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Nutrients utilization and biomass production by microalgae culture development in wastewater

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Abstract

The growth of microalgae for production of biofuels is beneficial when sewage wastewater is used as a growth media because of two reasons; wastewater treatment and cost effective biomass generation as compared to artificial growth medium cultivation. In this study, three microalgae species (*Chlorella vulgaris*, *Selenastrum* sp. and *Chlorococcum humicola)* were isolated from freshwater as well as wastewater sample and were purified. Treatment of sewage was carried out for effective utilization of nutrients by these strains, whereas biomass and lipid productivity was measured accordingly. The wastewater treatment by *C. vulgaris* shows 98% COD and more than 90% TN removal efficiency. Total phosphorus and ammonia was removed almost 100% by all of the strains. The treatment efficiency and biomass productivity of *Selenastrum* sp. was less as compared to other strains while *C. vulgaris* shows higher lipid productivity. According to results the biomass productivity can be further enhanced by addition of external nitrogen and carbon source to wastewater media. While treatment of domestic wastewater along with production of biomass is a key strategy to produce many biobased co-products including biodiesel.

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Introduction

Growing demand of energy has led to an unbalanced resource of fuel in the past several years. Many energy sources like petroleum and coal are estimated to become exhausted or too expensive to extract (Benemann *et al.*, 1996). Alternative demand of fuel has been increased from many years. The main sources of energy are biorenewable, solar, geothermal, hydro and wind; among them the renewable energy from algae has gained much interest due to the production of less greenhouse gases as compared to fossil fuels (Dincer, 2008). Various types of biofuels can be produced from microalgal biomass such as bioethanol, biodiesel, biomethane, biosyngas, bio-hydrogen and jet fuels. In order to develop cost effective biofuels production strategy, more research in downstream processing and cultivation of microalgae is expected (Chinnasamy *et al.*, 2012). Third generation biofuels is favorable and depend on algae which are simple microorganisms, live in water and cultivated hydroponically. The microalgae does not need land and soil and can grow in salty brackish water. In order to understand the phytoplankton fate in natural environment, the physiology of microalgae is very essential for optimization of biomass production at commercial scale with applications in bioenergy, aquaculture and cosmestic industry (Milledge, 2011)

The growth of microalgae on wastewater media has received attention and different wastewaters support microalgal cultivation which was considered as a tertiary treatment by removal of phosphorus, nitrogen and other nutrients. Moreover, large amount of biomass can be produced by this process which could be a source of biodiesel and other valuable products (Droop, 1955; Kobayashi *et al.*, 1992; Miao and Wu, 2006). Algae can be cultivated in various wastewaters such as agricultural, industrial, municipal and other eutrophic waters containing high concentration of nutrients (Crites and Technobanoglous, 1998). Some unicellular species of microalgae like *Scenedesmus* and *Chlorella* genus mostly tolerate sewage culturing conditions and numerous studies reported the cultivation of these strains (Lau *et al.*, 1995; Shi *et al.*, 2007; Bhatnagar *et al.*, 2010; Ruiz-Marin *et al.*, 2010).

Studies also revealed that microalgae have ability to utilize nutrients during growth in sewage wastewater. For example, 80% phosphorus and 90% total nitrogen has been removed by *Chlorella vulgaris* from primary treated municipal wastewater (Lau *et al.*, 1995). The aim of this study is to treat sewage wastewater using isolated microalgae species by indoor mixotrophic cultivation conditions. Furthermore, nutrient removal efficiency as well as biomass and lipid productivity of each strain was also evaluated during experiment.

Materials and methods

Sample collection

Fresh water sample for isolation of microalgae was collected from Rawal Lake, Islamabad and wastewater sample from local wastewater resources that contain algal growth. The samples were collected carefully and brought to the laboratory at the same day for analysis. Similarly, sewage water sample was obtained from domestic wastewater streams and used as a growth media for cultivation of microalgae. Prior to use, it was filtered by using glass microfiber filter 0.45µm (Whatman) in order to remove large particles (Wang *et al.*, 2010). The sample was characterized for various biological and physico-chemical parameters.

Enrichment in growth medium and cultivation conditions

Ten ml of each water sample was inoculated into 500ml conical flask containing sterilized BG-11 medium (Feng *et al.*, 2011) which contain following components: $NaNO_3$ (1500mg/L), Na_2CO_3 (20mg/L), ferric ammonium citrate (6mg/L), citric acid_$1H_2O$ (6mg/L), $CaCl_2$_$2H_2O$ (36mg/L), $MgSO_4$_$7H_2O$ (75mg/L), Na_2Mg EDTA (1mg/L), K_2HPO_4 (30.5mg/L), $MnCl_2$_$4H_2O$ (1.81mg/L), $ZnSO_4$_$7H_2O$ (0.222 mg/L), H_3BO_3 (2.86mg/L), $CuSO_4$_$5H_2O$ (0.079mg/L), $CoCl_2$_$6H_2O$ (0.050mg/L) and $NaMoO_4$_$2H_2O$ (0.391mg/L). Two conical flasks were used at a time, one for freshwater and other for wastewater sample. These were then incubated for two to three weeks on a rotary shaker at 150 rpm and a temperature of 25°C under continuous illumination of white fluorescent light (intensity, 38µ mol m^{-2} s^{-1}) until dark green color appears.

Strain isolation, purification and identification

Microalgae species were isolated from enriched sample cultures by serial dilution and streak plate method. Subcultures were prepared by inoculating culture sample (50µl) from culturing flask into petri plates having solidified BG-11 media (1.5% w/v bacteriological agar) by quadratic streaking method. Similar procedure was carried out for both culturing flasks. The petri plates kept for incubation under continuous illumination at 25°C for two weeks. Strain purification was confirmed by repeating streak plating method. Strains were carefully observed under microscope and were identified by using the manual (Prescott, 1962; Lee, 1999). The purified cultures were then maintained in BG-11 medium both in broth and slant cultures under optimized culturing conditions.

Algal growth and biomass productivity

The cultivation of three microlagal species was carried out in flasks (250 ml) containing sewage wastewater media upto 150ml and placed in a rotary shaker in the presence of white fluorescent lamp (intensity, 120µmol m^{-2} s^{-1}). Algal inoculum (for each specie) of 0.2g/L was added in wastewater media. Growth rate of three strains were examined everyday by measuring the TVSS (total volatile suspended solids) by following method (Zhou *et al.*, 2012). Five ml culture suspension was taken out and filtered by 0.45µm glass microfiber filter which was dried overnight in an oven at 105 °C. The filtered sample was placed in an oven at 105°C for 10h and then ignited at 550°C for 20-30min. The difference of weight between ignited (M_2) and dried (M_1) sample was known as biomass (g L^{-1}). All experiments were carried out in triplicate. The equation for microalgal growth on the basis of TVSS is as follows:

$$R_{TVSS} = \frac{\text{Ln}\,(TVSS_t) - \text{Ln}(TVSS_0)}{t} \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

R shows the growth rate of microalgae on the basis of TVSS whereas, $TVSS_t$ is TVSS at day t and $TVSS_o$ is TVSS at day 0. Time interval (*t*) represents number of days. The productivity of biomass $P_{Biomass}$ can be calculated by following equation (Feng *et al.*, 2011).

$$P_{Biomass}\ (g\ L^{-1}\ day^{-1}) = (DW_x - DW_1).\ (T_x - T_1)^{-1} \ldots\ldots (2)$$

DW_x and DW_1 represent the dry weight (g L^{-1}) concentrations of biomass on days T_1 (start of cultivation) and T_x (last day of cultivation) respectively.

Nutrients determination and removal efficiency

Liquid samples for analysis of nutrients consumption were collected from each flask after every day and centrifuged at 5000 rpm for 15 min. The supernatant was separated for analysis of COD (chemical oxygen demand), TP (total phosphorus), NH_3-N (ammonia) and TN (total nitrogen) according to manual of Hach DR 500 spectrophotometer (Hach, 2008). Removal efficiency (%) of nutrients was calculated according to equation (Ji *et al.*, 2014).

$$Ri = Sio\text{-}Sit \quad \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Si_o represents nutrient removal efficiency (i.e. COD, TP, $NH3^+$ -N and TN). Si_o and Si_t are the nutrient concentrations (mg/L) at day 0 and last day of cultivation period. The pH during cultivation was measured by pH meter (Fisher brand FE 150, Fisher Scientific, New Hampshie, USA). Metals quantification was determined by microwave plasma (agilent 4200) atomic emission spectrophotometer.

Carbohydrate, protein and chlorophyll contents

Carbohydrate content in each microalgal biomass sample was assessed according to phenol sulphuric acid method. Calibration curve was generated by using carbohydrate standard (glucose) and absorbance was determined by Spectrophotometer at 490nm (Ramsundar *et al.*, 2017). Protein content in microalgae was analyzed by Bradford assay. Protein standard (BSA known as Bovine Serum Albumin) was used for the generation of calibration curve. Microalgae sample was prepared according to previously published method (Guldhe *et al.*, 2015) by adding 100µl of 1M NaOH to algal biomass (10mg). The mixture was placed in an incubator for upto 10min at 80°C followed by the addition of distilled water (900µl). The mixture was centrifuged for 10min at 12,000g and supernatant was separated to be used for protein assay. The absorbance was recorded by Spectrophotometer (595nm).

The photosynthetic pigments *e.g*. chlorophyll a and b in harvested microalgae biomass were usually extracted by using acetone. These contents were determined and calculated by recording absorbance at 662nm and 645nm respectively by following method (Dere *et al*., 1998).

Extraction of total lipid

Before oil extraction, algal cells were harvested by centrifuged at 5000 rpm upto 15 min and dehydrated by placed into freeze dryer followed by a vacuum dryer. The extraction of total lipids was carried out by extraction procedure of one step as explained by (Sires and Brillas, 2012) with minor modification. Dry algae powder (40mg) was measured into 25ml glass tube and a mixture of chloroform: methanol (2:1) was added upto 2ml. Oil was extracted by using water bath at 100 rpm for 15min. The algal residues were separated from organic solution by centrifugation at the end of reaction. The process of extraction was repeated thrice. Organic solution was separated by using Nitrogen evaporator (NEVAP). The lipid was weighed after drying. The lipid content on the basis of dry biomass was calculated by following equation.

$$LW\ (g/g) = (m_2\text{-}m_0) \times V / 3 \times m_1 \quad (4).$$

LW is content of lipid on the basis of dry weight, m_0, m_1 and m_2 are the weights of empty glass tube, tube with algae powder and tube containing dried lipids respectively and *V* is known as the volume of lower layer after being washed (Zhou *et al*., 2012). The percentage of oil yield and lipid productivity was calculated by equation 5 (AOAC, 2000) and 6 (Hempel *et al*., 2012) respectively.

$$\text{Percentage oil yield (W/W)} = \frac{\text{Weight of oil}}{\text{Weight of algae powder}} \times 100 \quad (5)$$

$$P_{lipids}\ (mg\ L^{-1}\ day^{-1}) = P_{Biomass} \,.\, C_f \quad (6)$$

Where, P_{lipids} is the productivity of lipids, $P_{Biomass}$ is the productivity of biomass and C_f is final lipid content and is given as percent dry weight.

Statistical analysis

Data obtained was analyzed by using ANOVA to get means values ± standard deviation *etc*.

Results and discussion

Identification of Microalgae

The three isolated microalgae strains capable of growing in BG-11 medium were identified by morphological examination under microscope as *Chlorococcum humicola* and *Selenastrum sp*. from freshwater and *Chlorella vulgaris* was isolated from wastewater resource. The reason for survival of these strains in laboratory culturing conditions is because of their tolerance in varying conditions.

Characterization of Wastewater

The sewage wastewater sample used for cultivation was divided into three replicates and was characterized in terms of its biological and physico-chemical characteristics with the following mean results as listed in table 1. Results show that the initial pH of wastewater was almost neutral i.e. 7.1. When wastewater was treated with microalgae strains (*C. vulgaris, Selenastrum sp*. and *C. humicola*) then the pH became alkaline because of photosynthetic activity. Similar results were reported by (Kshirsagar, 2013). The amount of BOD (98mg/l) was near to national environmental quality standard's BOD (80mg/l) and COD (225mg/l) in our sample was little high as compared to national environmental quality standards i.e. (150mg/l) (Anon., 2000).

The high COD could provide a very good source of carbon for algal growth. The concentration of T-N, T-P, and NH_3-N (37.5mg/l, 10.46mg/l and 30.7mg/l respectively) is enough for successful growth of microalgae. Similarly, *Chlorella vulgaris* was cultivated in various types of municipal wastewaters but higher growth was recorded in Wastewater # 4 (sludge) containing high concentration of COD and other nutrients (Wang *et al*., 2010). It is noteworthy that various metal ions such as sodium ($Na^{2+)}$), calcium ($Ca^{2+)}$), cobalt ($Co^{2+)}$), potassium ($K^{+)}$) and magnesium ($Mg^{2+)}$) which are necessary for physiology of microalgae are also identified in wastewater selected in present study. Microalgae have been considered as more effective in removing metal ions from solution as compared to fugal and bacterial cultures (Khoshmanesh *et al*., 1996).

Table 1. Physico-chemical Analysis of Wastewater Used as a Growth Media for Algae Cultivation.

Parameters	Concentration
Total Nitrogen (T-N)*	37.5± 1.97
Total Phosphorus (T-P)*	10.46 ± 0.06
Chemical Oxygen Demand (COD)*	225 ± 6.05
Biological Oxygen Demand (BOD)*	98 ± 1.8
Ammonia (NH_3-N)*	30.7 ± 0.94
Total Dissolved Solid (TDS)**	41 ± 0.01
Turbidity ***	14.55 ± 0.25
pH	7.1
Conductivity ****	4.5 ± 0.01
Metallic ions	
Magnesium (Mg^{2+})*	19.12 ± 3.218
Potassium (K^{+})*	24.9 ± 1.08
Manganese (Mn^{2+})*	1.37 ± 0.07
Cobalt (CO^{2+})*	72.4 ± 4.93
Aluminium (Al^{3+})*	0.009 ± 0.01
Cadmium (Cd^{2+})*	< 0.001
Calcium (Ca^{2+})*	63.85 ± 3.62
Boron (B^{3+})*	0.25 ± 0.031
Copper (Cu^{2+})*	0.041 ± 0.01
Zinc (Zn^{2+})*	0.082 ± 0.003
Sodium (Na^{+})*	144.30 ± 7.17

Data represented as Mean ± SD (standard deviation). Whereas *, **, *** and **** represent mg L^{-1}, g L^{-1}, NTU and µScm^{-1} respectively.

Algal Growth in Wastewater and Biomass Productivity

The microalgae can grow well in wastewater media with no lag phase was observed in all of the three species (Fig.1), suggesting that these local isolated strains had good capability to adjust in sewage wastewater. Although, the exact mechanism for shortage of lag phase by strains was not clear, similar results were reported in another study by growing *C. vulgaris* in swine manure wastewater (Hu *et al.*, 2012). Among three selected species, *C. vulgaris* grow more rapidly and there is a smooth increase in biomass yield and its exponential phase lasted for 4 days with a biomass concentration of 1.494 ± 0.09g/L at day 4th followed by stationary phase in next 3 days. While, in case of other two strains exponential phase remains for 6 days before entering into stationary phase.

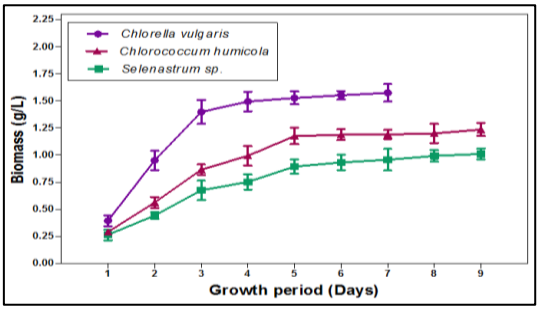

Fig. 1. Growth curve of microalgae in wastewater media.

Moreover, it was observed that biomass productivity of *C. vulgaris* (229 ± 7.3mg/ L/day) was higher than *C. humicola* (129 ± 3.94mg/ L/day) followed by *Selenastrum sp.* (101.25 ± 8.41mg/ L/day) although, these two strains have a growth period for 9 days. The highest growth rate and biomass productivity of *C. vulgaris* is because of its better acclimatization as it was selected among the top-performing microalgae strains in wastewater (Zhou *et al.*, 2011) and may be due to its natural habitat in wastewater which coincide with the previous study reported that the algae isolated from real water or wastewater treatment plant can adjust in same culturing conditions from where they are found (Perez *et al.*, 2004).

In this study, non-autoclaved wastewater media was used for simultaneous cultivation of algae and bacteria in batch cultivation mode. It was reported that during co-culture, *C. vulgaris* will compete with bacteria for various nutrients such as organic carbon and NH_4^+-N because the strain was cultivated in mixotrophic cultivation mode like our experimental conditions (Ma *et al.*, 2016). Bacteria also have a positive effect on algal growth because they promote the growth of microalgae by secretion of some viscous substances which would disintegrate into various products as cultivation time increases. Bacteria grow rapidly at early stage and then the growth was decreased followed by retained at very low level because some substances have been produced by algae to control the growth of bacteria (Ma *et al.*, 2014; Deng *et al.*, 2017).

Nutrients Assimilation by Microalgae species and pH Variation

The removal efficiency (%) of COD (chemical oxygen demand), TP (total phosphorus), NH_3-N (ammonia) and TN (total nitrogen) by three microalgae species in sewage wastewater media (SWM) for batch cultivation mode was studied and illustrated in Fig. 2. During cultivation of microalgae, nitrogen plays an essential role because many microorganisms use it to synthesize various substances like proteins, peptides, enzymes and transfer molecules of energy, genetic materials and chlorophylls (Park *et al.*, 2010).

Ammonium is usually considered as a preferred form of nitrogen because it could absorbed without redox reaction and in the presence of less energy (Maestrini *et al.*, 1986). However, the wastewater containing high ammonium concentration can be very effective to grow microalgae. In sewage wastewater the concentration of NH_3-N decreased rapidly and its removal efficiency was approximately 100% in two species except *C. humicola* (93%) while T-N removal efficiency ranged from 91.68%, 80.61% and 74.37% in *C. vulgaris*, *Selenastrum sp.* and *C. humicola* respectively. The reason for fast removal of ammonia is its low concentration in wastewater. The removal of T-N was slightly lower as compared to ammonia representing that some organic nitrogen must be there, produced during microalgae cultivation and wastewater treatment process (Oswald *et al.*, 1957).

Phosphorus plays an essential role in metabolism of energy for microalgae and present in proteins, lipids, nucleic acids and as intermediate during metabolism of carbohydrate. Two forms of inorganic phosphates such as HPO_4^{2-} and $H_2PO_4^-$ are important for mixotrophic cultivation of microalgae (Martinez *et al.*, 2000). There is a proper range of N/P ratio to use effectively both phosphorus and nitrogen in a media (Li *et al.*, 2010). In this study, the ratio of inorganic N/P is 2.93 not in optimum range which is 6.8-10 for growth of freshwater algae (Darley, 1982; Reynolds, 1984; Martin *et al.*, 1985). Phosphorus removal rate was slow as compared to nitrogen and it may be due to the reason that nitrogen is a limiting factor in selected wastewater sample. Phosphorus can be reduced by biotic process (absorption of phosphorus by biomass) as well as abiotic process in which phosphorus precipitates (Godos *et al.*, 2009). In a study conducted by (Nurdogan and Oswald, 1995) it was reported that high pH (11) is responsible for abiotic phosphorus removal because of the precipitation of orthophosphate. So, this removal mechanism could not take place in this study because maximum pH is 9. The phosphorus removal efficiency is near to 100 % for all of the species and in comparison to other studies, the removal efficiency of phosphorus by *Chlorella sp.* was 20-100% (Aslan and Kapdan, 2006; Khan and Yoshida, 2008; Ruiz-Marin *et al.*, 2010; Wang *et al.*, 2010).

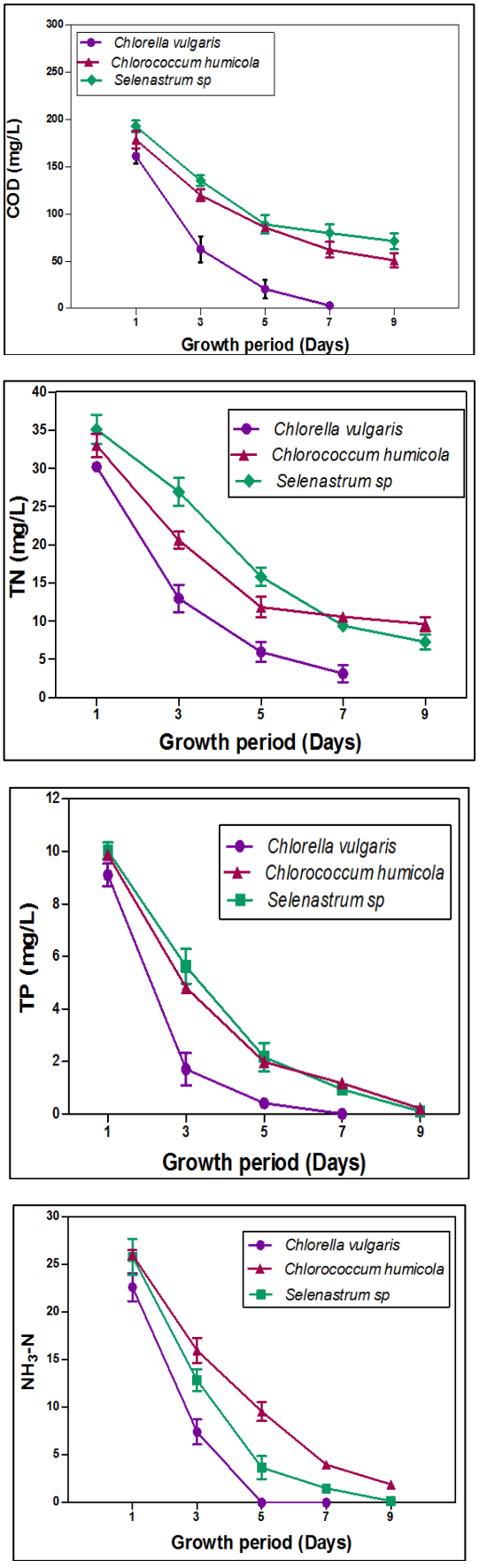

Fig. 2. Nutrients removal rate of microalgae species in wastewater media (a) COD (b) TN (c) TP and (d) NH_3-N.

The removal efficiency of COD much varied among different species and highest removal rate of 98.6% was achieved for *C. vulgaris* followed by *C. humicola* (77.37%) and *Selenastrum* sp. (68.35%). There is a rapid removal of carbon during first 4-5 days and COD removal rate was slow after that and this study was in line according to (Gutzeit *et al.*, 2005). This might be because of less concentration of carbon and the remaining carbon source in a media may be slowly biodegradable material. Generally, carbon was considered as a limiting factor when algae were cultivated in sewage wastewater.

During growth of microalgae, pH affects the concentration of nutrients. Extreme variation in pH can alter phosphates and ammonium solubility in medium and high level of pH cause precipitation of phosphate or ammonia stripping. The pH value ranged from 9 to 11 induced phosphorus precipitation such as calcium phosphate (Laliberte *et al.*, 1997). In the present study, pH of the media was adjusted to 7.0 before inoculation with microalgal strain. After inoculation, pH was not controlled during cultivation period and its variability with time was measured and represented in Fig. 3. Results show that pH increase gradually from 7.0 to 9.0. Various factors that affect the culture pH are growth of microalgae (rise in pH because of CO_2 uptake) nitrification of ammonia (decrease in pH as a result of H^+ release) and secretion of basic and acidic metabolites from biodegradation of organic matter (Gonzalez *et al.*, 2008b).

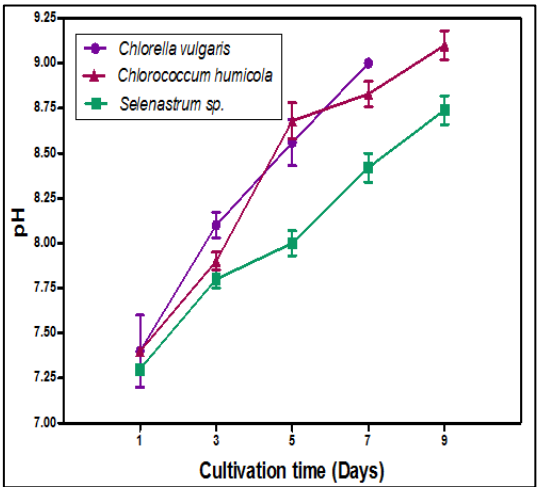

Fig. 3. Variation in pH during biomass production in microalgae.

Chemical Composition of Microalgal Biomass and their Productivities

The chemical composition of microalgae usually depends upon cultivation conditions. Table 2. shows the changes in various components in different species grown in sewage wastewater. The pigment contents (Chlorophyll a and b) in this study were lower in all of the species because of low nitrogen level. These results are in accordance with another study (Berges *et al.*, 1996) that starvation of nitrogen decreased the pigments synthesis such as carotenoid and chlorophyll. Mostly, microalgal biomass comprised of protein (6-52%), carbohydrate (5-23%) and lipids (7-23%) (Zhu, 2015). The protein content of microalgae species grown on wastewater ranged from 13% to 15%. The main reason for low concentration of protein in biomass is rapid depletion of ammonia during cultivation. However, carbohydrate contents were high in *Selenastrum sp* (23.96%) followed by *C. vulgaris* (18.23%) and *C. humicola* (8.67%). The highest lipid contents were observed in *C. vulgaris* (32.41%) as compared to other two species, this may be because of fast utilization of T-N as well as ammonia by this specie as a result of nitrogen starvation.

Table 2. Composition of Microalgae Grown in Sewage wastewater growth media in batch mode under mixotrophic cultivation conditions.

Chemical composition	*Chlorella vulgaris*	*Selenastrum sp*	*Chlorococcum humicola*
Biomass concentration [a]	1.574± 0.20	1.01± 0.15	1.235± 0.06
Biomass productivity [b]	229 ± 7.3	101.25 ± 8.41	129 ± 3..94
Lipid content [c]	32.41 ± 2.4	21.62 ± 1.9	25.5 ± 3.1
Lipid productivity [b]	74.19 ± 4.6	21.89 ± 1.8	32.89 ± 1.9
Chlorophyll a content [c]	14.2 ± 0.5	8.5 ± 3.87	12.76 ± 4.9
Chlorophyll b content [c]	2.90 ± 2.3	2.18 ± 0.01	5.9 ± 3.0
Carbohydrate content [c]	18.23 ± 3.94	23.96 ± 0.90	8.67 ± 1.5
Productivity of carbohydrate [b]	78.57 ± 12.60	44.39 ± 2.56	21.67 ± 8.2
Protein content [c]	13.12 ± 4.9	15.67 ± 3.6	13.78 ± 2.1
Productivity of protein [b]	56.54 ± 0.09	26.79 ± 5.4	34.45 ± 0.6

Data represented as Mean ± SD (standard deviation). Whereas a, b and c represent g L^{-1}, mg L^{-1} day^{-1} and % respectively.

Conclusion

This study has demonstrated that it is feasible to grow microalgae in sewage wastewater under batch cultivation. *Chlorella vulgaris* grow best in wastewater media and was capable of complete depletion of T-N, TP, NH_3-N and COD as compared to *Selenastrum sp* and *Chlorococcum humicola*.

The biochemical composition revealed that the carbohydrate and protein contents are present in normal range in all of the species and lipid contents were comparatively high in *C. vulgaris*. The limiting factor for growth of microalgae was NH_3-N and COD deficiency. So, more biomass productivity can be achieved by adding nitrogen and carbon in growth media which will ultimately increase the lipid production. This can be more effective method to overcome the microalgal cultivation cost and production of valuable products and the results could serve as a basis for cultivation of microalgae for biomass as well as biodiesel production.

References

Anonymous. 2000. National Environmental Quality Standards (NEQS). The Gazette of Pakistan. Ministry of Environment, Local Government and Rural Development, Government of Pakistan.

AOAC (Association of Official Analytical Chemists). 2000. Official methods of analysis of AOAC International. 17th Ed. Gaithersburg, MD, Washington, USA.

Aslan S, Kapdan IK. 2006. Batch kinetics of nitrogen and phosphorus removal from synthetic wastewater by algae. Ecological Engineering **28,** 64-70.

Benemann JR, Oswald WJ. 1996. Systems and economic analysis of microalgae pond for conversion of CO_2 to biomass. Final report, United States Department of Energy.

Berges JA, Charlebois DO, Mauzerall DC, Falkowski PG. 1996. Differential effects of nitrogen limitation on photosynthetic efficiency of photosystems I and II in microalgae. Plant Physiology **110,** 689-696.

Bhatnagar A, Bhatnagar M, Chinnasamy S, Das K. 2010. *Chlorella minutissima* – promising fuel alga for cultivation in municipal wastewaters. Applied Biochemistry and Biotechnology **161,** 523-536.

Chinnasamy S, Rao PH, Bhaskar S, Rengasamy R, Singh M. 2012. Algae: a novel biomass feedstock for biofuels. In: Arora R, Ed. Microbial Biotechnology: Energy and Environment 224-239.

Crites R, Technobanoglous G. 1998. Small and decentralized wastewater management systems, McGraw-Hill. New York. USA.

Darley WM. 1982. Algal biology: A physiological approach. Basic microbiology **9,** 168. Oxford: Blackwell scientific publications.

Deng XY, Gao K, Zhang RC, Addy M, Lu Q, Ren HY, Chen P, Liu YH, Ruan R. 2017. Growing *Chlorella vulgaris* on thermophilic anaerobic digestion swine manure for nutrient removal and biomass production. Bioresource Technology **243,** 417-425.

Dere S, Gunes T, Sivaci R. 1998. Spectrop hotometric determination of chlorophyll-a, b and total carotenoid contents of some algae species using different solvents. Turkish Journal of Botany **22,** 13-17.

Dincer K. 2008. Lower emissions from biodiesel combustion. Energy Sources: Part A **30,** 963-8.

Droop M. 1955. Carotenogenesis in *Haematococcus pluvialis*. Nature **175,** 42.

Feng P, Deng Z, Hua Z, Fanc L. 2011. Lipid accumulation and growth of *Chlorella zofingiensis* in flat plate photobioreactors outdoors. Bioresource Technology **102,** 10577-10584.

Feng Y, Li C, Zhang D. 2011. Lipid production of *Chlorella vulgaris* cultured in artificial wastewater medium. Bioresource Technology **102,** 101-105.

Godos I, Blanco S, Garcia-Encina PA, Becares E, Munoz R. 2009. Long-term operation of high rate algal ponds for the bioremediation of piggery wastewaters at high loading rates. Bioresource Technology **100,** 4332-4339.

Gonzalez C, Marciniak J, Villaverde S, Leon C, Garcia-Encina PA, Munoz R. 2008b. Efficient nutrient removal from swine manure in a tubular biofilm photo-bioreactor using algae bacteria consortia. Water Science and Technology **58,** 95-102.

Guldhe A, Misra R, Singh P, Rawat I, Bux F. 2015. An innovative electrochemical process to alleviate the challenges for harvesting of small size microalgae by using non-sacrificial carbon electrodes. Algal Research **19,** 292-298.

Gutzeit G, Lorch D, Weber A, Engels M, Neis U. 2005. Bioflocculent algal-bacterial biomass improves low-cost wastewater treatment. Water Science and Technology **52,** 9-18.

Hach 2008. DR 5000 Spectrophotometer Procedures Manual. Edition 2. Hach Company, Colorado, USA.

Hempel N, Petrick I, Behrendt F. 2012. Biomass productivity and productivity of fatty acids and amino acids of microalgae strains as key characteristics of suitability for biodiesel production. Journal of Applied Phycology **24,** 1407-1418.

Hu B, Min M, Zhou W, Du Z, Mohr M, Chen P, Zhu J, Cheng Y, Liu Y, Ruan R. 2012. Enhanced mixotrophic growth of microalga *Chlorella sp*. on pretreated swine manure for simultaneous biofuel feedstock production and nutrient removal. Bioresource Technology **126,** 71-79.

Ji F, Liu Y, Hao R, Li G, Zhou Y, Dong R. 2014. Biomass production and nutrients removal by a new microalgae strain *Desmodesmus sp*. in anaerobic digestion wastewater. Bioresource Technology **161,** 200-207.

Khan M, Yoshida N. 2008. Effect of L-glutamic acid on the growth and ammonium removal from ammonium solution and natural wastewater by *Chlorella vulgaris* NTM06. Bioresource Technology **99,** 575-82.

Khoshmanesh A, Lawson F, Prince IG. 1996. Cadmium uptake by unicellular green microalgae. The Chemical Engineering Journal and the Biochemical Engineering Journal **62,** 81-88.

Kobayashi M, Kakizono T, Yamaguchi K, Nishio N, Nagai S. 1992. Growth and astaxanthin formation of *Haematococcus pluvialis* in heterotrophic and mixotrophic conditions. Journal of Fermentation and Bioengineering **74,** 17-20.

Kshirsagar AD. 2013. Bioremediation of wastewater by using microalgae: An experimental study. International Journal of life sciences Biotechnology and Pharma Research **2,** 339-346.

Laliberte G, Lessard P, Delanoue J, Sylvestre S. 1997. Effect of phosphorus addition on nutrient removal from wastewater with the cyanobacterium *Phormidium bohneri*. Bioresource Technology **59,** 227-33.

Lau PS, Tam NFY, Wong YS. 1995. Effect of algal density on nutrient removal from primary settled wastewater. Environmental Pollution **89,** 59-66.

Lee RE. 1999. Phycology. 3rd Ed. Cambridge University Press, Cambridge, UK, 50 pp.

Li X, Hu HY, Gan K, Sun YX. 2010. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. Bioresource Technology **101,** 5494-500.

Ma X, Zheng H, Addy M, Anderson E, Liu Y, Chen P, Ruan R. 2016. Cultivation of *Chlorella vulgaris* in wastewater with waste glycerol: strategies for improving nutrients removal and enhancing lipid production. Bioresource Technology **207,** 252-261.

Ma X, Zhou W, Fu Z, Cheng Y, Min M, Liu Y, Zhang Y, Chen P, Ruan R. 2014. Effect of wastewater-borne bacteria on algal growth and nutrients removal in wastewater-based algae cultivation system. Bioresource Technology **167,** 8-13.

Maestrini SY, Robert JM, Leftley JW, Collos Y. 1986. Ammonium thresholds for simultaneous uptake of ammonium and nitrate by oyster-pond algae. Journal of Experimental Marine Biology and Ecology **102,** 75-98.

Martin C, De la Noun J, Picard G. 1985. Intensive cultivation of freshwater microalgae on aerated pig manure. Biomass **7,** 245-259.

Martinez ME, Sanchez S, Jimenez JM, Yousfi EL, Munoz L. 2000. Nitrogen and phosphorus removal from urban wastewater by the microalga *Scenedesmus obliquus*. Bioresource Technology **73,** 263-272.

Miao X, Wu Q. 2006. Biodiesel production from heterotrophic microalgal oil. Bioresource Technology **97,** 841-846.

Milledge JJ. 2011. Commercial application of microalgae other than as biofuels: a brief review. Reviews in Environmental Science and Biotechnology **10,** 31-41.

Nurdogan Y, Oswald WJ. 1995. Enhanced nutrient removal in high-rate ponds. Water Science and Technology **31,** 33-43.

Oswald WJ, Gotaas HB, Golueke CG, Kellen WR, Gloyna EF, Hermann ER. 1957. Algae in waste treatment. Sewage and Industrial Wastes **29,** 437-457.

Park J, Jin HF, Lim BR, Park KY, Lee K. 2010. Ammonia removal from anaerobic digestion effluent of livestock waste using green alga *Scenedesmus* sp. Bioresource Technology **101,** 8649-8657.

Perez MVJ, Castillo PS, Romera O, Moreno DF, Martínez CP. 2004. Growth and nutrient removal in free and immobilized planktonic green algae isolated from pig manure. Enzyme and Microbial Technology **34,** 392-398.

Prescott GW. 1962. Algae of the Western great lakes area. WMC Brown Company publishers, Dubuque, Iowa. 997.

Ramsundar P, Guldhe A, Singh P, Bux F. 2017. Assessment of municipal wastewaters at various stages of treatment process as potential growth media for *Chlorella sorokiniana* under different modes of cultivation. Bioresource Technology **227,** 82-92.

Reynolds CS. 1984. The ecology of freshwater phytoplankton. Cambridge: Cambridge University Press 157-191.

Ruiz-Marin A, Mendoza-Espinosa LG, Stephenson T. 2010. Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater. Bioresource Technology **101,** 58-64.

Shi J, Podola B, Melkonian M. 2007. Removal of nitrogen and phosphorus from wastewater using microalgae immobilized on twin layers: an experimental study. Journal of Applied Phycology **19,** 417-423.

Sires I, Brillas E. 2012. Remediation of water pollution caused by pharmaceutical residues based on electrochemical separation and degradation technologies: a review. Environment International **40,** 212-229.

Wang L, Li Y, Chen P, Min M, Chen Y, Zhu J, Ruan R. 2010. Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae *Chlorella* sp. Bioresource Technology **101,** 2623-2628.

Wang L, Min M, Li Y, Chen P, Chen Y, Liu Y, Wang Y, Ruan R. 2010. Cultivaton of green algae *Chlorella sp.* in different wastewaters from municipal wastewater treatment plant. Applied Biochemistry and Biotechnology **162,** 1174-1186.

Zhou W, Li Y, Min M, Hu B, Chen P, Ruan R. 2011. Local bioprospecting for high-lipid producing microalgal strains to be grown on concentrated municipal wastewater for biofuel production. Bioresource Technology **102,** 6909-6919.

Zhou W, Min M, Li Y, Hu B, Ma X, Cheng Y, Liu Y, Chen P, Ruan R. 2012. A hetero-photoautotrophic two-stage cultivation process to improve wastewater nutrient removal and enhance algal lipid accumulation. Bioresource Technology **110,** 448-455.

Zhu LD. 2015. Biorefinery as a promising approach to promote microalgae industry: an innovative framework. Renewable and Sustainable Energy Reviews **41,** 1376-1384.