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Culture of *Chlorella vulgaris* in press mud media as sugar mill waste

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Abstract
Chlorella vulgaris was cultured in different concentrations of press mud medium ($PMM_{0.5\ g/l}$, $PMM_{1.0\ g/l}$, $PMM_{1.5\ g/l}$ and $PMM_{2.0\ g/l}$) and in Bold basal medium (BBM) as control. Maximum cell number of *Chlorella vulgaris* was recorded on 8th day in different treatment where $PMM_{1.0\ g/l}$ obtained the highest rank and $PMM_{2.0\ g/l}$ obtained the lowest. Specific growth rate (SGR) and total biomass of cells indicate that maximum *Chlorella vulgaris* were grown in $PMM_{1.0\ g/l}$. The maximum SGR of *Chlorella vulgaris* was determined 0.55 in $PMM_{1.0\ g/l}$ followed by 0.53 in BBM, 0.52 in $PMM_{0.5\ g/l}$, 0.46 in $PMM_{1.5\ g/l}$, and 0.43 in $PMM_{2.0\ g/l}$ which were supported by cell number, total biomass and chlorophyll a content while optical density showed slightly higher in BBM may be due to cell nutrition and media turbidity. Maximum protein was found of *Chlorella vulgaris* grown in $PMM_{1.0\ g/l}$ followed by that grown in BBM, and then other concentrations of PMM. Lipid content of *Chlorella vulgaris* grown in all the concentrations of PMM were found significantly ($P<0.01$) higher than that of BBM.

Keywords: *Chlorella vulgaris*, press mud, chlorophyll a

1. Introduction
The sugar mills are the biggest agro-industries in Bangladesh under Bangladesh Sugar and Food Industries Corporation (BSFIC) which engaged in particularly for production of sugar with good infrastructure. In spite of this, this very useful industry causes some environmental pollution discharging a large quantity of untreated solid and liquid waste and effluents. The biota of the polluted water bodies are exposed to potential danger. It was observed that a large number of different species of fish and some mollusks died within five days following resumption of sugar production during production season [5]. Researchers worked on the sugar mills pollution and found that sugar mills effluent always increase water temperature, chemical oxygen demand, hardness, total suspended solids, total dissolved solids, conductivity, chloride, total sulphide and ammonia [23, 30]. Recently similar trends were recorded investigating toxicity of sugar mill effluent [29].

During the present investigation, it was observed in Jheel Bangla Sugar Mill, Dewangonj, Jamalpur that some sorts of solid mill discharges stacked in very large heap and also small ball like structured material in the sugar mill complex under open air which is called press mud. This press mud is the regular refusal wastes (scum) of the sugar processing plant. It has some economic importance as it has some utilities in the factory area. This is used as organic fertilizer around the sugar mill area. It is already proved a good kitchen burner like bagasse another, very large cellulose solid sugarcane waste. But this press mud makes the sugar mill complex polluted with dirt and unpleasant appearance in the production period. Observing the situation, the present study was undertaken to investigate the productivity performance of this solid effluent to prepare culture medium for microalgae *Chlorella vulgaris* which may promote it an effective inexpensive organic medium for algal culture and use in aquaculture system.

2. Materials and Methods
Sample Collection and Preparation of Culture Media
Press mud sample was collected from Jheel Bangla Sugar Mill, Dewangonj, Jamalpur, Bangladesh. It was smashed into powder and four different concentrations of dry press mud powder such as 0.5 g/l, 1.0 g/l, 1.5 g/l and 2,0 g/l were dissolved in distilled water hold in separate buckets. Then these were allowed to ferment for 10 days in the laboratory. Then the supernatant were collected and screened out by fine cloths to prepare press mud media (PMM) in different concentrations. 260 mg/l urea was added in each of the press mud medium for nitrogen enrichment and autoclaved to sterilize the media at 120°C steam heat. Four treatments of PMM were designed after a series of laboratory trial for culture of *Chlorella vulgaris* with a control as Bold basal medium (BBM). Chemical composition of BBM is shown below (Table1):

Table 1: Chemical Composition (g/l) of Bold Basal Medium (BBM)

No.	Stocks of Chemicals	g/litre
1.	$NaNO_3$	25.00
2.	$MgSO_4$. $7H_2O$	7.50
3.	NaCl	2.50
4.	K_2HPO_4	7.50
5.	KH_2PO_4	17.50
6.	$CaCl_2$. $2 H_2O$	2.50
7.	Trace elements:	
	$ZnSO_4$. $7 H_2O$	4.42
	$MnCl_2$. $4 H_2O$	1.44
	MoO_3	0.71
	$CuSO_4$. $5 H_2O$	1.57
	$Co (NO_3)_2$. $6 H_2O$	0.49
8.	H_3BO_3	11.40
9.	EDTA-KOH solution:	
	EDTA Na_2	50.00
	KOH	31.00
10.	$FeSO_4$. $7 H_2O$ with 1.0 ml Concentrated H_2SO_4	4.98

For preparation of 01 (one) litre BBM for culture medium 10 ml of each of the stock solutions from serial# 1 -6 and 1.0 ml from each of the stock solutions serial no# 7 -10 (Table1) were pipetted to make one litre volume with distilled water in a volumetric flask.

Culture of *Chlorella vulgaris*

Chlorella vulgaris (No.001) was cultured in 0.5 g/l, 1.0 g/l, 1.5 g/l and 2,0 g/l press mud media and in BBM at Live Food Culture Laboratory, Department of Aquaculture, Bangladesh Agricultural University. Mymensingh. *Chlorella vulgaris* were inoculated to make a 10% suspension (optical density at 620 nm= 0.02) in all the culture treatment. A 12h:12h light : dark system for 12 days were maintained in the laboratory under light intensity of 2000 at 18lux/m^2/s. Continuous aeration also was maintained providing electric aerator connected by plastic tubes in culture bottles. Three replications were taken for each culture. The cell count of *Chlorella vulgaris* was done in every alternate day using improved Neubuer ruling Haemacyto-meter under a light microscope. The cell number, optical density, chlorophyll-a, pH, free CO_2, dissolved oxygen, light intensity, temperature, alkalinity, hardness, phosphate- phosphorus and ammonia-nitrogen were measured every alternate day following standard methods[7].

Estimation of chlorophyll-a content

Optical densities of the prepared sample were analyzed at 664, 647 and 630 nm wave length operating UV-spectrophotometer [7]. A blank in selective tube with 100% acetone was allowed to run simultaneously. Chlorophyll-a content was calculated by the following formula:

Chlorophyll-a (mg/litre)

= 11.85 (OD 664) -1.54 (OD 647) - 0.8 (OD 620)

Specific growth rate (mg/day) of *C. vulgaris* on the basis of cell and chlorophyll-a content and the total biomass on the basis of chlorophyll-a content were also determined following standard methods [7].

Specific growth rate (SGR)

The specific growth rate (mg/day) of the cultured microalga was computed using following equation [7]:

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

Where,

X_1 = biomass concentration of the end of selected time interval;

X_2 = biomass concentration at beginning of selected time interval; and

$t_1 - t_2$ = time elapsed between the selected time in the day

Proximate composition analysis

The microalgae were harvested before stationary phase and placed in vials to centrifuge at 5000 rpm for five minutes to separate the microalgae. To prevent salt from the filtered samples, ammonium formate (32 g/l) was used to rinse the samples. Then the microalgae were cleaned with distilled water and separated with repeated centrifugation. The separated microalgae firstly kept at 0º C for three days and then dried in the oven at 40º C. The dry samples were preserved in the freeze at -10º C for study the proximate composition. The prepared samples of *C. vulgaris* were analyzed to estimate crude protein, lipid, moisture, crude fibre and nitrogen free extract (NFE) (in the Nutrition Lab of Department of Aquaculture, Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh) following the standard methods [13].

Analysis and interpretation of data

Differences within the measured parameters and the treatment means were determined using one way ANOVA and Duncan's Multiple Range Test following MSTAT statistical package [34].

3. Results and Discussions

Maximum cell number (185.73x10^5)/ml of *Chlorella vulgaris* (Figure 1) was recorded on 8th day cultured in PMM $_{1.0\ g/l}$ followed by (179.45X10^5)/ml in BBM and minimum cell number (51.47x10^5)/ml when grown in PMM $_{2.0\ g/l}$. Similar findings were also observed in the cases of chlorophyll a content (Figure 2) of different press mud media and BBM containing *Chlorella vulgaris*. The maximum SGR of *Chlorella vulgaris* was determined 0.55 when grown in PMM $_{1.0\ g/l}$ followed by 0.53 in BBM, 0.52 in PMM $_{0.5\ g/l}$, 0.46 in $PMM_{1.5\ g/l}$, and 0.43 in $PMM_{2.0\ g/l}$ which has similarity with cell number, chlorophyll a content and total biomass (Table 2). But optical density (OD) was found slightly higher in BBM media, may be due to vary in cell nutrition and turbidity of the media.

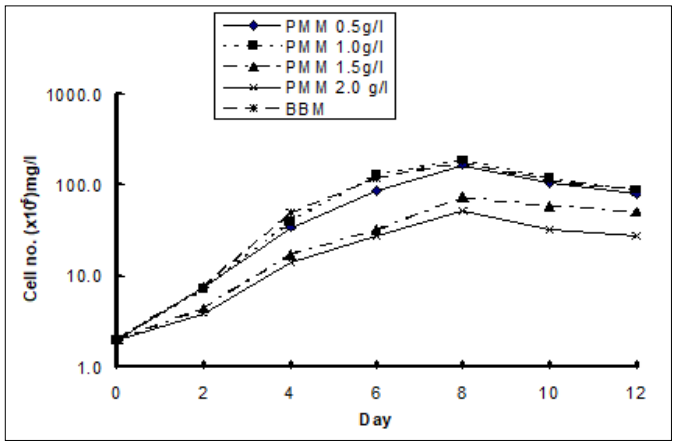

Fig 1: Semilogarithmic growth curve based on cell number (× 10^5)/ml of *Chlorella vulgaris* grown in different concentrations of press mud media (PMM) and bold basal medium (BBM)

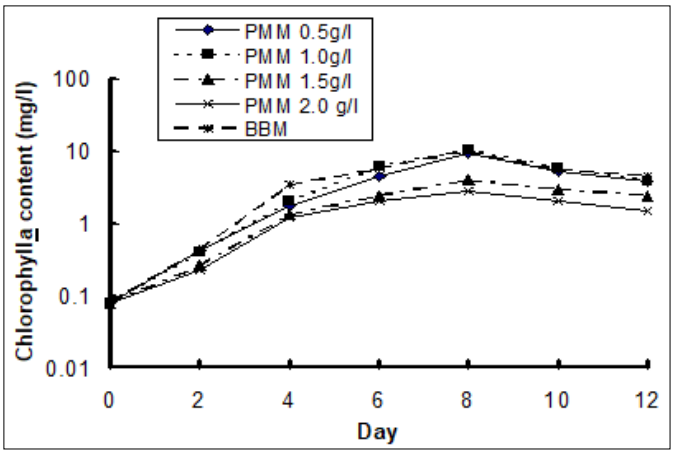

Fig 2: Semilogarithmic growth curve based on chlorophyll a content (mg/l) of *Chlorella vulgaris* grown in different concentrations of press mud media (PMM) and Bold basal medium (BBM)

Table 2: Specific growth rate (μ/day) of cell, chlorophyll a (chlo-a) and total biomass of *Chlorella vulgaris* grown in different concentration of press mud media (PMM) and Bold basal medium (BBM)

Parameters	$PPM_{0.5\ g/l}$	$PMM_{1.0\ g/l}$	$PMM_{1.5\ g/l}$	$PMM_{2.0\ g/l}$	BBM
SGR of cell	$0.52^{b} \pm 0.01$	$0.55^{a} \pm 0.00$	$0.46^{c} \pm 0.00$	$0.43^{d} \pm 0.02$	$0.53^{a} \pm 0.01$
SGR of chlo-a	$0.52^{a} \pm 0.01$	$0.55^{a} \pm 0.03$	$0.46^{b} \pm 0.01$	$0.43^{c} \pm 0.02$	$0.53^{a} \pm 0.03$
Total biomass (Chlo-a × 67)*	630.47± 9.38	707.30 ± 38.57	272.02 ± 29.18	196.53 ± 20.83	689.21 ± 37.28

Means (±SD) with different superscripts in each row indicate significant differences (P<0.01) *mg/l

The specific growth rates (SGRs) of cell and chlorophyll a and total biomass of *Chlorella vulgaris* grown in PMM $_{1.0\ g/l}$ were found higher than those grown in BBM and other press mud media (Table 2). *Chlorella vulgaris* grown in $PMM_{1.0\ g/l}$ contained higher amount of crude protein (46.68%) and lipid (16.61%) than that cultured in other media (Table 3). Soluble carbohydrate (NFE) content of *Chlorella vulgaris* in different treatment showed almost inverse trend to those of lipids. It has similarity with findings of other agro-industrial research [10, 17]. The researchers found higher specific growth rate of *Chlorella vulgaris* grown in different organic effluent media than that of control [10, 17]. These might be due to adequate nutrient available in the media [11, 17] which act as heterotrophic culture media [6], appropriate colour of the media, sufficient light penetration and continuous aeration in the culture [3, 25]. The ash content which is considered directly related with the ash concentration of the effluent indicates that minerals bioaccumulation was directly related with the concentration of the effluents[11,33] were found higher in *Chlorella vulgaris* grown in all the concentration of the PMM than that of grown in BBM.

Table 3: Proximate composition (amount % dry matter) of *Chlorella vulgaris* grown in different concentration of press mud media (PMM) and BBM as control

Components	PMM0.5 g/l	PMM 1.0 g/l	PMM1.5 g/l	PMM 2.0 g/l	BBM
Moisture %	$8.62^{c} \pm 0.08$	$7.06^{d} \pm 0.04$	$9.36^{b} \pm 0.08$	$10.12^{a} \pm 0.06$	$6.21^{e} \pm 0.09$
Crude protein	$44.60^{c} \pm 0.37$	$46.67^{a} \pm 0.30$	$42.44^{d} \pm 0.11$	$41.27^{e} \pm 0.12$	$45.44^{b} \pm 0.11$
Crude lipid	$16.25^{a} \pm 0.10$	$16.61^{a} \pm 0.11$	$15.87^{a} \pm 0.30$	$16.25^{a} \pm 0.17$	$10.49^{b} \pm 0.12$
Crude fiber	$5.30^{c} \pm 0.08$	$4.50^{d} \pm 0.16$	$5.48^{c} \pm 0.09$	$5.63^{b} \pm 0.06$	$10.43^{a} \pm 0.12$
NFE*	$21.30^{c} \pm 0.27$	$21.30^{c} \pm 0.30$	$23.41^{a} \pm 0.44$	$22.96^{b} \pm 0.18$	$23.45^{ab} \pm 0.22$
Ash	$12.55^{a} \pm 0.12$	$10.92^{b} \pm 0.26$	$12.79^{a} \pm 0.10$	$12.90^{a} \pm 0.09$	$10.49^{b} \pm 0.12$

*NFE was calculated by adding percentage values of crude protein, crude fat, crude fiber and ash on dry basis and subtracting it from 100%. Means (± SD) with different superscripts in each row indicate significant differences (P<0.01).

Proximate analysis (Table 3) indicates that maximum protein were found in *Chlorella vulgaris* cultured in $PMM_{1.0\ g/l}$ followed by that grown in BBM, and then other concentrations of PMM. Lipid content of *Chlorella vulgaris* cultured in all the media of PMM were found significantly (P<0.01) higher than that of BBM. Crude fiber and NFE of this micoalgae grown in BBM were determined significantly (P<0.01) higher than those of algae cultured in different concentrations of press mud media. Ash was found maximum in PMM $_{2.0\ g/l}$ and minimum in BBM with a little bit variation to $PMM_{1.0\ g/l.}$

Physico-chemical parameters (Table 4) investigated every alternate day may have direct or indirect influence on the growth of *Chlorella vulgaris.* Maximum cell growth was determined at pH level 8.01. The present pH level was supported by findings of many investigators [2, 17, 28, 32]. They observed maximum cell growth of different microalgae at the pH range of 6.84 to 8.38. An MS researcher [8] recorded

highest cell density of *Scenedesmus* sp. at pH 7.72. Other academic researchers [26] reported similar pH level 7.2 with treated effluent. During present investigation minimum temperature fluctuation was recorded ranged from 27.5-30.01°C. Some other researchers [1, 15, 17] recorded almost similar temperature (26.8 - 30°C) in microalgae culture. In 1990's a few researchers [19, 22, 31] investigated microalgae culture and also found almost similar temperature in their culture system. The maximum cell growth of *Chlorella vulgaris* at an optimum temperature of 32.4°C was also observed [21]. Dissolved oxygen level was found between 3.42 mg/l and 4.85 mg/l throughout the culture period. Researchers [1, 22] in two investigations recorded maximum DO level 4.49 mg/l and 5.46 mg/l respectively during culture of *Chlorella* sp. in different inorganic media. Another study it was found similar DO level ranged 3.09-5.21 during culture of *Scenedesmus obliquus* in sweetmeat waste medium [32]. Phosphate-phosphorus (PO_4-P) was found maximum 5.91 mg/l on the first day and the minimum were recorded 0.94 mg/l on 10th day of culture. Similar trend was reported by some researchers [14, 1, 32] in different inorganic and organic media cultured with *Chlorella* and other microalgal species.

Table 4: Optical density of media and physico-chemical parameters recorded on maximum cell growth of *Chlorella vulgaris* on 8th day in different concentration of PMM and BBM as control

Parameters	Different concentration of PMM				BBM
	0.5mg/l	1.0mg/l	1.5mg/l	2.0mg/l	
Optical density	1.40 ± 0.03	1.52 ± 0.07	0.76 ± 0.05	0.63 ± 0.05	1.81 ± 0.06
Light intensity	1970	1960	1940	1850	1910
pH	8.00 ± 0.06	8.01 ± 0.06	8.02 ± 0.06	8.06 ± 0.07	8.59 ± 0.07
Temperature °C	28.63± 0.02	28.64± 0.02	28.66± 0.02	28.63± 0.02	28.65± 0.02
DO mg/l	4.01 ± 0.03	4.11 ± 0.03	3.84 ± 0.04	3.73 ± 0.03	4.87 ± 0.04
PO_4-P mg/l	0.81± 0.04	0.95± 0.04	1.01± 0.05	1.06± 0.04	2.65± 0.05
NH_3- N mg/l	0.52 ± 0.06	0.47 ± 0.03	0.64 ± 0.04	0.72 ± 0.04	0.11 ± 0.02
NO_3- N mg/l	1.10± 0.05	1.01± 0.03	1.10± 0.05	1.15± 0.04	7.95± 0.44
NO_2- N mg/l	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.17 ± 0.03	0.06 ± 0.02
Alkalinity (mg/l)	142.5 ± 9.87	171.0 ±17.1	176.7 ± 9.87	176.7 ± 9.87	153.8 ± 17.1
CO_2 mg/l	25.0 ± 5.0	30.0 ± 5.0	23.33 ± 2.89	23.33 ± 2.89	38.33 ± 2.89
Hardness (mg/l)	159.6 ± 9.87	159.6 ± 19.75	159.6 ± 19.75	165.3 ± 9.87	45.6 ± 9.87

Ammonia-nitrogen (NH_3-N) and nitrite-nitrogen (NO_2-N) were found minimum at beginning of the culture and maximum on death phase. But in case of nitrate-nitrogen the highest level was found at the beginning and lowest after the stationary phase on 10th day with a higher level in BBM ranged 14.51 slows down to 4.14. An MS researcher [14] determined similar trends in ammonia, nitrate and nitrite-nitrogen level. Alkalinity were found more or less higher throughout the culture period ranged from 113.7 to 199.5 mg/l always increasing trend in all the treatments of PMMs and the control BBM. In hardness determination its level was found minimum at the beginning and maximum at the end with inverse findings in BBM. It indicates that sugarmill effluent increases the hardness [23, 30].

Microalgae-derived biomass is recognized as an alternative source for a wide variety of bioproducts, such as biofuels, especial oils, pigments and polymers [24]. These photosynthetic microrganisms present higher growth rates and lower land area requirements compared to terrestrial crops commonly used for biofuels production. However, the production of microalgal biomass is still not economically viable due to high costs of cultivation, harvesting and processing [27]. Significant cost reductions can be achieved if CO2, nutrients and water for microalgae cultivation are obtained at low cost [4]. A significantly large volume of waste is generated during the manufacture of sugar and contains a high amount of pollution load, particularly in terms of suspended solids, organic matter, pressmud, and bagasse and air pollution [26]. Sugar mill effluents, press mud and sugar mill by-product such as molasses are inexpensive and easily available ingredients in Bangladesh. These agroindustrial wastes and byproduct may be used as organic media for algal culture to fore commercial live food development and simultaneously the country will be free from aquatic pollution partially [18].

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