C. minutissima* biomass showed presence of carbon (48.02%), nitrogen (7.62%), hydrogen (7.43%), sulphur (0.36%), and oxygen (36.56%). The elemental composition of *C. minutissima* oil includes carbon (75.9%), nitrogen (0.33%), hydrogen (11.18%), sulphur (0.008%), and oxygen (12.57%). Glucose supplemented culture (GSC), glycerol supplemented culture (GlSC) and succinate supplemented culture (SSC) showed remarkable increase in growth as shown in the biomass curve (Fig. 1E). The biomass production results matched with those presented by Liang et al. (19). The biomass increased from 3.6 g/L in control cultures to 17.15 g/L, 16 g/L and 11.35 g/L in GSC, GlSC and SSC. MSC and PSC showed very low biomass productivity which reached its maximum towards the end of exponential phase. This may be because of the presence of high concentrations of some micronutrients like copper and zinc in aqueous extract of press mud. Also, molasses being highly viscous resulted in increased viscosity of the nutrient medium that may have affected growth and thus biomass accumulation. 4.4 g/L of biomass was produced in stationary phase in control cultures unlike CSC. A dramatic increase in the chlorophyll content was recorded as compared to control culture. Chlorophyll content was found to be low in case of GSC, GlSC and SSC in the early exponential phase and increased sharply by the end of late exponential phase. The maximum increase in

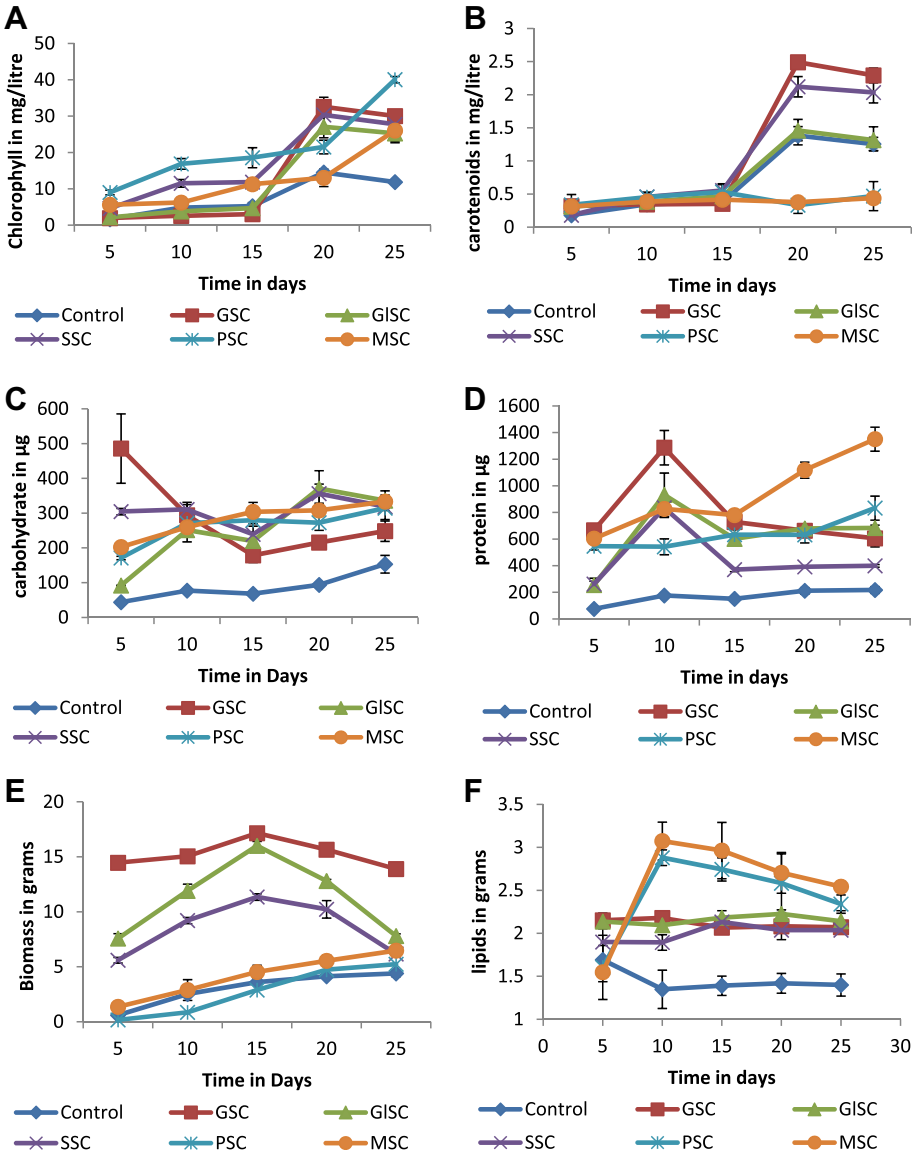

FIG. 1. (A) Chlorophyll content, (B) carotenoid content, (C) carbohydrate (soluble sugar) content, (D) soluble protein content, (E) total biomass content, and (F) lipid content in autotrophic (control) and glucose, glycerol, succinate, molasses and press mud supplemented mixotrophic cultures in *Chlorella minutissima*. GSC, glucose supplemented culture; GlSC, glycerol supplemented cultures; SSC, succinate supplemented culture; PSC, press mud supplemented cultures; and MSC, molasses supplemented cultures.