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Culture of the Microalga *Chlorella vulgaris* on Different Proportions of Sugar Mill Effluents

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Abstract. *Chlorella vulgaris* was cultured in four different dilutions of sugar mill effluent media (SMEM). Bold's basal medium (BBM) was used as the control under laboratory conditions. Maximum cell growth and chlorophyll-**a** content were obtained on 10th day of the culture in 50% diluted SMEM, followed by those grown in BBM, and 75, 25 and 100% SMEM at stationary phase. The specific growth rate (µg/day) of cells and chlorophyll-**a** of *C. vulgaris* grown in 50% SMEM varied significantly ($p < 0.01$) from those of *C. vulgaris* cultured in BBM, followed by other SMEM concentrations. Total biomass of *C. vulgaris,* cultured in 50% SMEM, was found to be significantly higher ($p < 0.01$) than that of *C. vulgaris* cultured in BBM, and 25, 75 and 100% SMEM concentrations. Similar trend was also observed in the case of optical density. Cell number and chlorophyll-**a** of *C. vulgaris* were highly ($p < 0.01$) and directly correlated with chlorophyll-**a** ($r^2 = 0.991$) of *C. vulgaris* and optical density ($r^2 = 0.989$) for the culture media containing *C. vulgaris*, respectively. Crude proteins and crude lipids of *C. vulgaris,* grown in 50% SMEM, were significantly ($p < 0.01$) higher than those of *C. vulgaris* cultured in other SMEM concentrations. Due to good growth performance exhibited in the 50% SMEM dilution, the sugar mill effluent may be used for efficient cultivation of *C. vulgaris* and possibly other microalgae.

Keywords: algal culture, *Chlorella vulgaris*, sugar mill effluents, *Chlorella* culture

Introduction

There are 17 sugar mills in Bangladesh, out of which 15 are operational. The sugar mills are the largest agroindustry in Bangladesh, which release huge amounts of waste effluents, particularly during the crushing season (November-April). The effluent contains washings from the sugarcane pulp press and condensers, and lime cake, molasses, press mud and oil from machinery. It contains large quantities of organic and inorganic compounds, some of which may produce toxic substances on decomposition and on undergoing 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 effluents in the oxidation ponds, it has been observed that aquatic weeds cannot survive during the period of several months. In rainy season, however, when heavy rainfall washes out the effluent, plants and animals can gradually start to grow and exist in these ponds. Chowdhury *et al.* (1998) observed that a large number of different species of fish and some mollusks died within five days, following the resumption of sugar production period, due to pollution caused by the effluent. It has been suggested, nevertheless, that due to the presence of nutrients in this waste effluent, it may be usable to grow and culture microalgae for recycling the nutrients (Kulkarni and Manissery, 1997).

Chlorella vulgaris Beijerinck is an autospore forming, fast growing and nutritionally rich microalga (Habib, 1998). It grows widely in different decomposed organic media. It achieves peak growth within 6-8 days. The cell mass contains high level of crude proteins (39-42%), crude lipids (12-19%), minerals (7-12%), and considerable amounts of carbohydrates. The microalga is rich in polyunsaturated fatty acids, specifically C18 : 2n-3 and C20 : 3n-3 (Habib *et al.,* 2003; Tan and Johns, 1991), and amino acids, particularly, lysine, leucine, threonine and methionine (Habib *et al.,* 2003).

Recent studies with rubber, palm oil and sugar mill effluents have shown that these effluents contain high organic load, which can cause high chemical oxygen demand (Habib *et al.,* 1998; 1997; Kulkarni and Manissery, 1997; Isa, 1993; Anton, 1992). Studies have also shown that these effluents are rich in nutrients, such as broken proteins, lipids, cellulose, carbohy-

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drates, amino acids, peptides, fatty acids, hydrocarbon, as well as some essential metabolites and minerals (Habib *et al.,* 1998; Phang, 1990). Due to the presence of a variety nutrients in these agroindustrial effluents, these may be used as autotrophic and heterotrophic culture media (Habib *et al.,* 2003; Vazhappilly and Chen, 1998; Chu *et al.,* 1995; Johns, 1994; Chui, 1993). However, studies on the nutritional aspects, as well as the growth performance of microalgae cultured in sugar mill effluents originating from agroindustries in Bangladesh, are very scanty. The present work was, therefore, undertaken to grow *Chlorella vulgaris* in different concentrations of sugar mill effluent media to study the growth performance of the alga in these constituted culture media and to determine the variabilities in their chemical composition as a result of such algal cultivations.

Materials and Methods

Algal culture conditions. The microalga, *Chlorella valgaris* (No. 001) was cultured in four different concentrations (25, 50, 75, 100%) of sugar mill effluent media (SMEM). Bold's basal medium (BBM) was used as the control under laboratory conditions. The composition of the Bold's basal medium (Nicholas and Bold, 1965) is given in Table 1. Efforts were made to produce a 10% algal culture (optical density at 620 nm = 0.02). The light intensity of 2000 ± 18 lux/m²/s was maintained in the laboratory at a 12 h - 12 h light-darkness cycle for 12 days. Continuous aeration was provided in culture bottles of 2 litre capacity using an aerator. During the experimental period, temperature was observed to range between 27-29 °C. The cell count of algal culture was done on every alternate day, using improved Neubauer ruling haemacyto-meter under a light microscope. The cell number, optical density, chlorophyll-**a**, pH, free CO_2, dissolved O_2, light intensity, temperature, alkalinity, hardness, phosphate-phosphorus, ammonia-nitrogen, nitrate-nitrogen, and nitrite-nitrogen were determined every alternate day following standard methods (Clesceri *et al.,* 1989).

Estimation of chlorophyll-a content. The estimation of chlorophyll-**a** content of the microalga 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/litre) = 11.85 (OD 664) – 1.54 (OD 647) – 0.08 (OD 630)

The specific growth rate (µg/day) of *C. vulgarsis* on the basis of cell and chlorophyll-**a** content, and the total biomass on the basis of chlorophyll-**a** content were also determined (Clesceri *et al.,* 1989).

Specific growth rate (SGR). The specific growth rate (µg/day) of cultured microalga was calculated from the following equation (Claeceri *et al.,* 1989):

$$SGR\ (\mu g/day) = Ln\ (X_1\text{-}X_2)/t_1\text{-}\ t_2$$

where:

X_1 = biomass concentration of the end-selected time interval
X_2 = biomass concentration at the begining of selected time interval
t_1 - t_2 = elapsed time between the selected time in the day

For preparation of one litre BBM, 10 ml of the stock solutions serial # 1-6 and 1.0 ml from stock solution serial # 7- 10 (Table 1) were pipetted to make 1 litre volume with distilled water in a volumetric flask.

Determination of pH and dissolved oxygen. pH and dissolved oxygen were determined following the methods of Clesceri *et al.* (1989).

Proximate composition analysis. The cultured microalga was harvested before the onset of stationary phase of growth and centrifuged at 5000 rpm for five min. Ammonium formate (32 g/ litre) was used to wash salts sticking with the centrifuged algal mass. The microalgal mass was then cleaned with

Table 1. Chemical composition (g/litre) of Bold's basal medium (Nicholas and Bold, 1965).

No.	Stocks of chemicals	g/litre
1.	$NaNO_3$	25
2.	$Mg.SO_4.7H_2O$	7.5
3.	NaCl	2.5
4.	K_2HPO_4	7.5
5.	KH_2PO_4	17.5
6.	$CaCl_2.2H_2O$	2.5
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 dissolve these chemicals	
8.	H_3BO_3	11.4
9.	EDTA-KOH solution:	
	a) $EDTANa_2$	50.0
	b) KOH	30.1
10.	$FeSO_4.7H_2O$ with 1.0 ml concentrated H_2SO_4	4.98

distilled water, repeatedly centrifuged and kept at 0 °C for three days, followed by drying in oven at 40 °C. The dry samples were preserved frozen at -10 °C until analyzed for proximate composition. The crude proteins, lipids, moisture, ash, crude fibre and nitrogen free extract (NFE) were determined following the standard methods (AOAC, 1984).

Data analysis and interpretation. Observations were recorded for triplicate samples. Correlation coefficient for the growth parameters was done following linear regression analysis. Differences within the measured parameters and the treatment means were determined using one-way ANOVA following Duncan's multiple range test. Statistical analyses were done following MSTAT programme (Zar, 1984).

Results and Discussion

Maximum cell growth of *Chlorella vulgaris,* measured as cell count (199.88 x 10^5/ml), was found significantly higher ($p < 0.01$) in 50% SMEM on the 10th day, followed by BBM with a lower cell count of 190.25 x 10^5/ml, which indicated better growth performance of *C. vulgaris* in the 50% sugar mill effluent medium (Fig. 1). Similar trend was observed for chlorophyll-**a** (Fig. 2), also signifying better performance of the microalga in 50% SMEM, which may be attributed to adequate nutrient availability in the medium at this dilution (Habib *et al.,* 2003; 1998). The high ($p < 0.01$) correlation coefficient values among cell count and chlorphyll-**a** of *C. vulgaris* with chlorophyll-**a** and optical density of media containing this alga indicate that the growth performances were positively interrelated (Fig. 3 and 4). Specific growth rate (SGR) of *C. vulgaris,* grown in 50% SMEM, showed significantly ($p < 0.01$) higher cell count on 10th day of the culture, followed by those grown in BBM and other concentrations of SMEM (Table 2). Total biomass of *C. vulgaris* grown in 50% SMEM was significantly higher ($p < 0.01$) than that grown on BBM and other concentrations of SMEM. The transparent medium permitted sufficient light penetration, and adequate supply of CO_2 from the air into the medium was ensured through aeration to overcome the deficiency of carbon in the medium (Anton *et al.,* 1994; Phang, 1991). Phang and Ong (1988) reported that C : N : P ratios in different heterotophic media, such as diluted raw latex, concentrated rubber, and standard Malaysian rubber effluent, indicated that the quantity of C was almost three-times less than the recommended ratio (56.30 : 8.60 : 1.20). This situation was partially overcome by adequate supply of filtered air (Johns, 1994), and CO_2 gas into the media (Geeta *et al.*, 1994). However, the recorded level of CO_2 in the present study was initially high in different treatments, which decreased slowly as the growth of alga progressed.

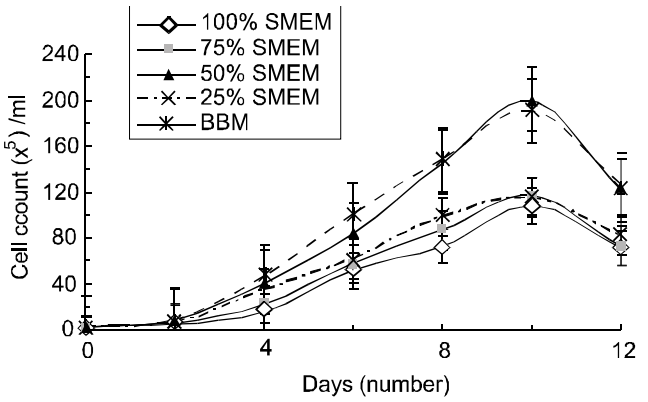

Fig. 1. Cell count of *Chlorella vulgaris* grown in different concentrations of sugar mill effluent media (SMEM) and Bold's basal medium (BBM) as the control.

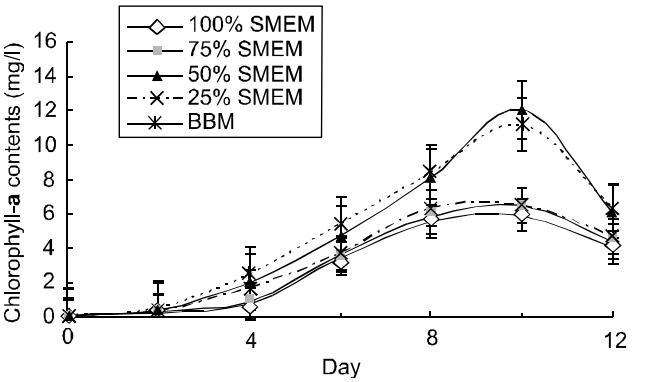

Fig. 2. Chlorophyll-**a** content found in 100%, 75%, 50%, 25% sugar mill effluent media (SMEM), and Bold's basal medium (BBM) as the control.

The amount of CO_2 was very low at the stationary phase, which reached minimum level at the death phase. This might be due to the maximum use of CO_2 for photosynthetic activity of *C. vulgaris* at the peak, as the cell count was maximum in the declining growth phase and the stationary phase. Continuous aeration was provided during the study using electric aerator to supply air in the culture bottles so as to meet the carbon needs for algal growth. Agitation has been reported to be essential for mixing CO_2 and nutrients in the media so that the growing algae can get the needed nutrients properly (Habib *et al.,* 1998; Oswald, 1988; Terry and Raymond, 1985). Agitation keeps both nutrients and cells evenly distributed, promoting uniform nutrient and light absorption. Mixing during culture through aeration yielded approximately 30% more algal growth than was achieved without aeration (Molina *et al.,* 1990). Bubbling air, as a means of mixing, may be more appropriate for small-scale cultures than in the large-scale operations (Persoone *et al.,* 1980).

Physicochemical properties of the culture media on which *C. vulgaris* was growing were recorded every alternate day. These characteristics of 50% SMEM on the 10th day of culture (highest growth stage) are presented in Table 3. A day-night (12 h -12 h) light-darkness cycle was provided under 2500 lux/m^2/s light intensity. Hoff and Snell (1989) have recommended a wide range of light intensity (lux/m^2/s), 1,000-10,000 with the optima range of 2,500-5,000 depending on culture volume and density maintaining a minimum photoperiod of 16-8 h light-darkness. Temperature was recorded, which ranged from 27.8 to 28.5 °C during the study, and has been reported as favourable for algal growth (Habib *et al.,* 2003). The optimum pH range for most of the algal species has been reported to be 7-9. The "most optimum" range of 8.2-8.7 was reported by Ukeles (1971). In the present study, the pH level ranged between 6.86 and 8.53 (Table 3), which is agreeable with the reported find-

Table 2. Specific growth rate (SGR, µg/day) of cell, chlorophyll-**a** (chlo-**a**), and total biomass of *Chlorella vulgaris* grown in 100%, 75%, 50% and 25% SMEM, and BBM as the control on 10th day of culture

Parameters	100% SMEM	75% SMEM	50% SMEM	25% SMEM	BBM
SGR of cell	$0.37^{c} \pm 0.01$	$0.37^{c} \pm 0.01$	$0.39^{a} \pm 0.00$	$0.36^{c} \pm 0.00$	$0.38^{b} \pm 0.01$
SGR of chlo-**a**	$0.38^{d} \pm 0.01$	$0.37^{cd} \pm 0.01$	$0.41^{a} \pm 0.02$	$0.38^{c} \pm 0.01$	$0.40^{b} \pm 0.00$
Total biomass (chlo-**a** x 67)*	398.21± 11.73	424.33 ± 11.49	809.14 ± 16.15	437.73 ± 6.04	750.18 ± 23.56

* mg/1; mean values (± SD) with different superscripts in each row indicate significant differences ($p < 0.01$); SMEM = sugar mill effluent media; BBM = Bold's basal medium (Nichlas and Bold, 1965)

Table 3. Optical density of media, and the physicochemical properties of four different media made from the sugar mill effluent (SMEM), and Bold's basal medium (BBM) used as the control, for the growth of *Chlorella vulgaris*

	Different concentration of SMEM				
Parameters	100%	75%	50%	25%	BBM
Optical density	0.02-1.08 (1.08)	0.02-1.21 (1.21)	0.02-2.14 (2.14)	0.02-1.30 (1.30)	0.02-1.98 (1.98)
Light intensity	1690-1920 (1890)	1660-1970 (1820)	1600-2000 (1850)	1690-1950 (1710)	1760-1950 (1830)
pH	7.26-8.50 (8.50)	7.16-8.38 (8.25)	7.05-8.30 (8.24)	7.06-8.53 (8.25)	6.86-8.30 (8.26)
Temperature (°C)	27.9-28.5 (28.5)	27.9-28.5 (24.4)	27.8-28.5 (28.4)	27.8-28.4 (28.3)	27.8-28.4 (28.3)
Free CO_2(mg/l)	15.0-35.0 (16.67)	13.33-35.0 (16.67)	16.67-35.0 (20.0)	10.0-26.67 (11.67)	18.33-35.0 (20.0)
Alkalinity (mg/l)	79.8-114.0 (108.3)	74.1-108.3 (102.6)	74.1-114.0 (108.3)	62.7-108.3 (102.6)	91.2-165.3 (165.3)
Hardness (mg/l)	34.2-74.1 (39.9)	34.2-74.1 (39.9)	34.2-68.4 (39.9)	27.5-57.0 (28.5)	39.9-74.1 (45.6)
Ammonia-N (mg/l)	0.21-2.26 (1.86)	0.12-3.31 (1.76)	0.05-2.16 (1.59)	0.13-2.20 (0.96)	0.02-0.95 (0.28)
-NO_2-N (mg/l)	0.01-0.20 (0.18)	0.01-0.18 (0.18)	0.01-0.19 (0.18)	0.01-0.19 (0.15)	0.01-0.15 (0.10)
-NO_3-N (mg/l)	0.69-3.51 (0.90)	0.64-3.65 (0.85)	0.61-3.75 (0.75)	0.45-2.93 (0.54)	1.97-15.19 (4.29)
-PO_4-P (mg/l)	0.98-4.54 (1.88)	0.88-4.36 (1.56)	0.81-4.52 (1.65)	0.79-4.44 (1.54)	3.01-5.39 (3.64)

figures in parenthesis indicate values at peak of growth of *Chlorella vulgaris*

ings of Habib (1998), Mayo (1997), Anaga and Abu (1996), and James *et al.* (1988). Dissolved oxygen (DO) was found to be maximum at the stationary phase in all the SMEM concentrations studied, with a range of 4.05 to 4.96 mg/litre. Fluctuations in the DO values throughout the culture period were minimum, and at death phase the DO value was at the lowest. This might be due to the maximum fixation of DO during photosynthesis. Hussain (2001), and Miah *et al.* (1999) determined maximum dissolved oxygen of 5.32 mg/litre and 4.49 mg/litre, respectively, when *Chlorella* sp. was grown in different inorganic and organic media.

Phosphate-phosphorus (PO_4-P) was found to be maximum at the beginning in different culture media and minimum was noted on the 8th day at the slow growth phase, which were noted to increase during the stationary phase (Table 3). Hussain (2001) found minimum phosphate level during the culture of *C. ellipsoidea* in the jackfruit seed powder medium and BBM on the 8th day at stationary phase, which has similarity with the present study. Ammonia-nitrogen (NH_3-N) was found to be maximum at the death phase, and was minimum before the culture was started. Similar trend was observed in the case of nitrite-nitrogen (NO_2-N). In the case of nitrate-nitrogen (NO_3-N), however, the trend was inverse. Nitrate level was found to be maximum before inoculation of algal cells and minimum at the death phase, having a very high level in BBM as compared with differently diluted effluent media. Hussain (2001) found similar trend in his study on *Chlorella* sp., when grown in organic media. Alkalinity and hardness of the media were observed to be inversely related, i.e., maximum alkalinity at the end of the culture period when hardness was minimum (Table 3). On the other hand, hardness was maximum at the beginning when the alkalinity was minimum.

Proximate composition analysis (Table 4) showed that *C. vulgaris* grown in BBM contained the highest amount of proteins (45.44%), followed by those grown in 50% SMEM

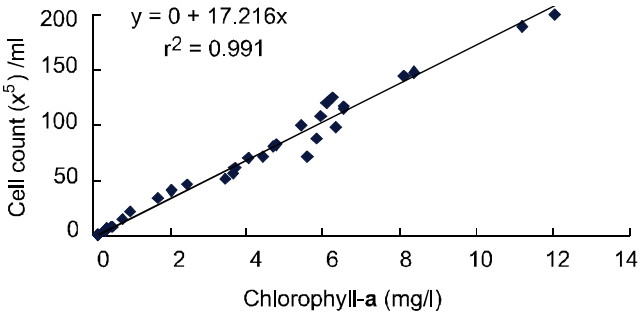

Fig. 3. Correlation between cell count and chlorophyll-**a** contents of *Chlorella vulgaris* grown in different concentrations of sugar mill effluent media (SMEM).

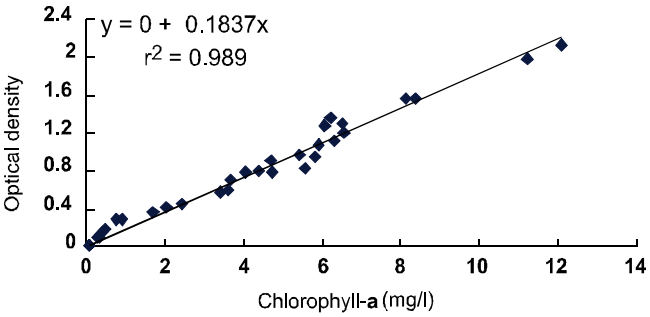

Fig. 4. Correlation between chlorophyll-**a** contents and optical density found in growth of *Chlorella vulgaris* grown in different concentration of sugar mill effluent media (SMEM).

Table 4. Proximate composition (% dry matter) of *Chlorella vulgaris* grown in 100%, 75%, 50% and 25% sugar mill effluent media (SMEM) and Bold's basal medium (BBM) (Nicholas and Bold, 1965) as the control

Components	100% SMEM	75% SMEM	50% SMEM	25% SMEM	BBM
Moisture	$9.17^{b} \pm 0.09$	$10.20^{a} \pm 0.04$	$8.36^{c} \pm 0.12$	$10.31^{a} \pm 0.04$	$6.21^{d} \pm 0.09$
Crude proteins	$40.46^{d} \pm 0.11$	$41.45^{c} \pm 0.09$	$43.69^{b} \pm 0.26$	$40.71^{d} \pm 0.10$	$45.44^{a} \pm 0.11$
Crude lipids	$16.32^{b} \pm 0.08$	$15.90^{b} \pm 0.12$	$17.27^{a} \pm 0.11$	$16.48^{b} \pm 0.08$	$10.49^{c} \pm 0.12$
Crude fibre	$6.27^{b} \pm 0.06$	$5.58^{c} \pm 0.06$	$5.12^{d} \pm 0.08$	$6.31^{b} \pm 0.04$	$10.43^{a} \pm 0.12$
NFE*	$23.07^{b} \pm 0.07$	$23.44^{a} \pm 0.09$	$22.67^{c} \pm 0.04$	$23.69^{a} \pm 0.12$	$23.45^{a} \pm 0.22$
Ash	$13.89^{a} \pm 0.08$	$13.63^{b} \pm 0.06$	$11.25^{d} \pm 0.12$	$12.81^{c} \pm 0.11$	$10.49^{e} \pm 0.12$

*nitrogen free extract (NFE) = 100 − (moisture + crude proteins + crude lipids + crude fibre + ash); mean values (± SD) with different superscripts in each row indicate significant differences ($p < 0.01$).

(43.51%), 25% SMEM (42.71%), 75% SMEM (41.71%), and the lowest amount was 40.46% when *C. vulgaris* was grown in 100% SMEM. Maximum lipid contents in *C. vulgaris* were noted when it was grown in 50% SMEM (17.27%), followed by those grown in 25% SMEM, 100% SMEM, 75% SMEM and BBM. Ash was found to be maximum (13.89%) in *C. vulgaris* when grown in 100% SMEM and minimum (10.49%) noted when cultured in BBM. The ash content was observed to be directly related with the ash concentration of effluents, which are referred to as the inorganic residues (FDS, 1994). This observation indicates that the mineral bioaccumulation was directly correlated with the concentration of effluents present in the medium (Habib *et al.,* 2003; Vymazal, 1995; Fabregas and Herrero, 1986).

Conclusion

The present studies indicate that sugar mill effluents (SMEM) can be used as a suitable culture medium for the cultivation of *Chlorella vulgaris*, thus replacing the otherwise standard but costly algal culture media, such as the Bold's basal medium. The SMEM may thus be used for mass culture of important microalgae, like *Chlorella vulgaris,* for use as nutritious feed for zooplankton species for generating important components in the food chain in aquaculture systems.

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