Sindh Univ. Res. Jour. (Sci. Ser.) Vol. 47 (4) 769-774(2015)

SINDH UNIVERSITY RESEARCH JOURNAL (SCIENCE SERIES)

Growth Kinetics of Algae Cells Under Heterotrophic Conditions

A. R. MEMON[++], N. SOOMRO*, K. L. KHATRI**

Department of Chemical Engineering, Mehran University of Engg. and Technology, Jamshoro, Pakistan

Received 1st April 2015 and Revised 3rd October2015

Abstract: Algae cells were cultivated both at micro and macro level under heterotrophic conditions in a prepared growth medium to analyze the cell response under the given conditions. Micro-cultivation of the cells was carried out separately in small-sized flasks, whereas mass cultivation of the cells was performed in a large-size tank. Pre-culturing of green algae *Chlorella vulgaris* (*C Vulgaris*) proved that the cells responded productively in the organic mode of growth medium. Saturated cycles of DO concentration in the range of 14 mg/L/d or above were observed during mass cultivation of *C Vulgaris* along with consequent occurrence of lowered pH values, which vindicated the optimum algae growth. The optimum *C Vulgaris* growth transpired that during the log phase every 1 g growth in *C Vulgaris* cell mass caused a COD reduction of 13%. Similarly, every 1g drop in *C Vulgaris* growth during stationary or death phase coincided with a COD increase of 730 mg l^{-1} likely caused by the release of soluble organic fraction of the decaying algae cells along with the generation of insoluble algae detritus in the cultivation medium.

Keywords: Kinetics, green algae, heterotrophic growth, Pseudomonas Putida, Cell cultivation

1. INTRODUCTION

The optimized preliminary cell culturing of algae species is an important stage in cultivating algae biomass at the macro scale. The growth environment during the culturing process can be either autotrophic (inorganic) or heterotrophic (organic) depending upon the nature of cells and their growth tendencies. Heterotrophic and mixotrophic algae are more capable of growing much faster with higher cellular oil content as compared to autotrophic algae cells (Memon *et al.*, 2014). However, heterotrophic algae require organic carbon sources like glucose or acetate as a sole source of carbon for growth, which are responsible for 80% of the medium costs (Debjani, *et al.*, 2012). The dissolved organic material ranges in size from 1 nm to 1 µm in diameter, thus it is directly taken up by the microorganisms (Racz and Goel, 2010). In addition to organics, the algae growth medium must contain considerable level of phosphates, ammonium nitrogen along with micronutrients such as minerals and metals for optimum propagation of algae cells (Bojcevska and Tonderski, 2007). Colosi (2009) hypothesizes that feeding algae with rich sources of both organic and inorganic carbon could boost the oil yield in grown algae mass to as much as 40 percent by weight. Microorganisms such as algae and bacteria are used to oxidise or convert the dissolved carbonaceous organic matter into simple products with additional biomass, which in case of algae mass is a potential source of proteins, carbohydrates, pigments, lipids and hydrocarbons (Hodaifa *et al.*, 2008). The biomass with a slightly higher specific gravity than that of water can be removed from the cultivation medium via gravity settling (Tchobanoglous *et al.*, 2003). Munoz *et al.* (2009) concluded that biomass retention in algal-based wastewater remediation processes showed a remarkable potential to maintain an optimum bacterial activity in addition to improving the final quality of the effluent. Roughly one kg of COD removed in an activated sludge process requires one kWh of electricity for aeration. By contrast, one kg of COD removed by photosynthetic oxygenation requires no energy inputs and produces enough algal biomass to generate renewable source of bioenergy that can produce one kWh of electric power (Rehman, 2011). Tarlan *et al.* (2002) also found utilization of algae cells as a monoculture effective in the treatment of wood-based pulp and paper industry wastewater.

The objective of this research was to optimize the use of sugar water as a sole source of organic carbon and for algae growth under heterotrophic conditions. In addition, the profile of operational variables such as pH, DO, COD, light incidence as well as biomass growth were studied on diurnal basis to assess the process performance.

2. EXPERIMENTAL

2.1 Sugar water

Sugar water was prepared by dissolving sugar (13g) in one liter distilled water in accordance with the COD value contained in sugar factory wastewater, which was 13000 mg/L. The word sugar comes from Indian

[++]Corresponding Author: Abdul Rehman email: enxarm@gmail.com
*Department of Textile Engineering, Mehran University of Engg. and Technology, Jamshoro, Pakistan
**Department of Civil Engineering, Mehran University of Engg. and Technology, Khairpur campus, Pakistan

sarkara, while its scientific name is Saccharides and the chemical name is sucrose. The sucrose molecule, which is a disaccharide made from glucose and fructose, contains 51.5% oxygen, 42% carbon, and 6.5% hydrogen. White sugar as a source of organic matter was used in the preparation of sugar water (SW), since in most cases it is the best organic substrate (Mayo and Noike, 1995).

2.2 Green algae

Green algae *Chlorella Vulgaris* (*C Vulgaris*) cells were selected for their tendency to grow rather well under heterotrophic conditions, their mixotrophic characteristics and biodegradation capability in organic rich conditions (Ono and Cuello, 2006). *C. Vulgaris* is one of the dominant green algae species, which are also used in oxidation pond systems (Sahinkaya and Dilek, 2009). *C Vulgaris* strain (CCAP No. 211/79) was purchased from Culture Collection for Algae and Protozoa (CCAP), Scotland, UK, in a 10ml liquid solution of the medium carrying 2-3ml of *C Vulgaris* cells.

2.3 Algae culturing unit

Photoheterotrophic mass cultivation of *C Vulgaris* in sugar water (SW) was carried out in algae culturing unit to assess the growth potential of algae cell mass solely based on the organic carbon present in SW along with the corresponding influence on the organic strength of SW. The propensity of algae cells feeding on the organic carbon was realized during preculturing of algae strain, when the addition of SW in the growth medium spurred the growth of the cells within 24 hr. The performance parameters during algae cultivation, like temperature, incident light (IL), optimized SW recirculation flow rate (RFR), DO and organic loading (COD) were determined on daily basis to analyze the process efficiency. All the results presented were a measure of three experimental results to express them as mean values. The results obtained are mostly presented in 3-D format and the rotation range while making the graphs for x and y axes along with perspective and depth values are mostly taken as same with respect to each parameter for the sake of relative comparison.

3. RESULTS AND DISCUSSION

3.1 Pre-culturing of *C Vulgaris*

Two culturing flasks of 100 ml were taken, each containing 3 ml of the starter algae culture along with 50 ml of BG-11 medium and were kept in the algae culturing unit (ACU). After 48 hr of culture propagation, one of the flasks showed growth, which was visible in the form of increased amount of algae cells. In addition, the pH of the grown culture in the flasks also registered an increase in pH in the range of 0.4-0.7, indicating the consumption of inorganic carbon by the cells supplied via air. After 10 days of preculturing, the contents of both the flasks were transferred for expansion or sub-culturing in a larger volume to check the viability of the cultured cells (Koussemon *et al.*, 2001). The sub-culturing bottles were already added with 500 ml of BG-11 medium each. (**Fig. 1)** shows the images taken during two weeks of sub-culturing. The image (a) was taken after 24 hr of the start of subculturing, while image (b) was taken after 7 days of subculturing showing visible growth of algae cells in the culturing bottle, inoculated with the propagated cells. The growth of the cells was measured daily in terms of algae cell concentrate volume by means of either sedimentation or filtration, which transpired that the cells were growing at the rate of 2.1 ml of algae concentrate per day. Image (c) was taken after 11 days when the culture cells showed a colonial growth after the addition of 2 ml of prepared SW the previous day, which increased the growth rate of algae cell volume up to 3.4 ml per day. This meant that the cell volume was increased by 1.3 ml or by 60% per day in comparison to the lower growth rate observed without the addition of SW. Image (d) was taken on day 12 of subculturing, when the cell growth volume rate was increased from 3.4 to 4.2 ml per day yielding total algae concentrate volume of 50.4 ml after 12 days of subculturing. The cell growth rate obtained after day 12 suggested that the cell mass volume was increased by 24% giving a growth reduction of 36% in contrast to previous day growth rate likely due to limiting sugar in the medium. The colonial as well as rapid growth of *C Vulgaris* after the addition of SW as observed on day 11 of subculturing indicates towards *C Vulgaris* tendency for organic carbon as a preferred feed for their growth. In contrast, with CO_2 as the sole feed for the growth of algae cells, the cells were always seen in scattered form suggesting that the cells were under stress, which either implied towards insufficient carbon availability in the medium or its decreased consumption.

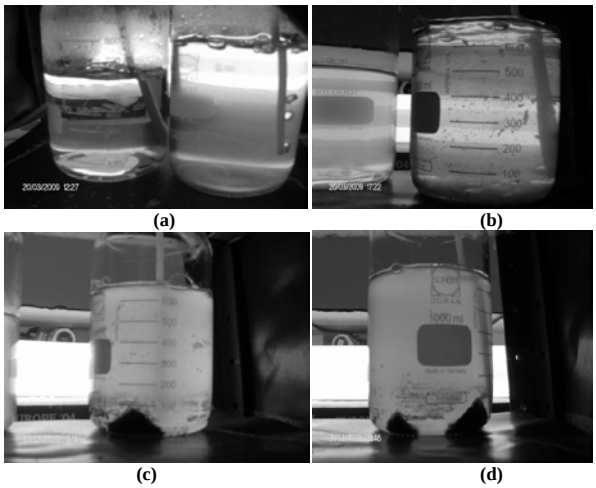


Fig. 1. Camera images showing *C Vulgaris* growth pattern during its sub-culturing. Photos taken after days: [a] = 1, [b] = 7, [c] = 11, and [d] = 12.

3.2 *C Vulgaris* mass cultivation

The cultured cells of *C Vulgaris* were then applied in a large sized tank for their mass cultivation with nutrient broth (BG-11). The sub-culturing of *C Vulgaris* transpired that the growth of the cells was triggered more by the introduction of SW into the culture medium than by the aeration. Hence, SW was considered as the direct source of organic carbon for the cultivation of *C Vulgaris* in a larger volume of culture medium. SW was prepared for a total working volume of 13 l with a ratio of 13 g sugar per l DW and was directly poured into the tank holding a depth of 5 cm in the tank. *C Vulgaris* concentrate volume of 40 ml was mixed with a solution of BG-11 broth medium prepared in 50 ml SW containing 22.1g of BG-11 medium (1.7g per l SW) along with corresponding trace metal mix solution volume of 13 l (1 ml per l SW). The prepared mix of *C Vulgaris* and BG-11 broth medium was transferred progressively (millilitre-wise) into the tank so as for the cells to stick to the surface of water in the tank. The inoculation was done at points 1 and 2 in the tank, yielding *C Vulgaris* cell volume to SW ratio of 1 ml: 260 ml and *C Vulgaris* cell volume to BG-11 broth ratio of 2.26 ml: 1g or that for every ml of *C Vulgaris* concentrate, 440 mg of the broth was available in the medium. Before transferring the prepared mix into LPT its pH, which was reading 2.9 was adjusted to 7.2 by adding 1.4 ml of 1M NaOH progressively. 100% BG-11 broth contained nitrogen with a total concentration of 116 mg l^{-1}, while total phosphorous concentration was 11 mg l^{-1}, giving an N and P ratio of around 10: 1 in the broth. SW recirculation flow rate was set at 40 ml min^{-1} to induce gentle mixing in the medium (Origin Oil, 2008). The luminance profile in the tank was set as continuous until the end of the experiment since continuous light to the cultures was preferred in case when the light flux was not variable and of lower intensity around 3000 lux as well (Andresen, 2005).

3.2.1 DO, pH, Temperature and incident light profile during mass cultivation of *C Vulgaris*

DO values obtained during nine days of *C Vulgaris* cultivation in SW are charted in **(Fig. 2)**. The graph depicts decreasing DO pattern and suggested that the lowest range of DO values between 0-2mg l^{-1}, highlighted by the occurred after day five. This implied that higher oxygen concentration was present in the culturing medium, likely due to the effect of algae growth. Towards the end of the cultivation run, the DO was again reduced to zero either owing to algae cell lysis, which brought a halt in the growth benthic *C. Vulgaris* or due to the increased oxygen consumption rate by the autochthonous (inherent) bacteria, This observation appeared to be consistent with the one made by Park *et al*. (2001).

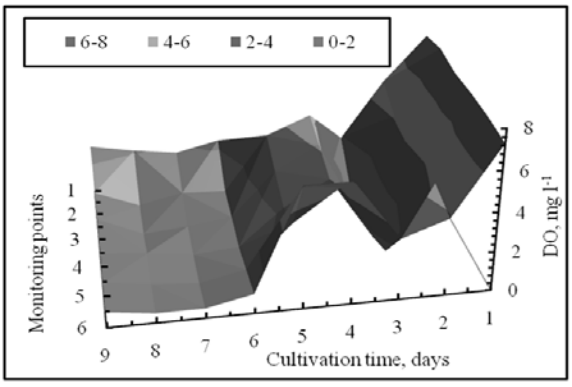


Fig. 2. DO at all monitoring points at the surface after 9 days of free cell cultivation of *C Vulgaris* with 100% BG-11 broth in SW (mean, n=3).

The data obtained for SW pH is shown in (**Fig. 3)** The graph shows that pH started to decrease after day two by 30%, which further decreased after day six by almost the same margin. This reduction might have been caused by the production of refractory (resistible to biodegradation) organic acids (Chang *et al.,* 2008). In addition, higher growth rate in algae cultures was also one of the factors in causing a pronounced decrease in the pH value of the cultivation medium (Hohe and Reski, 2005).

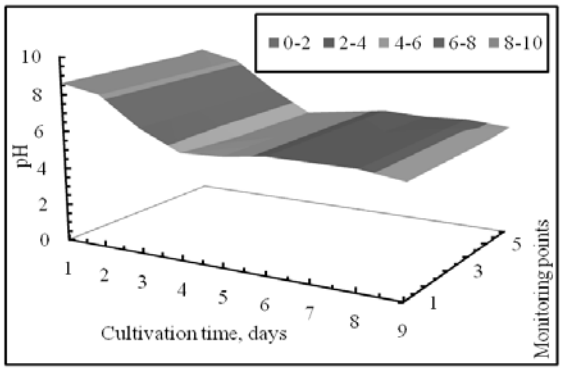


Fig. 3. pH at all monitoring points at the surface after 9 days of free cell cultivation of *C Vulgaris* with 100% BG-11 broth in SW (mean, n=3).

Incident light (IL) values at all the monitoring points during nine days of *C Vulgaris* cultivation are highlighted in **(Fig. 4).** The insolation (light incidence) profile suggested that maximum decrease in the luminance occurred at points 2 and 5, the high intensity points, as the reduction of around 28% was recorded at these points. The higher IL reduction was likely caused by the presence of higher amount of BG-11 broth in the cultivation medium, which probably led to the turbid conditions all over the tank thus decreasing the incidence of light. The reduction in insolation at the surface was lesser as compared to the bottom of the tank, as the algae cells were mostly settled at or near the bottom of the tank. The IL at point 2 showed maximum

of 15% reduction, whereas it was 19% at point 5 due to higher accumulation of biomass there. The decrease in IL at the surface started to occur after day 4 of *C Vulgaris* cultivation, when the algae cells had probably entered into the log phase of their growth. In actuality, the IL attenuation observed at the surface was probably higher than that observed at the bottom, as IL availability at the bottom was already reduced by 18% due to increase in the light path by 5 cm. Thus IL attenuation at the surface was probably higher as compared to the bottom during the log growth phase of *C Vulgaris*, which could be related with the growth of the biomass of their size and density thereby allowing the cells to suspend near the surface of the tank.

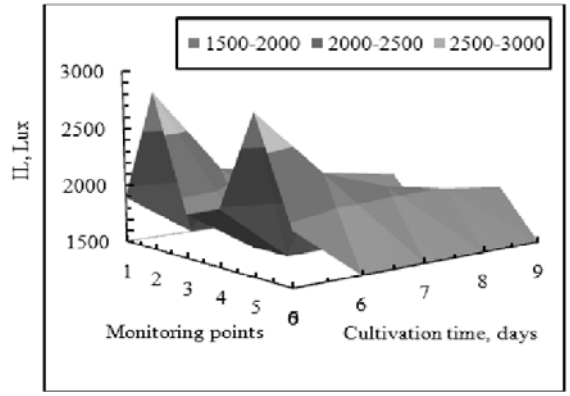


Fig. 4: Incident light profile at the surface at all monitoring points during 9 days of *C Vulgaris* cultivation with 100% BG-11 in SW (mean, n=3).

(Fig. 5) depicts diurnal *C Vulgaris* growth as against the COD values obtained during free cell cultivation *C Vulgaris* in SW. The study of the curves in the graph suggested that there is a correlation between the two lines as increase in the growth percentage of *C Vulgaris* seemed to be proportional to the simultaneous reduction in SW COD. This was fittingly highlighted by the steep rise in *C Vulgaris* growth after day four, which coincided with the simultaneous decrease in SW COD until day six and similarly by the drop in *C Vulgaris* growth after day six resulting in the corresponding increase in the COD value from the same day. The COD results in the graph suggested that maximum cell growth occurred during days 3 and 7, which resulted in 66% of the COD removal during this time. However, the last 24hr saw an increase in the COD by around 44% likely due to the release of organic fraction of cell detritus in the medium caused by the cell decay, which was probably induced by IL attenuation in the tank (Chisti, 2007). *C. Vulgaris* biomass data vindicated the argument that *C Vulgaris* cells started to multiply at faster rate after three days of cultivation with a maximum doubling time of almost 2 in 24 hr. This growth pattern continued until the end of day six with an overall doubling time of 1.2 for the six days. The last two days of cultivation showed dwindling growth of *C Vulgaris* likely due to cell decay, which could have been caused by limiting growth conditions in the tank. This condition was likely caused by the IL attenuation due to mushroom cell growth in the medium as well as possible generation of inherent bacterial mass as well as the non-removal of saturated DO from the cultivation medium (Chisti, 2008). On the whole, it was determined that during the log phase growth of *C Vulgaris* every 1 g growth in *C Vulgaris* cell mass caused a COD reduction of 13%. Similarly, the drop in *C Vulgaris* growth observed after day six transpired that every 1g drop in *C Vulgaris* growth coincided with a COD increase of 730 mg l^{-1}, which was probably caused by the release of soluble organic fraction of the decaying algae cells along with the suspension of insoluble algae detritus in the cultivation medium.

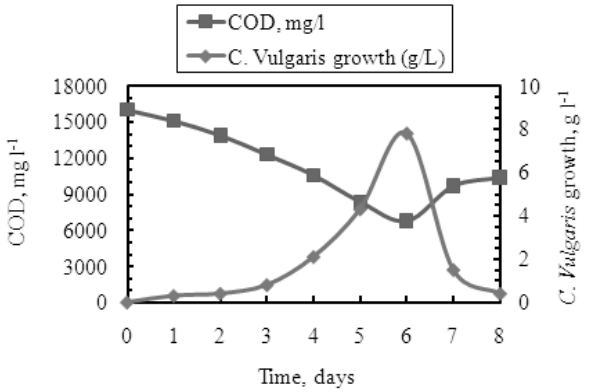


Fig. 5: Diurnal growth profile of *C Vulgaris* versus COD removal obtained during *C Vulgaris* cultivation in SW with 100% BG-11 (mean, n=3).

With respect to benthic mode of *C Vulgaris* cultivation, two factors can be put forward as an argument: First, the photosynthesis process took longer time of three days or more to take effect as reflected by the slower growth of the cells; second and as a resultant effect of the first, the cell-doubling time was perhaps too high (3.3 days) to cause substantial impact on the COD reduction. As observed during the baseline work (data not shown here), the aerobic degradation of organic carbon in SW started to occur after 40 hr, thereby depleting DO in the process. However, during *C Vulgaris* free cell cultivation in SW, it was observed that DO became a limiting factor within the first three days when it was reduced to zero, indicating that until that time or during lag phase of *C Vulgaris* growth there was actually no substantial evolution of photosynthetic oxygen in the cultivation medium (De Schryver *et al.*, 2008). This drastic decline in DO was perhaps triggered by the addition of higher concentration of BG-11 broth exacerbating the oxygen depletion due to probably rapid

growth of autochthonous or indigenous bacteria, resulting in the faster consumption of DO in the medium. The argument concerning the DO replenishment by *C Vulgaris* until after three days of free cell cultivation appeared to be consistent with that made by Irvine (2003), who stated that *C Vulgaris* respiratory or photosynthetic rates were clearly stimulated by sugar addition in the culture medium and that it took 72 hr for *C Vulgaris* to enter into their log phase of growth.

4. CONCLUSION

This research employed heterotrophic conditions for the cultivation of *C Vulgaris* cells in prepared sugar water without the pre-requisite supply of inorganic form of carbon such as CO_2. The given type of algae was found to show comparative tendency towards the organic growth medium. The growth pattern of *C Vulgaris* during its sub-culturing suggested that the cell growth was stimulated after the addition of SW into the culture medium, which showed *C Vulgaris* affinity towards sugar-oriented culture medium and hence its applicability for its mass cultivation in the organics-rich growth medium. The mass algae growth resulted in saturated concentrations of DO (14 mg/L/d) particularly during log phase of growth along with concomitant reduction in organic loading of the growth medium, when every 1 g growth in *C Vulgaris* cell mass caused a COD reduction of 13%.

REFERENCES:

Asadi, M., (2007). *Beet-Sugar Handbook*, John Wiley & Sons, Inc., USA.

Andresen, R. A., (2005). *Algal Culturing Techniques*, Elsevier, UK.

Bojcevska, H., and K. Tonderski, (2007). Impact of loads, season, and plant species on the performance of a tropical constructed wetland polishing effluent from sugar factory stabilization ponds, *Ecological Engineering*, 29, 66-76.

Chang, E. E., H. Hsing, P. Chiang, M. Chen, and J. Shyng, (2008). The chemical and biological characteristics of coke-oven wastewater by ozonation, *Journal of Hazardous Materials*, 156, 560-567.

Colosi, L., (2009). *Algae: biofuel of the future*, e-newsletter, University of Virginia, USA.

Chisti, Y., (2007) Biodiesel from microalgae, *Biotechnology advances*, 25, 294-306.

Chisti, Y., (2008) Biodiesel from microalgae beats bioethanol, *Trends in Biotechnology*, 26, 126-131.

De Schryver, P., R. Crab, T. Defoirdt, N. Boon, and W. Verstraete, (2008). The basics of bio-flocs technology: The added value for aquaculture, *Aquaculture*, 277, 125-137.

Debjani, M., J. V. Leeuwen, and L. Buddhi, (2012). Heterotrophic/mixotrophic cultivation of oleaginous Chlorella vulgaris on industrial co-products, *Algal Research*,1, 40–48.

Hohe, A., and R. Reski, (2005) Control of growth and differentiation of bioreactor cultures of Physcomitrella by environmental parameters, *Plant Cell, Tissue and Organ Culture*, 81, 307-311.

Hodaifa, G., M. E. Martinez, and S. Sanchez, (2008). Use of industrial wastewater from olive-oil extraction for biomass production of *Scenedesmus obliquus*, *Bioresource Technology*, 99, 1111-1117.

Koussemon, M., Y. Combet-Blanc, B. K. C. Patel, J. Cayol, P. Thomas, J. Garcia, and B. Ollivier, (2001). *Propionibacterium microaerophilum sp. nov*., a microaerophilic bacterium isolated form olive mill wastewater, *International Journal of Systematic and Evolutionary Microbiology*, 51, 1373-1382.

Memon, A. R., J. Andresen, M. Habib, and M. Jaffar, (2014). Simulated sugar factory wastewater remediation kinetics using algal-bacterial raceway reactor promoted by Polyacrylate polyalcohol, *Bioresour. Technol.* 157, 37–43.

Martinez, F., and M I. Orus, (1991) Interactions between glucose and inorganic carbon metabolism in *Chlorella Vulgaris strain UAM 101*, *Plant Physiology*, 95, 1150-1155.

Munoz, R., C. Kollner, and B. Guieysse, (2009). Biofilm photobioreactors for the treatment of industrial wastewaters, *Journal of Hazardous Materials*,161,29-34.

Mayo, A. W., and T. Noike, (1995). Effects of temperature and pH on the growth of heterotrophic bacteria in waste stabilization ponds, *Water Research*, 30, 447-455.

Ono, E., and J. L. Cuello, (2006). Feasibility assessment of microalgal carbon dioxide sequestration technology with photobioreactor and solar collector, *Biosystems Engineering*, 95, 597-606.

OriginOil, (2008). *A breakthrough technology to transform algae into oil*, OriginOil, Inc. India.

Park, J. I., Y. Yun, and J. M. Park, (2001). Oxygen-limited decomposition of food wastes in a slurry bioreactor, *Journal of Industrial Microbiology and Biotechnology*, 27, 67-71.

Racz., L. A., and R. K. Goel, (2010). Fate and removal of estrogens in municipal wastewater, *Journal of Environmental Management*, 12, 58-70.

Rehman, A., (2011). Lagooning and Bio-consortium Optimization for Secondary Level Remediation of Simulated Sugar Factory Wastewater, *PhD Thesis*, The University of Nottingham, UK.

Sahinkaya, E., and F. B. Dilek, (2009). The growth behaviour of *Chlorella Vulgaris* in the presence of 4-chlorophenol and 2, 4-dichlorophenol, *Ecotoxicology and Environmental Safety*, 72, 781-786.

Tchobanoglous, G., F. L. Burton, and H. D. Stensel, (2003). *Wastewater Engineering*, McGraw Hill, New York, USA.

Tarlan, E., F. B. Dilek, and U. Yetis, (2002). Effectiveness of algae in the treatment of wood-based pulp and paper industry wastewater, *Bioresource Technology*, 84, 1-5.