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A two step sequential treatment of ethanol distillation bottom liquid by bacterial fermentation and subsequent *Chlorella vulgaris* culture under continuous illumination of various lights

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Abstract

Bottoms liquid from an ethanol distillation of spent sugarcane juice media was treated using a twostep process. First, a *Clostridium* strain isolated from a wastewater treatment pond was grown for 6 days on diluted distillation bottoms liquid. This treatment reduced sugar concentration from 10.7%(w/v) to 2.6%(w/v) and accomplished a COD removal of 68.58%. It increased concentrations of NO_3^-, P, K, Na, Ca and Mg from 183, 2218, 285, 237, 304 and 224 mg/L to 616, 2901, 1600, 3863, 389 and 472 mg/L, respectively. Subsequently, mineral removal was accomplished using a biotreatment by *Chlorella vulgaris*. *Chlorella vulgaris* was cultured in ethanol distillation bottoms liquid diluted 1:100 with water. Experimental conditions were an initial pH of 5, incubation at room temperature with continuous illumination using white, red and green light at the intensities of 3,000 and 5,000 Lux for 10 days. It was found that *Chlorella vulgaris* had the highest biomass (cell dry weight) of 0.94g/day under white light with illumination at the intensity of 5,000 Lux. *Chlorella vulgaris* grown in Basal Medium under same conditions (control treatment) produced less biomass (0.183 g/day) than the tested conditions. After the *Chlorella vulgaris* biotreatment, concentrations of NO_3, P, K, Na, Ca and Mg were low enough for discharging into the environment. The NO_3^-, P and K removal was especially pronounced.

Keywords: Chlorella vulgaris, ethanol distillation residue, wastewater, sugarcane

1. Introduction

Industrial and agricultural wastewater is primarily composed of organic substances such as carbohydrates, proteins and fats. If these are released into an environment, they can cause adverse environmental impacts. Sugarcane juice for industrial ethanol production has a high level of sugar. Sugarcane juice has become an important raw material for fuel ethanol production in Udon Thani Province (Thailand). Approximately 2.5 tonnes of sugarcane is needed to produce 1,000 L of juice containing 200-230 g/L of total sugar [1]. After fermentation, the distillation process produces 150 L of ethanol and 750 L wastewater also known as bottoms liquid [1]. The bottoms liquid contains organic materials that are harmful it is released into the environment. It also contains lot of

nutrients including sugar. These can be used as nutrients to produce biogas. Anaerobic digestion has been utilized to treat waste water containing high organic matter to significantly reduce chemical oxygen demand (COD). Bio-conversion of wastewater sludge to biogas is limited. Anaerobic digestion of wastewater sludge was done using a *Clostridium* strain isolated from the sludge as an inoculum. Anaerobic conversion of biomass has also been demonstrated as a technically feasible way of generating hydrogen and methane [2]. Anaerobic digestion of organic materials and uptake of some minerals can support microalgae growth.

Carbon, nitrogen and phosphorus are the three most important nutrients influencing microalgal growth [3]. Microalgae have long been used in tertiary sewage treatment to eliminate N and P compounds after COD removal by conventional secondary treatment [4-5]. Industrial wastewater from olive-oil extraction was used for *Scenedesmus obliquus* biomass production [6]. *Chlorella vulgaris* can reduce COD of wastewater from cassava ethanol fermentation resulting in increased biomass at pH 6.0 at 27 ^{o}C under, continuous illumination at 3,000 Lux [7]. Microalgae are photosynthetic organisms with relatively simple growth requirements. Through the process of photosynthesis, microalgae convert water and carbon dioxide into oxygen and biomass using minerals in their metabolic pathway. Nutrient requirements for growth of microalgae can be found in many industrial wastes.

The current study investigated a sequential twostep wastewater treatment. In the first step, anaerobic fermentation of ethanol distillation bottoms liquid by a strain of *Clostridium* was done to reduce sugar levels. The second step involved nutrient removal by *Chlorella vulgaris* under continuous illumination of three light colors and intensities. This was done for the purpose of reducing the mineral contents of the wastewater.

2. Materials and Methods

2.1 Strain and cultivation

The first microorganism used in this study was a *Clostridium* strain isolated from sludge collected from a wastewater pond at Udon Thani Rajabhat University (UDRU). It was cultivated in 500 ml flasks containing 200 ml of culture medium at 35 °C for 24 h.

The microalgae used in this study was *Chlorella vulgaris* from the biotechnology laboratory of the Thailand Institute of Scientific and Technology Research (TISTR). It was cultivated in 2,000 ml fermentation flasks containing 1,800 ml of culture medium at 25 °C under continuous illumination by white light at an intensity of 3,000 Lux for 7 days. The inoculum concentration was about 10^{10} cells per liter of medium. The biomass was harvested using a centrifuge at 3,000 rpm for 10 min.

The nutrient broth (NB) used in this study consisted of 5 g peptone and 3 g beef extract per liter of distilled water.

N8Y basal medium (BM) [8], consisted of 1 g KNO_3, 0.74 g KH_2PO_4, 0.207 g Na_2HPO_4, 0.013 g $CaCl_2.H_2O$, 0.01 g FeNaEDTA, 0.025 g $MgSO_4$, 0.1 g yeast extract and 1 ml micronutrient solution per liter of distilled water. The micronutrient solution contained 3.58 g $Al_2(SO_4)_3.18H_2O$, 12.98 g $MnCl_2.4H_2O$, 1.83 g $CuSO_4.5H_2O$, 3.2 g $ZnSO_4.7H_2O$ per liter of distilled water.

Ethanol distillation bottoms liquid was obtained from an industrial ethanol process (JSP Chemical Plant, Udon Thani Province). It was stored at -20 °C prior to use.

2.2 Pre-treatment wastewater and analysis

Ethanol distillation bottoms liquid was the substrate in these experiments. The initial pH of the bottoms liquid was 5. The initial reducing sugar concentration was determined by the 3,5 dinitrosalicylic acid method [9]. Total sugar was measured using the phenol sulfuric method [10]. Nitrate (NO_3^-) [11] and phosphorus (P) [12] concen-

trations were determined using a spectrophotometer (UV-1800, Shimadzu, Japan). Potassium (K), sodium (Na), calcium (Ca) magnesium (Mg) contents were measured using atomic absorption spectroscopy (AAS-3110, Perkin-Elmer, USA) [13] and COD was determined by the titration method.

2.3 Inoculums

The inoculum was isolated from wastewater sludge. It was pretreated by heating at 105 ๅ C for 3 h to inactivate methanogenic bacteria. Ten mg of dried sludge was added to 200 ml NB and incubated at 35 °C for 24 h under anaerobic conditions. Then microbial cells were cultured on NB agar at 35 °C for 24 h to obtain pure cultures. Three strains were isolated in pure culture and preliminary sugar fermentation tests done. The strain consuming the most sugar was the inoculum used in this study. Morphological examination and biochemical tests indicate that the microorganism was a member of the *Clostridium* species.

2.4 Fermentation and testing

One liter of pretreated substrate was mixed with 50 ml of inoculum suspension of *Clostridium* and incubated anaerobically at 35 °C for 6 days without stirring. Samples were collected to determine the concentrations of reducing sugars, total sugars, nitrate (NO_3^-), phosphorus (P), potassium (K), sodium (Na), calcium (Ca) magnesium (Mg) [13] and COD.

2.5 Cultivation of *C. vulgaris* and analysis

Chlorella vulgaris was cultured in ethanol distillation bottoms liquid. Bottoms liquid with an initial pH=5 was diluted 1:100 with water. *Chlorella vulgaris* was grown aerobically for 6 days at 25 °C. The initial pH was 5, incubation at room temperature with continuous illumination using white, red and green light at intensities of 3,000 and 5,000 Lux for 10 days. Samples were collected daily for analysis, Biomass, nitrate, phosphorus, potassium and calcium contents were determined.

2.6 Biomass determination

Samples were collected every day. Optical density was measured at 682 nm. Biomass concentration was determined by correlating the optical density (OD_{682}) with the dry weight of biomass in the culture medium. The relationship between biomass concentration and optical density was found to be the following:

$$Cb\ (\text{mg/L}) = 216.1 OD_{682}\ (R^2=0.99)$$

where *Cb* is the biomass concentration in mg/L and OD_{682} is optical density at 682 nm. The above relationship was derived by measuring OD_{682} and determining the corresponding biomass concentration. This was done for OD_{682} values over the range of 0.101.00. Biomass concentration was determined by centrifugation at 5,000 rpm for 10 minutes followed by washing with deionized water. This was repeated and followed by drying at 80 °C for 6 h [14].

3. Results and Discussion

The research investigated a sequential two step process for wastewater treatment. In the first step, reducing sugar and COD concentrations of ethanol distillation bottoms liquid was reduced by bacterial fermentation. In the second step, *Chlorella vulgaris* was cultured for biomass production on the sugar and COD depleted bottoms liquid of step 1. This second culture was done under continuous white, red and green lighting at the intensities of 3,000 and 5,000 Lux. Medium was reduced of the mineral contents.

3.1 Removal of COD and minerals from ethanol distillation bottoms liquid by *Clostridium*

Removal of reducing sugars, total sugars, COD, P, NO_3^-, Ca, Mg, K and Na from ethanol distillation bottoms liquid was investigated. It was found that the initial concentration of reducing sugars and total sugars was 22,000 and 104,000 mg/L respectively. COD, P, NO_3^-

, Ca, Mg, K and Na concentrations were found to be 380,000, 2,218, 183, 304, 224, 285 and 237 mg/L, respectively (Table 1). These values represent high nutrient content. This is especially true with regard to total sugars, reducing sugars and COD. Ethanol distillation bottoms liquid was inoculated with a species of *Clostridium* and anaerobically incubated at 35 °C for 6 days. It was observed that the concentrations of total sugars, reducing sugars and COD were reduced to 23,000, 2,000 and 120,000 mg/L, respectively (Figures 1 and 2). These values represent 77.9 90.9 and 68.5% removal. However, the concentrations of other nutrients were increased over their initial levels. Concentrations of NO_3^-, P, K, Na, Ca and Mg increased from 183, 2,218, 285, 237, 304 and 224 mg/L to 616, 2,901, 1,600, 3,863, 389 and 472 mg/L, respectively (Figures 3 and 4). Microorganism used carbon source from COD and released mineral contents during fermentation. This material, with reduced levels of organic carbon and enhance levels of mineral nutrients was then used as a substrate to culture algal biomass, *Chlorella vulgaris*.

Table 1. Concentration of minerals in basic medium and ethanol distillation wastewater

Parameter	Concentration (mg/L)	
	Basic medium	ethanol distillation bottoms liquid
Reducing sugar	-	22,000
Total sugar	-	104,000
COD	-	380,000
P	293	2,218
NO_3^-	620	183
Ca	52	304
Mg	6	224
K	670	285
Na	97	237

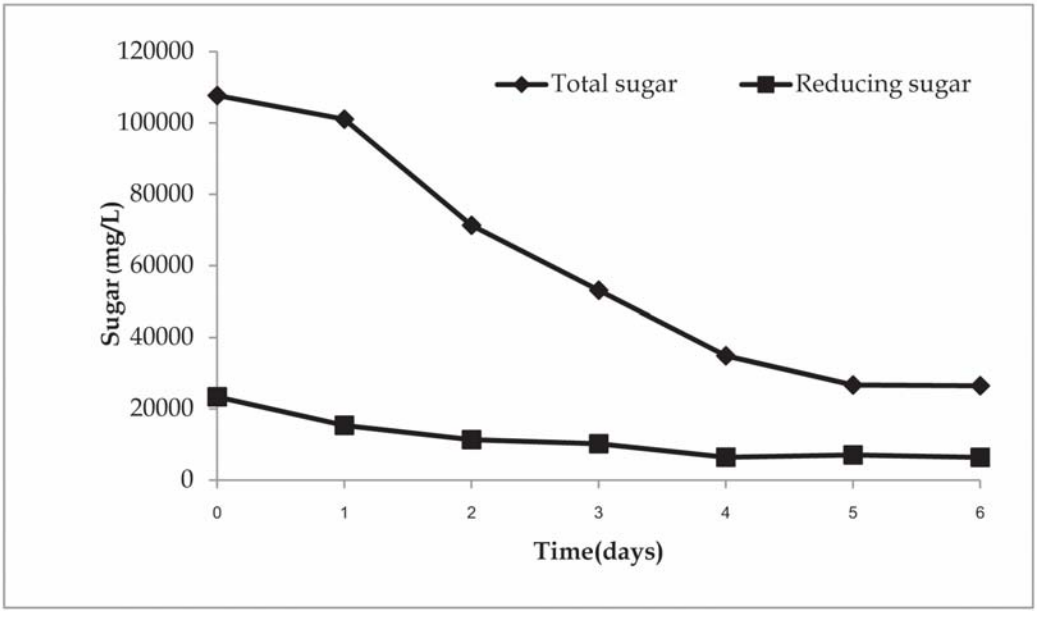

Figure 1. Concentration of a) reducing sugars and b) total sugars in ethanol distillation wastewater during fermentation

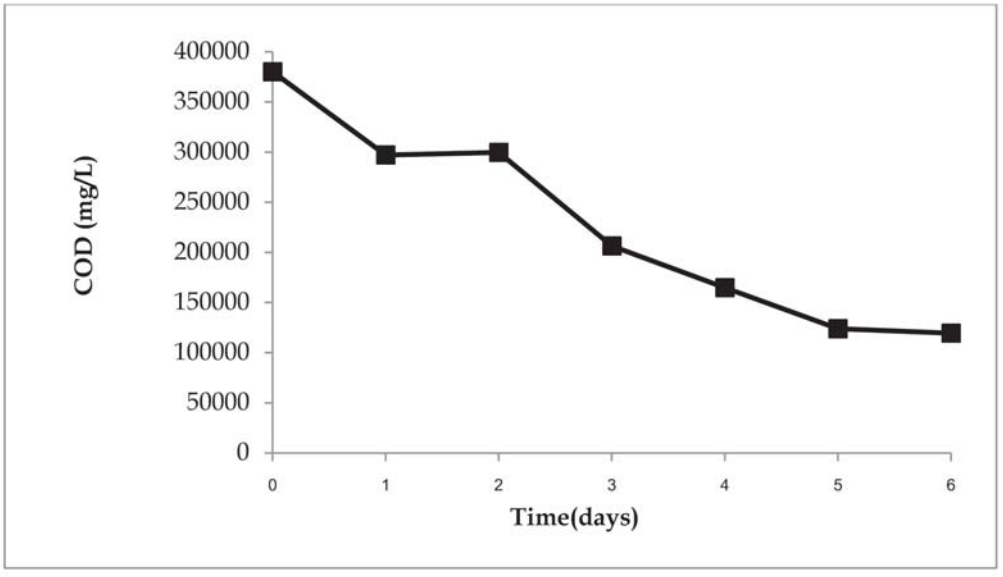

Figure 2. Concentration of COD in ethanol distillation wastewater during fermentation

3.2 Removal of NO_3^-, Ca, Mg, P, K and Na from ethanol distillation bottoms liquid by *Chlorella vulgaris* under continuous illumination of various colors

Chlorella vulgaris was cultured under white, red and green light at intensities of 3,000 and 5,000 Lux on BM and sugar and COD depleted ethanol distillation bottoms liquid. As shown in Table 2, concentrations of P, NO_3^-, Ca, Mg, K and Na in bottoms liquid decreased to low values.

Four culture conditions produced high levels of *Chlorella vulgaris* biomass. These were 1) growth on BM under white light at an intensity of 3,000 Lux (194 mg/L), 2) growth on BM under red light at an intensity of 5,000 Lux (162 mg/L), 3) growth on distillation bottoms liquid under white light at an intensity of 3,000 Lux (867 mg/L), and, 4) growth on BM under white light at an intensity of 5,000 Lux (940 mg/L). This is seen in Figures 5(a), 5(b), 6(a) and 6(b), respectively. The results indicated that *C. vulgaris* can grow on ethanol distillation bottom wastewater better than on BM.

Biomass of *C. vulgaris* grown on BM under white, red and green lighting showed similar patterns. *C. vulgaris* grown on BM reached peak levels of biomass after 10 days of culture. Cultures of *C. vulgaris* showed higher levels of biomass production when cultivated under a light intensity of 3,000 Lux than at 5,000 Lux. Biomass of *C.vulgaris* grown on distillation bottoms liquid was approximately 5 times that grown on BM. Additionally, peak biomass production on distillation bottoms liquid was seen at 5 days, compared to 10 days when grown on BM.

Biomass production in distillation bottoms liquid culture under white and red light showed similar patterns at both intensities. *C. vulgaris* culture under green light had the lowest biomass. The algae did not grow well under green light at both light intensities. It was seen that the highest biomass was produced under white light. It has been reported that there is an optimum light intensity and color for growth of each microalgae species [15]. This is referred to as its light saturation point. Light of excessive intensity cannot be fully utilized by algae. In this case, there would be unnecessary production costs. Inadequate light intensity limits microalgal growth [16]. For example, the effect of cultivation conditions on *Chlorella pyrenoidosa* was that light saturation point was closer to 3,000 Lux than 5,000 Lux [6].

Algae photosynthetically convert water and carbon dioxide into oxygen and biomass and use mineral elements in their metabolism. The mineral requirements for growth of *Chlorella vulgaris* may be available in process waste streams. The results of the current study show that removal of P, NO_3^-, K and Ca is accomplished in *C. vulgaris* culture. This was seen under continuous illumination of all colors and intensities (Table 2). However, Na and Mg were not removed by *C. vulgaris* culture (data not shown).

As can be seen in Table 3, when grown on BM, levels of P, NO_3^- and K removal under red light by *C. vulgaris* were 47.44%, 54.00% and 69.00%, respectively. These values were observed at 3,000 Lux for P and 5,000 Lux for NO_3^- and K. Ca removal under white light at the intensity of 3,000 Lux was 38.09%. NO_3^- levels were reduced by 36.46% under white light at an intensity of 5,000 Lux. P and Ca removal under red light at intensities of 3,000 and 5,000 Lux were 21.72% and 27.27%, respectively. P, NO_3^-, K and Ca are important nutrients influencing *Chlorella vulgaris* growth. These results suggest that a two-step process can be employed for wastewater treatment. The first step is done to utilize COD, reducing and total sugars. The second step is done to eliminate P, NO_3^-, K and Ca. Distillation bottoms liquid has the necessary nutrients to support the growth of algal biomass. The optimum levels of other nutrients for biomass production will be further investigated in the future.

Table 2. Mineral use by *Chlorella vulgaris* cultured in basal medium and ethanol distillation bottoms liquid under white red and green light at intensities of 3,000 and 5,000 Lux

Mineral		Basal medium (mg/L)				Ethanol Distillation Bottoms Liquid (mg/L)			
		3,000 Lux		5,000 Lux		3,000 Lux		5,000 Lux	
		Initial	Final	Initial	Final	Initial	Final	Initial	Final
P	White	4947	4244	7011	5240	5663	5198	4968	4841
	Red	4167	2190	4367	3911	7060	5526	6485	5510
	Green	5989	5068	4789	3792	6233	5451	5361	5335
NO_3^-	White	150	112	150	139	201	129	181	115
	Red	150	111	150	99	152	141	162	145
	Green	150	60	150	128	190	177	172	154
K	White	123	106	115	101	49	44	67	56
	Red	107	47	100	31	60	43	62	44
	Green	127	104	113	113	60	40	60	29
Ca	White	21	13	15	14	9	9	11	11
	Red	17	15	15	14	10	10	11	8
	Green	18	16	17	14	10	9	11	10

Table 3. Percentage of mineral removal from basal medium and ethanol distillation bottoms liquid by *Chlorella vulgaris*

Mineral		Basal medium (%)		Ethanol distillation wastewater (%)	
		3,000 Lux	5,000 Lux	3,000 Lux	5,000 Lux
P	White	14.21	25.26	8.21	2.55
	Red	47.44	10.44	21.72	15.03
	Green	15.37	20.81	12.54	0.48
NO_3^-	White	25.33	7.33	35.82	36.46
	Red	26.00	34.00	7.23	10.49
	Green	16.60	14.6	6.84	10.46
K	White	13.82	12.17	10.20	16.41
	Red	56.07	69.00	28.33	29.03
	Green	18.11	0.00	33.33	51.66
Ca	White	38.09	6.60	0.00	0.00
	Red	11.76	6.60	0.00	27.27
	Green	11.11	17.64	10.00	9.09

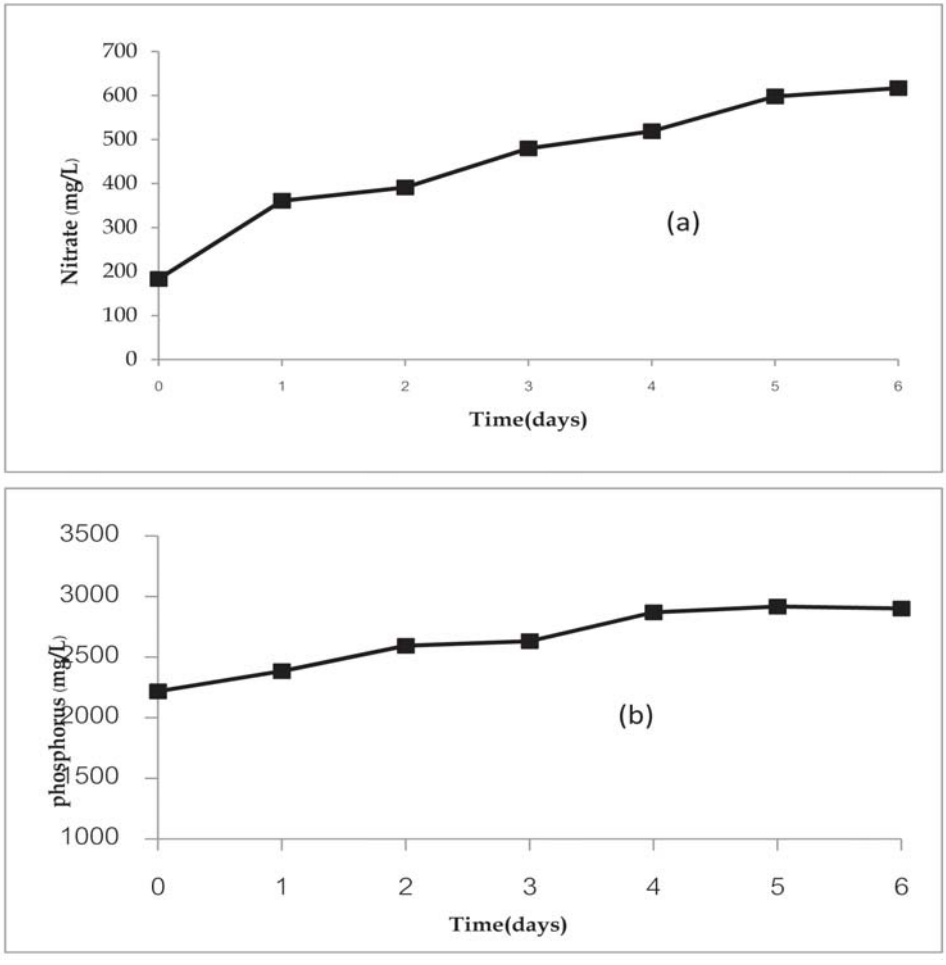

Figure 3. Concentration of (a) nitrate and (b) phosphorus in ethanol distillation wastewater during fermentation

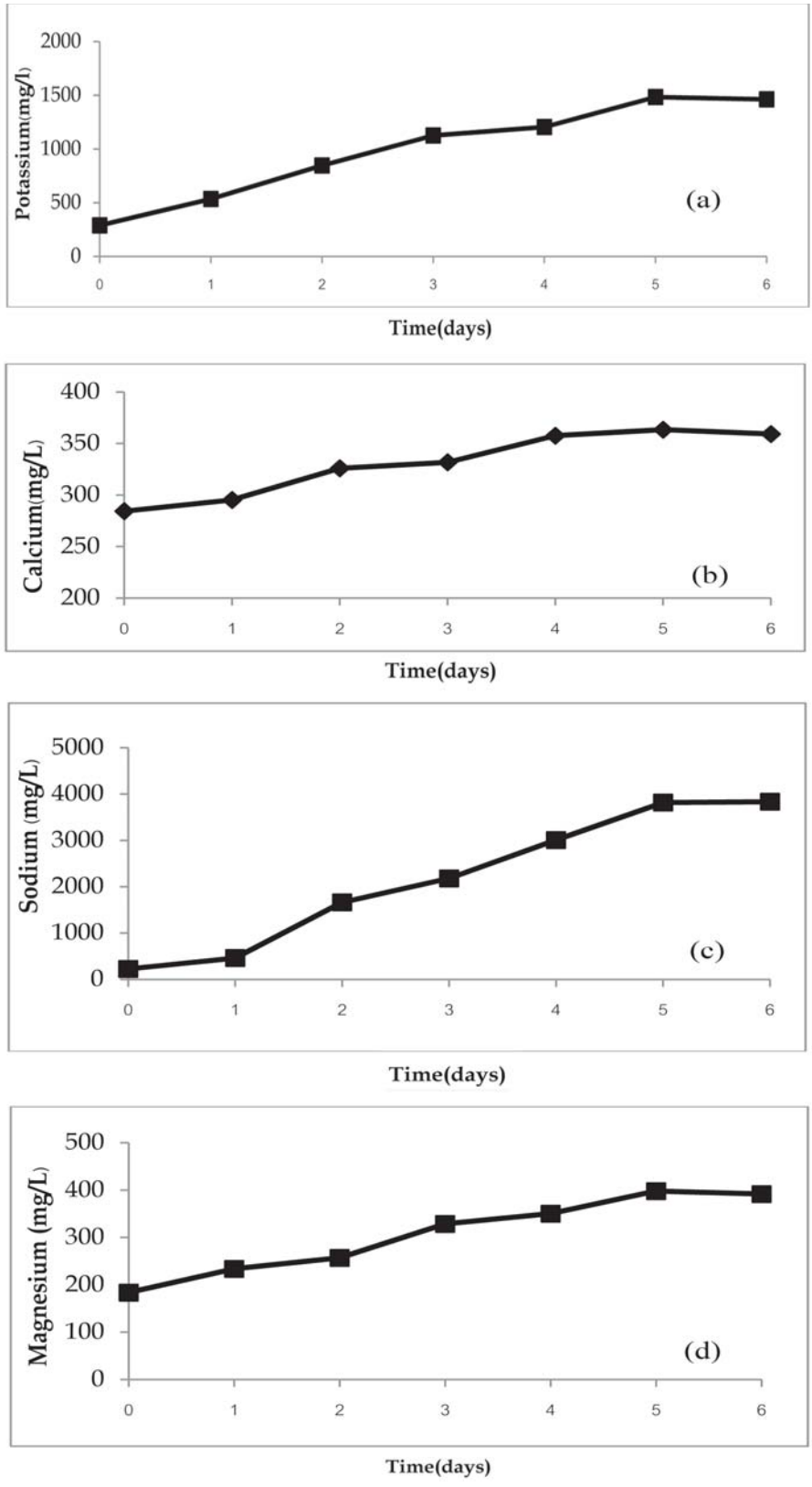

Figure 4. Concentration of (a) potassium, (b) sodium (c) calcium and (d) magnesium in ethanol distillation wastewater during fermentation

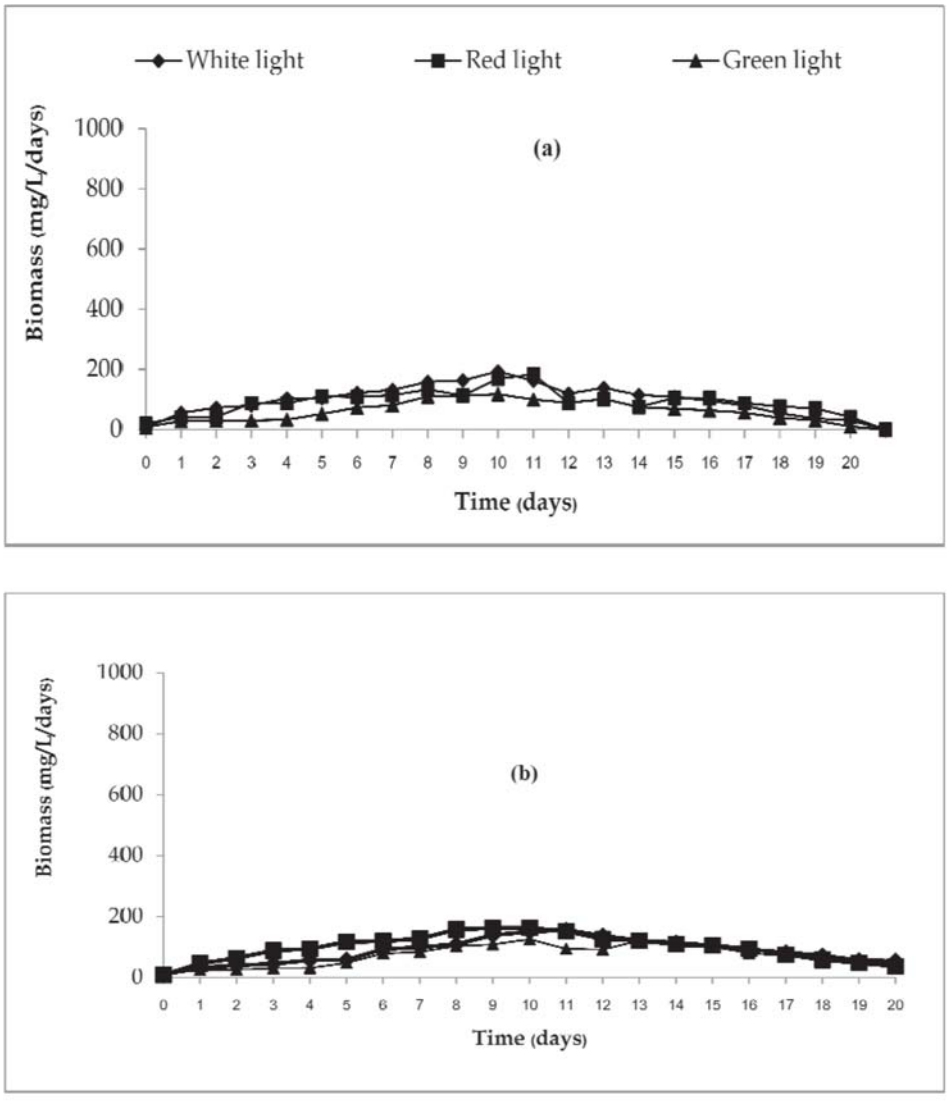

Figure 5. Biomass of *Chlorella vulgaris* culture in BM under white red and green light at intensities of (a) 3,000 and (b) 5,000 Lux

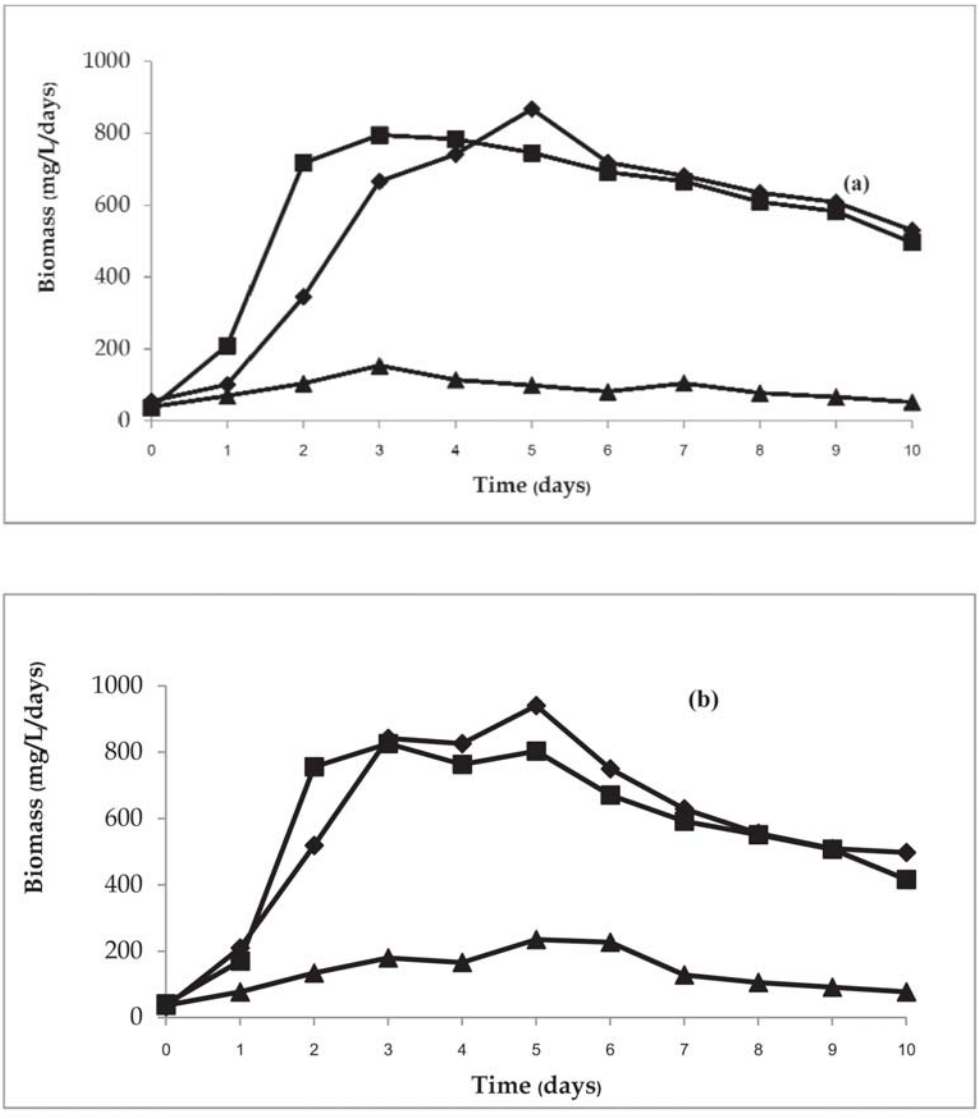

Figure 6. Biomass of *Chlorella vulgaris* culture in distillation bottoms liquid under white red and green light at intensities of (a) 3,000 and (b) 5,000 Lux

4. Conclusion

Organic materials in ethanol distillation bottoms liquid were treated using bacterial fermentation. Organic carbon sources in this wastewater were used as substrates. After this primary treatment, the total sugar, reducing sugar and COD were removed at levels of 77.7, 90.9 and 68.5%, respectively. Concurrently, mineral contents increased for phosphorus, nitrate, potassium, sodium, calcium and magnesium. These minerals were consumed by subsequent *Chlorella vulgaris* culture under continuous illumination of various colors. *Chlorella vulgaris* used P, NO_3^-, K and Ca in wastewater for biomass production. White light at an intensity 5,000 Lux exhibited highest response to biomass production. A wastewater treatment coupling a process using a *Clostridium* ssp. and *Chlorella vulgaris* is feasible. Such a process may be used to simultaneously reduce pollution, production costs for microalgae, increase biomass production and reduce costs of wastewater treatment.

5. Acknowledgement

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