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Growth performance of *Chlorella vulgaris* in different concentrations of red sugar medium easily available in Bangladesh

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Abstract
Chlorella vulgaris was cultured in different concentrations of red sugar medium ($RSM_{0.4\ g/l}$, $RSM_{0.8g/l}$, and $RSM_{1.2\ g/l}$) and in Bold basal medium (BBM) as control. Maximum cell growth ($\times 10^5$)/ml of *Chlorella vulgaris* was found 200.21 on 8th day in red sugar medium ($RSM_{0.4}$ g/l) followed by BBM and other RSMs. Similar trends were observed in case of chlorophyll a content and optical density of *C. vulgaris* grown in red sugar media (RSM) and BBM. Specific growth rates (SGRs) of cell number and chlorophyll a content were found maximum on 8th day of *Chlorella vulgaris* grown in $RSM_{0.4\ g/l}$ followed by other media of RSM and the BBM. Total biomass followed the similar trend. Proximate composition analysis of *Chlorella vulgaris* grown in different concentrations of RSM and BBM showed maximum protein 46.11% in $RSM_{0.4\ g/l}$ followed by 45.44% in BBM and all RSM media. Crude protein and crude lipid of the cultured *C. vulgaris* were found significantly ($P<0.01$) higher grown in $RSM_{0.4\ g/l}$ indicates that this food item may possibly be a promoting source of low cost culture medium for *Chlorella vulgaris* or any other microalgal species.

Keywords: Biomass, chlorophyll a, optical density, crude protein

1. Introduction
During investigation of growth performance of sugarmill wastes as microalga culture media, red sugar (RSM) locally known as 'gur' was also considered to test for microalgae culture medium as easily available in Bangladesh countryside. There are two types of red sugar available in Bangladesh – one made from sugarcane juice and another made from date tree juice. These sugars are found in solid state (block) with some particular structure, such as, small sized half ball, large size half ball and medium ball size, although sugarcane sugars sometimes found in powdered form. Sugarcane are crushed by local crushing machine as well as motor driving crushing machine in sugar mill command area to prepare red sugar in traditional methods. The sugarcane farmers produce thousands of tons of red sugar around the mill areas and other places of the country. However, around Jheel Bangla Sugarmill area 63,845 MT red sugars were produced in 1999-2000 production season [16]. According to Annual Report of Bangladesh Sugar and Food Industries Corporation (2015-2016) [4] the total sugarcane production was 2101008 MT where 964228 MT (45.89%) supplied to sugarmill and produced 58219 MT sugar, 737469 MT (35.10%) sugarcane used for gur production, 156971 MT (7.47%) used for seedlings and 242340 MT (11.53%) for domestic and other purposes respectively. The corporation also produced 36392 MT molasses, 336269.85 MT bagasse and 28900 MT pressmud in 2015-2016 periods. Due to discharge of these wastes and byproduct the sugar industries always face environmental pollution. This red sugar (gur) is less costly and very popular sweetening item to the rural areas in comparison to refined sugar from sugar mills. Date sugar is costly and its availability is very limited and not related to sugarcane industry. As the overall investigation deals with sugar mill wastes for culture of microalgae, red sugar from sugarcane may be an ingredient to use in culture media for *Chlorella vulgaris*. Mill sugar contains 99.74% sucrose and molasses contains 36.01% sucrose [16]. Rasappan *et al.*, (2015) [33] observed molasses contains 30-35% sucrose and 10-20% glucose and fructose which indicates that red sugar also contains high level of sucrose. *Chlorella* is produced both through the autotrophic and mixotrophic metabolic routes, i.e. in addition to carbon dioxide supplied during the day, acetic acid or glucose are supplied and greatly increase production

per unit volume of culture medium [34]. Microalgae-derived biomass is recognized as an alternative source for a wide variety of bioproducts, such as biofuels, especial oils, pigments and polymers [27]. In Japan more than a dozen species of microalgae are mass cultivated at various fish hatcheries as larval diets of shellfish and as diets for living animal feeds, mostly rotifers, which are in turn used for diets of fish larvae [25]. *Chlorella sp.* contains 40-50% protein. *Chlorella sp*. has been successfully cultured in agro-based organic nutrient such as rubber and palm oil mill effluent, ripe and unripe been seed powder etc [29, 10, 17]. Significant cost reductions can be achieved if CO_2, nutrients and water for microalgae cultivation are obtained at low cost [6]. The researchers continuously investigating different low cost ingredients like sugarmill effluent, sweetmeat effluent, sugarmill wastes like molasses, pressmud, municipal wastes, swine wastes, even green crops (cabbage) powder etc. [18, 32, 39, 26, 19, 37, 15] to find out suitable microalgae culture media as well as to make the environment pollution free. Considering these factors, the present study was undertaken to investigate the growth performance of *C. vulgaris* in different concentrations of red sugar media to establish a low cost and easily available source of microalgae culture media.

2. Materials and Methods

2.1 Sample Collection and Preparation of Culture Media: Red sugar was collected from local market and red sugar media (RSM) were made in different concentrations to inoculate *Chlorella vulgaris* in volumetric flask with a blank in Bold Basal Medium (BBM). Different media concentrations of red sugar $RSM_{0.4\ g/l}$, $RSM_{0.8\ g/l}$ and $RSM_{1.2\ g/l}$ were developed for culture of *C. vulgaris* in laboratory condition. Red sugar was measured and diluted in distilled water to make the solutions of RSM $_{0.4g/l,}$ RSM $_{0.8g/l}$ and RSM $_{1.2g/l}$ in different glassware's. 260 mg/l urea was added in each of the red sugar medium to enhance the nitrogen level and all the flasks were autoclaved to sterilize the media at 120°C steam heat. Three treatments of RSM 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 stock solutions is shown below in Table 1.

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 (Table 1) were pipetted to make one litre volume with distilled water in a volumetric flask.

2.2 Culture of *Chlorella vulgaris*: *Chlorella vulgaris* (No.001) was cultured in RSM $_{0.4g/l,}$ RSM $_{0.8g/l}$ and RSM $_{1.2g/l}$ and in BBM at Live Food Culture Laboratory, Department of Aquaculture, Bangladesh Agricultural University, Mymensingh. *Chlorella vulgaris* were inoculated from a stock culture of six days to make a 10% suspension (optical density at 620 nm= 0.02) in all the culture treatments [10]. 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 was also 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, and some other physicochemical parameters were measured every alternate day following standard methods [8].

2.3 Estimation of chlorophyll-*a* content: Optical densities of the prepared sample were analyzed at 664, 647 and 630 nm wave length operating UV-spectrophotometer [8]. 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) and the total biomass of *C. vulgaris* were also determined on the basis of cell and chlorophyll-*a* content following standard methods [8].

2.4 Specific growth rate (SGR): The specific growth rate (mg/day) of the cultured microalga was computed using following equation [8]:

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

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

2.5 Proximate composition analysis: The microalgae cultured in different concentrations of RSM and BBM 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 0^o C for three days and then dried in the oven at 40^o C. The dry samples were preserved in the freeze at -10^o C for study the proximate composition. The prepared samples of *C. vulgaris* were analyzed in triplicate 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 [12].

2.6 Analysis and interpretation of data: Data were statistically analyzed and interpreted to determine the differences within the measured parameters and the treatment means using one way ANOVA and Duncan's Multiple Range Test following MSTAT statistical package [40].

3. Results

Maximum cell growth of *Chlorella vulgaris* was found 200.21 ($\times 10^5$)/ml on 8th day (Figure 1) in red sugar medium ($RSM_{0.4}$ g/l) followed by 185.63 ($\times 10^5$)/ml, 140.46 ($\times 10^5$)/ml, 75.76 ($\times 10^5$)/ml cultured in BBM, $RSM_{0.8\ g/l}$ and $RSM_{1.2\ g/l}$. Similar trends were observed in case of chlorophyll a (mg/l) content (Figure 2) 11.18 in $RSM_{0.4g/l}$, 10.36 in BBM, 7.72 in $RSM_{0.8\ g/l}$ and 3.80 in $RSM_{1.2\ g/l}$. Optical density (Figure 3) of *C. vulgaris* grown in various culture media found 2.21, 2.02, 1.44 and 0.69 were supported the trends of cell number and chlorophyll *a* content. Specific growth rates (SGRs µ/day) of cell number determined 57, 54, 49, 52 in different culture media on highest growth of C. vulgaris on 8th day which were complied by chlorophyll a content (Table 3) of the cultured microalga in different media of RSM and BBM. Total biomass showed a mild difference trend but signified the $RSM_{0.4g/l}$ as the highest biomass producing medium.

The physico-chemical parameters of different concentrations of RSM and BBM were determined every alternate day. On the basis of the recorded data the different values of parameters during highest cell growth performance of red sugar medium ($RSM_{0.4mg/l}$) on 8day and the values of various parameters during whole culture periods ranged in different concentrations of RSM and BBM are shown in table 2 and the pH of different medium during culture period ranged 6.78-8.96 described in Figure 4.

Table 2: Physico-chemical parameters recorded during *Chlorella vulgaris* culture in different concentrations of RSM and BBM.

Parameters	Quantity on 8th day in RSM $_{0.4g/l}$ with maximum cell growth	Quantity ranged in different concentrations of RSM and BBM in whole culture period
Light intensity (lux/m^2/s)	1890	1700-1950
P^H	8.17	6.78-8.96
Temperature °C	28.11	27.10-29.25
DO mg/l	4.13	3.25-4.57
PO_4-P mg/l	0.86	5.42-0.6
NH_3- N mg/l	0.45	0.02-1.18
NO_3- N mg/l	0.91	16.52-0.08
NO_2- N mg/l	0.09	0.01- 0.31
Alkalinity (mg/l)	68.40	39.9-159.2
CO_2 mg/l	36.67	16.67-40.0
Hardness (mg/l)	22.80	22.8-91.2

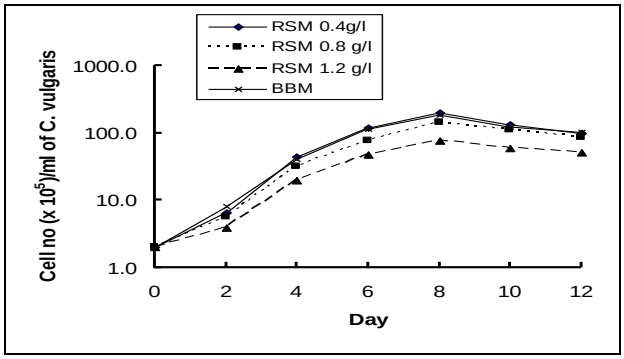

Fig 1: Semilogarithmic growth curve based on cell number (×105)/ml of media contained *Chlorella vulgaris* grown in different concentrations of red sugar media (RSM) and Bold basal medium (BBM)

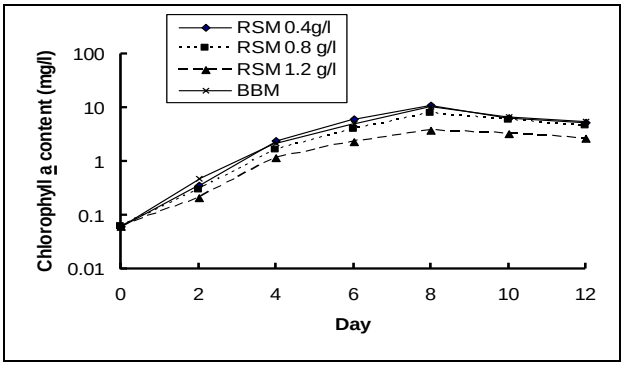

Fig 2: Semilogarithmic growth curve based on chlorophyll a content (mg/l) of media contained *Chlorella vulgaris* grown in different concentrations of red sugar media (RSM) and Bold basal medium (BBM)

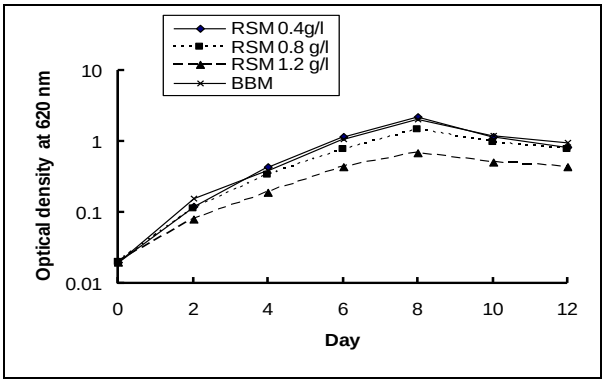

Fig 3: Semilogarithmic growth curve based on optical density at 620 nm of media contained *Chlorella vulgaris* grown in different concentrations of red sugar media (RSM) and Bold basal medium (BBM

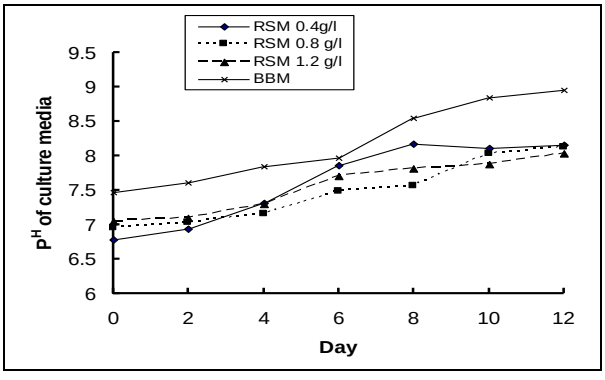

Fig 4: Growth curve based on hydrogen ion concentration (pH) of media containing *Chlorella vulgaris* grown in different concentrations of red sugar media (RSM) and Bold basal medium (BBM) as control

Table 3: Specific growth rates (μ/day) of cell, chlorophyll a (chlo-a) and total biomass of *Chlorella vulgaris* grown in different concentrations of red sugar media (RSM) and Bold basal medium (BBM)

Parameters	$RSM_{0.4\ g/l}$	$RSM_{0.8\ g/l}$	$RSM_{1.2\ g/l}$	BBM
SGR of cell	$0.57^{a} \pm 0.02$	$0.54^{b} \pm 0.02$	$0.49^{d} \pm 0.01$	$0.52^{c} \pm 0.03$
SGR of chlo-a	$0.58^{a} \pm 0.02$	$0.54^{b} \pm 0.02$	$0.49^{d} \pm 0.01$	$0.52^{c} \pm 0.04$
Total biomass (Chlo-a × 67)*	748.84a ± 31.92	517.91c ± 18.31	254.82d ± 6.71	694.34b ± 18.72

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

Proximate composition analysis of *Chlorella vulgaris* grown in different concentrations of RSM and BBM showed that maximum protein 46.11% was found in $RSM_{0.4\ g/l}$ followed by 45.44% in BBM, 44.46% in $RSM_{0.8\ g/l}$ and 42.23% in $RSM_{1.2\ g/l}$ (Table 4). Maximum crude lipid 14.25% of *C. vulgaris* grown in $RSM_{0.4\ g/l}$ was estimated followed by 12.77%, 12.35%, and 10.47% lipid of the same cultured in $RSM_{0.8\ g/l}$, $RSM_{1.2\ g/l}$ and BBM respectively. In case of crude fiber the trend was inverse, ie. the minimum crude fiber was found in the algae cultured in $RSM_{0.4\ g/l}$ and maximum of that in BBM. Maximum NFE of *C. vulgaris* was recorded that grown in $RSM_{1.2\ g/l}$ followed by BBM and the minimum was found that grown in $RSM_{0.4\ g/l}$. Maximum ash was determined in the microalgae cultured in $RSM_{1.2\ g/l}$ and the minimum when grown in BBM.

Table 4: Proximate composition (amount% dry matter) of Chlorella vulgaris grown in different concentrations of red sugar media (RSM) and Bold basal medium (BBM)

Composition	RSM 0.4 g/l	RSM 0.8 g/l	RSM 1.2 g/l	BBM
Moisture	$9.34^{a} \pm 0.09$	$8.75^{b} \pm 0.09$	$9.46^{a} \pm 0.08$	$6.21^{c} \pm 0.09$
Crude protein	$46.11^{a} \pm 0.08$	$44.46^{c} \pm 0.11$	$42.23^{d} \pm 0.11$	$45.44^{b} \pm 0.11$
Crude fat	$14.25^{a} \pm 0.10$	$12.77^{b} \pm 0.09$	$12.35^{c} \pm 0.10$	$10.47^{d} \pm 0.12$
Crude fiber	$6.11^{d} \pm 0.04$	$7.52^{c} \pm 0.10$	$7.90^{b} \pm 0.06$	$10.43^{a} \pm 0.12$
NFE	$22.17^{c} \pm 0.08$	$23.06^{b} \pm 0.18$	$25.01^{a} \pm 0.25$	$23.45^{b} \pm 0.22$
Ash	$11.37^{b} \pm 0.10$	$12.30^{a} \pm 0.10$	$12.52^{a} \pm 0.08$	$10.49^{c} \pm 0.12$

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

4. Discussions

Maximum cell growth of *Chlorella vulgaris* (200.21×10^{5}/ml) grown in red sugar medium ($RSM_{0.4\ g/l}$) was significantly (*P<0.01*) higher than that grown in all other treatments of RSM and BBM which complying the significance of better performance of the studied $RSM_{0.4g/l}$ as a microalga culture media (Figure 1). Similar trends were observed in case of chlorophyll a content (Figure 2) and optical density (Figure 3) of *C. vulgaris* grown in red sugar media (RSM) and BBM. These also signify the better growth performance of the media $RSM_{0.4\ mg/l}$, which may be attributed to adequate nutrient availability in this dilution of the medium [11, 9]. Chlorophyll a and optical density of the cultured media found to be positively interrelated with the cell growth [10, 18, 39].

Specific growth rate (SGR) of *Chlorella vulgaris* cultured in $RSM_{0.4g/l}$ were found significantly (*P<0.01*) higher than that grown in all other treatments including the control BBM. Similarly chlorophyll a content and total biomass of *C. vulgaris* grown in $RSM_{0.4\ g/l}$ were also found significantly (*P<0.01*) higher than those cultured in other RSM and BBM (Table 3). Similar findings were recorded by crude protein and lipids of C. vulgaris cultured in $RSM_{0.4\ g/l}$ were higher than those of this alga grown in other media (Table 4). This might be due to that the *Chlorella vulgaris* cultured in $RSM_{0.4\ g/l}$ could bioaccumulate maximum nutrient than those grown in other concentrations of RSM and BBM. The $RSM_{0.4\ g/l}$ might contains adequate nutrients available in the medium [9, 11] which act as heterotrophic culture media [7], appropriate color of the media which permitted sufficient light penetration in the media and supply of adequate carbon dioxide through air to mix into the media to overcome the deficiency of carbon in the media [28, 5, 11].

In the present study *C. vulgaris* grown in $RSM_{0.4g/l}$ contained the highest level of protein than the microalga grown in all other red sugar media (RSM) and BBM. Crude lipid found higher of *C vulgaris* grown in all the red sugar media (RSM) than that of the control medium BBM. Habib (1998) [10] found similarly higher lipid and protein contents of *Chlorella vulgaris* grown in different effluent media of latest concentrate of rubber industry (LCRE), standard Malaysian rubber effluent (SMRE) and palm oil mill effluent media (POMED) than that grown in the control media NPK.

All the physico-chemical parameters such as light intensity ranged 1700-1950 lux/m^2/s, pH ranged 6.78-8.96, temperature ranged 27.10-29.25°C, dissolved oxygen ranged 3.25-4.57 mg/l, orthophosphate ranged 5.42-0.86 mg/l, ammonia nitrogen ranged 0.02- 1.18mg/l, nitrate nitrogen ranged 16.52-0.08 mg/l, nitrite nitrogen ranged 0.01- 0.31mg/l, carbon dioxide ranged 16.67-40.0 mg/l were found in normal ranges (Table 2) throughout the culture system. Landau (1991) [22]; Thinh (1994) [38]; Anaga and Abu (1996) [3]; Khan (1996) [21]; Miah *et al*., (1999) [24]; Alam *et al*., (2003) [1]; Khan *et al*., (2006) [18, 19]; Mayo (1997) [23] found almost similar ranges of physico-chemical parameters of microalgae culture media agreed the present findings.

Tadashi *et al*. (2018) [37] investigated three microalgae, *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, and *Euglena gracilis*, were cultured in two municipal wastewater effluents and one swine wastewater effluent with and without indigenous bacteria for 7 days. All microalgae grew better in all effluents with indigenous bacteria than without bacteria. Biomass production of *C. reinhardtii*, *C. vulgaris*, and *E. gracilis* increased > 1.5, 1.8–2.8, and > 2.1-fold, respectively, compared to the axenic cultures of each microalga. These results suggest that the three microalgae produced and supplied organic carbon that supported bacterial growth in the effluent. They recorded initially the water qualities of the effluents. They found pH level 7.1-7.7, TOC (total organic carbon) 10.2-56.4mg/l, ammonia nitrogen (NH_4-N) 2.2-57.4mg/l, nitrous nitrogen (NO_2-N) 0.1- 2.0 mg/l, nitrate nitrogen (NO_3-N) 1.8-5.4 mg/l and orthophosphate (PO_4-P) 2.1-23.4 mg/l. The physicochemical parameters they studied

partly agreed the present investigation and as obtained very high level of ammonia nitrogen in waste water mostly unlike but their biomass production was satisfactory. This might be due to waste water quality improved afterwards reducing the ammonia nitrogen and nitrous nitrogen.

5. Conclusion

In Bangladesh red sugar is locally known as 'gur' and very popular sweetening item abundantly available in sugarcane industry area. More than 35% sugarcane used for gur production. In India it is widely known as JAGGERY/GUR. Jaggery/Gur production in India is about 7-10 million tons (mt) per annum, while its per capita consumption is about 5 kg. It is the most ancient sweetening agent in India. This is a low grade non-centrifugal sweetener consumed in India, Pakistan, Bangladesh, Africa, Myanmar, China and other countries (Amit & Narendra, 2017) [2]. They found 75% of Jaggery/Gur goes to alcohol production, rest goes for ayuvedic, herbal product, animal feed, eating purpose etc in some area of UP. In present investigation the red sugar medium (RSM $_{0.4g/l}$) found a good microalgal culture media even showed significantly better growth performance than the control Bold basal medium (BBM). For microalgal biomass productions [27], microbial transglutaminase [36, 26], extraction of β-carotene, polyunsaturated fatty acids and astaxanthin [22, 35], this RSM medium may contribute in a great extend as gur being a low cost [6] and easily available ingredient in many sugarcane producing countries. Hope this traditional food item may be an important source for biomass production to accumulate biofuels, especial oils, pigments and polymers [27] in future.

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