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Growth performance of *Chlorella ellipsoidea* as biomicrocapsule using supernatant of digested sugar mill waste effluent

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Abstract
Biomicrocapsules mean microscopic living organisms which carry important nutrients very essential for the growth and development of aq
uatic organisms as well as other animals. Among these biomicrocapsules, *Chlorella ellipsoidea*, an important green microalga (Chlorophyceae) which contains 40-45% crude protein, 12-16% crude lipid, 14-15% minerals, colour pigments, vitamins and carotene.

The microalga, *C. ellipsoidea* was cultured in four different dilutions of supernatant of digested sugar mill effluent (DSME) i.e. 25, 50, 75 and 100% DSME and Bold basal medium (BBM) as control in laboratory condition. Maximum cell growth and chlorophyll a content of *C. ellipsoidea* were obtained on 10th day of culture in supernatant of 50% diluted DSME followed by those of this biomicrocapsule grown in BBM, and 75, 25 and 100% DSME at stationary phase. Cell number had highly ($p<0.01$) direct correlation with chlorophyll a ($r = 0.889$) of *C. ellipsoidea*, and optical density ($r = 0.926$) of media. Chlorophyll a was also highly ($p<0.01$) and directly correlated with optical density ($r= 0.877$) of media. The specific growth rates (μ/day) of cell and chlorophyll a of *C. ellipsoidea* grown in supernatant of 50% DSME were significantly ($p < 0.01$) varied from those of *C. ellipsoidea* cultured in BBM followed by other DSME. Total biomass of *C. ellipsoidea* cultured in supernatant of 50% DSME was found significantly ($p<0.01$) higher than that of this microalga cultured in BBM, and supernatant of 25, 75 and 100% DSME. Similar trend was also observed in the case of optical density. The physico-chemical properties of media were varied with the growth of cell of this microalga. It was recorded that cell number, chlorophyll a of biomicrocapsule, and optical density of media were highly ($p<0.01$) and directly correlated with pH, hardness and alkalinity, and inversely correlated with nitrate-N. Crude protein and crude lipid of *C. ellipsoidea* grown in supernatant of 50% DSME were significantly ($p<0.01$) higher than those of *C. ellipsoidea* cultured in other DSME and BBM. Due to best growth performance exhibited by this microalga grown in supernatant of 50% DSME, it may be used to grow in supernatant of 50% DSME to get more essential nutrients than that cultured in supernatant of other DSME media.

Keys words : *Chlorella ellipsoidea*, Biomicrocapsules, Sugar mill waste

Introduction

There are 17 sugar mills out of which 15 are running in Bangladesh which are releasing a huge amount of waste effluents every year during operation (Nov-April). It was estimated that about 7000 m ton of press mud and 12000 m ton waste effluent are discharging from these sugar mills every year. These wastes are discharged in the surrounding aquatic bodies which creating serious pollution, as a consequence no living aquatic organism can grow. The nutrients present in the wastes will be recycled through the production of microalgae as biomicrocapsules which may be used to feed fish as live food and or used to supplement fish protein in the diets of fish, poultry and domestic animals.

The wastes of sugar mill may produce toxic substances by decomposition and chemical changes (Baliarsingh *et al.* 1992). Sugar mills also release sulphur compounds along with effluents which are acted upon by reducing bacteria to produce hydrogen sulphide (H_2S), a gas highly toxic to fish and other aquatic organisms (Banerjea and Motwani 1960). During treatment of effluent in oxidation ponds, it is observed that the aquatic weeds cannot survive for several months but in rainy season when heavy rainfall washout the effluent, the plant and the animal can gradually start to grow and to exist. Chowdhury *et al.* (1998) observed that a large number of different species of fish and some mollusks died within five days following resumption of sugar production due to pollution caused by effluent discharged from sugar mill. Due to the presence of nutrients in this waste effluent, it may be usable to grow and to culture microalgae for recycling nutrients (Kulkarni and Manissery 1997). Two types of benefits will be gained in the country due to this work: 1) nutrients will be recycled from waste to consumable form through *Chlorella ellipsoidea* as an important biomicrocapsule and 2) aquatic environment will be at least partially free from pollution. This microalga *C. ellipsoidea* will be used as food for fishes, shrimp and mollusks as well as aquaculture. Some researchers on the use of nutrients of waste effluents released from rubber, palm oil, sago and sugar mills for the growth and production of different important microalgae have done (Anton 1992, Isa 1993, Kulkarni and Manissery 1997, Habib *et al.* 1998). But, the information regarding the nutritional aspects as well as growth performance of this biomicrocapsule (microalga) cultured in sugar mill effluent media originated from these agro-industries in Bangladesh are very scanty. Therefore, the present work was undertaken to grow this biomicrocapsule, *Chlorella ellipsoidea* in supernatant of digested sugar mill effluent media in different concentrations to study the growth performance of the media, to analyze chemical composition and its possibility for mass culture.

Materials and methods

The sugar mill effluent discharged by the sugar mills as sugar mill waste was collected from Dewanganj Sugar mills, Jamalpur. It was aerobically digested in 5.0 L glass jar for a period of 12 days in ambient temperature in the laboratory. The physico-chemical properties of effluent such as colour, smell, total solids (total suspended solids

and total dissolved solids), pH, alkalinity, hardness, chemical oxygen demand, ammonia-N, nitrate-N and phosphate-P (Table 1) were analyzed following the methods of Clesceri *et al.* (1989).

Table 1. Average physico-chemical properties (mg/l except colour, smell and pH) of sugar mill effluent

Physico-chemical properties	Quality & Quantity
Colour	Blackish red
Smell	Bad odour
Total suspended solids (TSS)	58
Total dissolved solids ((TDS)	6600
pH	7.18
Alkalinity	225
Hardness	600
Chemical oxygen demand (COD)	22500
Nitrate-N	65
Phosphate-P	25

The biomicrocapsule, *Chlorella ellipsoidea* was cultured in four different dilutions of supernatant of digested sugar mill effluent (DSME) i.e. 25, 50, 75 and 100% DSME, and Bold basal medium (BBM) as control (Table 2) in the laboratory condition in triplicates.

Table 2. Chemical composition (g/L) of Bold basal medium (BBM) (Phang and Chu 1999)

Sl. No	Stocks of chemicals	g /L
1	$NaNO_3$	25
2	$Mg.SO_4.7H_2O$	7.50
3	NaCl	2.50
4	K_2HPO_4	7.50
5	KH_2PO_4	17.50
6	$CaCl_2.2H_2O$	2.50
7	Trace elements solution:	
	a) $ZnSO_4.7H_2O$	4.42
	b) $MnCl_2.4H_2O$	1.44
	c) MoO_3	0.71
	d) $CuSO_4.5H_2O$	1.57
	e) $Co(NO_3)_2.6H_2O$	0.49
	Autoclaved to dissolved these chemicals	
8	H_3BO_3	11.40
9	EDTA-KOH solution:	
	a) $EDTANa_2$	50.00
	b) KOH	30.10
10	$FeSO_4.7H_2O$ with 1.0 ml concentrated H_2SO_4	4.98

The microalga was inoculated in 2.0 L flasks from a monoculture containing 10% (optical density at 620 nm= 0.20) algae. The light intensity of 1980 ± 15 lux/m^2/s was maintained in the laboratory at a 12 h: 12 h light: dark cycle for 12 days. Continuous aeration was provided by aerator in culture bottles of 2.0 L capacity. During the experiment, temperature was observed 29°C. The cell count of biomicrocapsule (microalga) culture was done on every alternate day using Improved Neubauer ruling Haemacytometer and a light microscope. The cell number, optical density, chlorophyll a, pH, light intensity, temperature, alkalinity, hardness, ammonia-N, nitrate-N and phosphate-P were determined every alternate day following standard methods (Clesceri *et al.* 1989).

For preparation of 1.0 L BBM, 10 ml from stock solutions 1 to 6 and 1.0 ml from stock solutions 7 to 10 were pipetted in 1.0 L volumetric flask and made the volume 1.0 L with distilled water.

Estimation of chlorophyll a content

The estimation of chlorophyll a content of biomicrocapsule as *C. ellipsoidea* was done at 664, 647 and 630 nm by UV Spectrophotometer (Clesceri *et al.* 1989). A blank with 100% acetone was run simultaneously. Chlorophyll a content was calculated by the following formula:

Chlorophyll a (mg/l) = 11.85 (OD 664) – 1.54 (OD647) -0.08 (OD 630)

The specific growth rate (μ/day) of *C. ellipsoidea* on the basis of cell and chlorophyll a content, and the total biomass on the basis of chlorophyll a content were analyzed (Classer *et al.* 1989).

Specific growth rate (SGR)

The specific growth rate (μ/day) of cultured biomicrocapsule was calculated from the following equation of Clasceri *et al.* (1989):

$$SGR\ (\mu/day) = \ln (X_1 - X_2)/t_1 - t_2$$

Where, X_1 = Biomass concentration at the end of selected time interval;
X_2 = Biomass concentration at the beginning of selected time interval;
and t_1-t_2 =Elapsed time between selected time in the day.

Proximate composition analysis

The cultured biomicrocapsule (microalga) was harvested before stationary phase and placed in vials to centrifuge at 5000 rpm for five minutes to separate the microalga. Then the microalga was cleaned with distilled water and separated with repeated centrifugation. The separated microalga first kept at 0°C for three days and then dried in oven at 40°C. The dry samples were preserved in the freeze at –10°C for the study of proximate composition. The proximate composition of biomicrocapsule (*Chlorella ellipsoidea*) such as crude protein, crude lipid, moisture, ash, crude fibre and nitrogen free extract (NFE) was analyzed following the standard methods (Horwitz 1984).

Data analysis and interpretation

Data were statistically analyzed and interpreted to measure the relationship between different parameters. Differences among the measured parameters and treatment means were analyzed using one way ANOVA following Duncan Multiple Range Test through MSTAT programme (Zar 1984). Linear correlation among the growth parameters and physico-chemical properties of the media were analyzed.

Results and discussion

Maximum cell growth of biomicrocapsules, *Chlorella ellipsoidea* (199.88×10^5/ml) was found significantly ($p<0.01$) higher when grown in supernatant of 50% digested sugar mill effluent (DSME) on 10th day than that cultured in BBM with a low cell number 190.25×10^5/ml which indicates the better growth performance of *C. ellipsoidea* in supernatant of 50% digested sugar mill effluent (Fig. 1). Similar trend of chlorophyll a (Fig. 2) and optical density (Fig. 3) satisfy the best growth performance of this micro-alga in supernatant of 50% DSME which might be due to the presence of adequate nutrients (Table 1 and Table 4) in the medium (Habib *et al.* 1998, Habib *et al.* 2003). Cell number was significantly ($p<0.01$) and directly correlated with chlorophyll a ($r = 0.889$) and optical density ($r=0.926$), and chlorophyll a also significantly ($p<0.01$) correlated with optical density ($r=0.988$) (Table 5). Significant correlation coefficients among these growth parameters again proved the best growth of this microalga in sugar mill effluent. The pH values were increased from initial value of 7.0 up to 8.80 at the point of final collection. Specific growth rate (SGR) of *C. ellipsoidea* grown in supernatant of 50% DSME showed significantly ($p<0.01$) higher cell numbers on 10th day of the culture than that cultured in BBM followed by those grown in other concentrations of supernatant of DSME (Table 3). Total biomass of cell of *C. ellipsoidea* grown in supernatant of 50% DSME showed significantly ($p<0.01$) higher than that of microalga cultured in BBM and other dilutions of supernatant of DSME. Therefore, the growth performances of this microalga in supernatant of 50% DSME signifies this medium as the best one among these DSME media.

Table 3. Specific Growth Rate (SGR, μ/day) of cell, Chlorophyll a (Chlo-a, mg/l) and total biomass of *Chlorella ellipsoidea* grown in supernatant of 100%, 75%, 50% and 25% digested sugar mill effluent and BBM as control on 10th day of culture

Parameters	100% SMEM	75% SMEM	50% SMEM	25% SMEM	BBM
SGR of cell	$0.39^{c}\pm0.01$	$0.39^{c}\pm0.01$	$0.41^{a}\pm0.00$	$0.35^{c}\pm0.00$	$0.38^{b}\pm0.01$
SGR of Chlo-a	$0.40^{d}\pm0.01$	$0.39^{cd}\pm0.01$	$0.43^{a}\pm0.02$	$0.38^{c}\pm0.01$	$0.40^{b}\pm0.00$
Total biomass (Chlo-a × 67)*	395.30 ± 15.60	519.25 ± 19.50	827.45 ± 26.50	455.60 ± 11.50	750.40 ± 20.70
Chlorophyll a	5.90	7.75	12.35	6.80	11.20

Means (± SD) with different superscripts in each raw indicates significant differences ($P < 0.01$), *mg/l

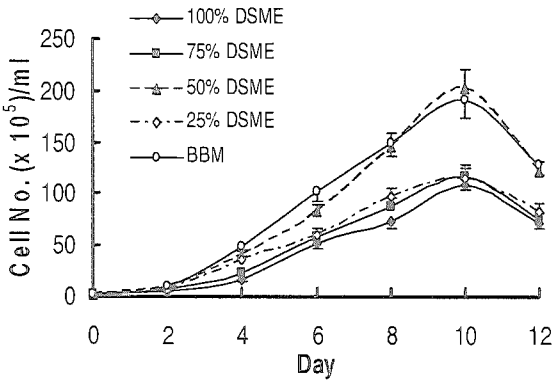

Fig. 1. Growth curve of cell of *Chlorella ellipsoidea* cultured in supernatant of digested sugar mill effluent (DSME) media, and BBM

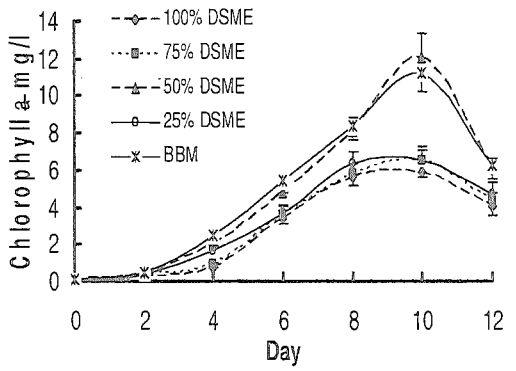

Fig. 2. Growth curve of chlorophyll a of *Chlorella ellipsoidea* cultured in supernatant of digested sugar mill effluent (SME) media, and BBM

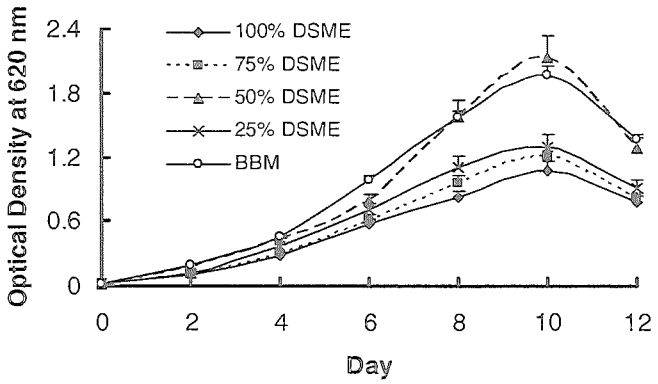

Fig. 3. Optical density of four different media of sugar mill effluent (SME), and BBM contained *Chlorella ellipsoidea*

The medium permitted sufficient light penetration and adequate carbon dioxide from the air to mix into the medium through aeration to overcome the deficiency of carbon in the medium (Phang 1991, Anton *et al.* 1994). Phang and Ong (1988) noted the C:N:P ratios in different heterotophic media like diluted raw latex concentrated rubber and standard Malaysian rubber effluents showed that the carbon was almost three times less than the recommended ratio (56.30:8.60:1.20). This situation was partially overcomed by adequate supply of filtered air (Johns 1994) and carbon dioxide gas into the media (Geetha *et al.* 1994). Continuous aeration was provided in culture flasks to add carbon for algal growth and to agitate the nutrients of media so that these biomicrocapsules can get nutrients properly (Terry and Raymond 1985, Oswald 1988, Habib *et al.* 1998). Aeration during culture increases yield approximately 30% more than without aeration (Molina *et al.* 1990) which agrees with the present observations.

Physico-chemical properties of these culture media contained *C. ellipsoidea* were recorded every alternate day. The average ranges of these characteristics of four different concentrations of supernatant of DSME and the values at peak on the 10th day (highest

growth attended) are presented in Table 4. The light intensity about 1995 lux/m^2/s was provided during culture which is little bid lower than the light used by Hoff and Snell (1989). Optimum temperature (27.8 to 28.5°C) of these cultures was observed during the study which was favourable for algal growth (Alam *et al.* 2003, Habib *et al.* 2003). The optimum pH range for media of DSME contained this microalgal species was between 7 and 8.80 which has similarity with the findings of Anaga and Abu (1996), Mayo (1997), Habib (1998) and James *et al.* (1988). The 'most optimum' range of pH from 8.2 to 8.7 for microalgae was reported by Ukeles (1971).

Table 4. Ranges of physico-chemical properties of four different dilutions of supernatant of decomposed sugar mill effluent media contained *Chlorella ellipsoidea*

Parameter	25% SMEM	50% SMEM	75% SMEM	100% SMEM
Light intensity (lux/m^2/s)	1970-1990 (1980)	1975-1995 (1990)	1970-1990 (1995)	1975-1995 (1990)
pH	7.02-8.70 (8.26)	7.02-8.80 (8.30)	7.02-8.70 (8.50)	7.02-8.60 (8.25)
Temperature (°C)	27.70-28.20 (28.20)	27.80-28.30 (28.25)	27.75-28.40 (28.25)	27.70-28.45 (28.30)
Hardness	27.30-60.0 (34.20)	35.50-69.00 (29.90)	34.70-74.50 (45.60)	34.20-74.10 (46.70)
Alkalinity (mg/l)	62.70-108.30 (95.20)	74.10-114.0 (97.50)	74.10-108.30 (95.20)	79.70-115.0 (103.50)
NH_3-N (mg/l)	0.12-2.20 (0.95)	0.08-2.15 (0.88)	0.11-3.30 (0.75)	0.18-2.20 (0.60)
NO_3-N (mg/l)	0.40-2.90 (0.75)	0.58-3.60 (1.10)	0.65-3.70 (1.20)	0.72-3.55 (1.25)
PO_4-P (mg/l)	0.85-4.40 (0.75)	0.88-4.50 (0.84)	0.90-4.35 (0.90)	0.96-4.50 (0.96)

Figures in the parentheses indicate the values on 10th day of culture (peak of growth before stationary phase)

Phosphate-P and nitrate-N of DSME media contained this biomicrocapsule as *C. ellipsoidea* were found maximum at initial day of culture and gradually decreased upto 10th day of culture (maximum growth of cell recorded) which was increased at stationary phase. But ammonia-N found maximum on the death phase and minimum before the culture started. Habib *et al.* (2003) found similar trend in the study on *C. vulgaris* in digested and diluted palm oil mill effluent. Alkalinity and hardness of these media followed the inverse trend with each other i.e. maximum alkalinity at the end of the culture when hardness was minimum and vice-versa. Cell number, chlorophyll a of *C. ellipsoidea*, and optical density of DSME media were highly ($p<0.01$) and directly correlated with pH, hardness and alkalinity of media, and inversely correlated with nitrate-N (Table 5). Only ammonia-N of media had simple ($p<0.05$) and direct correlation with cell number and chlorophyll a of *C. ellipsoidea* and optical density of media. These highly significant correlation values indicate the influence of inorganic nutrients for the growth of *Chlorella ellipsoidea* (Habib *et al.* 2003).

Table 5. Correlation coefficient values (r) of growth parameters with physico-chemical parameters of media contained *Chlorella ellipsoidea*

Parameters	Cell Number (No/ml)	Optical Density at 620nm	Chlorophyll a (mg/l)
pH	0.723**	0.737**	0.751**
Carbon dioxide (mg/l)	0.558**	0.572**	0.542**
Hardness (mg/l)	0.609**	0.613**	0.619**
Alkalinity (mg/l)	0.643**	0.623**	0.663**
Nitrate-N (mg/l)	-0.610**	-0.628**	-0.605**
Ammonia-N	0.415*	0.411*	0.427*
Phosphate-P	0.568**	0.218	0.149
Cell number	-	0.926**	0.926**
Optical density	-	-	0.877**

* $P < 0.05$, ** $P < 0.01$, df = 28

Proximate composition analysis showed that *C. ellipsoidea* grown in 50% DSEM contained highest amount of protein (42.70%) followed by that of algae cultured in supernatant of 75% DSME (40.50%), 100% DSME (40.50%), 75% DSME (40.60%) and the lowest amount was 37.20% when *C. ellipsoidea* grown in BBM (Table 6). Maximum lipid was found in *C. ellipsoidea* when grown in supernatant of 50% DSME (17.50%) followed by that grown in supernatant of 75%, 100% and 25% DSME media, and BBM. Ash was found maximum 14.40% in *C. ellipsoidea* when grown in supernatant of 100% DSME medium and minimum 10.65% when cultured in BBM. The ash content was directly related with the ash concentration of effluents which refers to the inorganic residue (FDS 1994). It indicates the direct minerals bioaccumulation related with the concentration of minerals in effluents (Fabregas and Herrero 1986, Vymazal, 1995, Habib *et al.* 2003).

Table 6. Proximate composition (% dry matter) of *Chlorella ellipsoidea* grown in supernatant of 100%, 75%, 50% and 25% digested sugar mill effluent, and BBM as control

Components	100% DSME	75% DSME	50% DSME	25% DSME	BBM
Moisture	7.15 ± 0.10	7.22 ± 0.06	7.38 ± 0.10	7.25 ± 0.06	7.25 ± 0.08
Crude protein	40.50^{b} ± 0.20	40.60^{b} ± 0.22	42.70^{a} ± 0.24	40.25^{b} ± 0.12	37.20^{c} ± 0.22
Crude lipid	15.30^{b} ± 0.08	15.50^{b} ± 0.13	17.50^{a} ± 0.10	14.55^{b} ± 0.07	12.75^{c} ± 0.10
Crude fiber	3.20^{b} ± 0.05	3.30^{b} ± 0.05	3.10^{b} ± 0.06	3.27^{b} ± 0.06	4.21^{a} ± 0.15
NFE*	19.37^{c} ± 0.07	19.24^{c} ± 0.09	17.17^{d} ± 0.04	22.69^{b} ± 0.12	27.85^{a} ± 0.22
Ash	14.40^{a} ± 0.14	14.10^{a} ± 0.19	12.08^{b} ± 0.13	11.80^{c} ± 0.15	10.65^{c} ± 0.10

*NFE was calculated by adding percentage values of crude protein, crude fat, crude fiber and ash on dry basis and subtracting it from 100%. Mean values (± SE) with different superscripts in each raw indicates significant differences ($p<0.01$).

Conclusions

Bold basal medium (BBM) is a recognized standard medium for algal culture but due to high cost of the medium it is necessary to find out alternate low cost organic matters

to grow microalgae. It is proved that supernatant of 50% digested sugar mill effluent would be a good low cost indigenous agro-industrial waste for algal culture. Therefore, agro-industrial waste like supernatant of decomposed sugar mill waste effluent may be used for mass culture of important microalga like *Chlorella ellipsoidea* and other important algal species to feed important zooplankton species for live food production in aquaculture system.

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