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Removal of Ammonia and Phosphate from Water Resources using Free and Immobilized Microalgae

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ABSTRACT

Laboratory experiments were performed to study nitrogen and phosphorus uptake by the green microalga *Scenedesmus quadricauda.* The treatment process was studied using different forms of microalga i.e. free, immobilized and co-immobilized microalga in sodium alginate beads. The study revealed that the maximum removal percentage of 1 mg/l ammonia and phosphate solutions reached up to 100% and 86%, respectively, after 4 days using free microalga; meanwhile, at 5mg/l ammonia and phosphate concentrations, the maximum removal percentage reached 91.8% after 4 days and 61% after 6 days, respectively. To solve the harvesting problem, immobilization process was carried out for the microalga and the removal percentage of immobilized microalga was nearly close to the removal percentage of free microalga. The removal percentage was enhanced by the addition of microalga growth promoting bacteria MGPB *Azotobacter chroococcum* and *Bacillus megatherium* where the removal capacity reached up to 100% for ammonia, and 80.4% for phosphate after the 3 and 6 days, respectively, as compared to 100% ammonia, and 61% phosphate after 4 and 6 days, respectively, by using the immobilized microalga.

Key words: Ammonia, phosphate, Scenedesmus, immobilized microalga, microalga growth-promoting bacteria

Introduction

The quick development of human activities has greatly increased the input of ammonia and phosphate into water bodies. This input induces eutrophication and causes deterioration in natural water quality. As such, the removal of nitrogen and phosphorus from wastewater is a fundamental way to prevent eutrophication and water bloom.

Effluents from secondary domestic and agricultural wastewater treatment plants contain high concentrations of inorganic nitrogen and phosphorus that may lead to eutrophication of the water bodies that they discharge (Martínez *et al*., 2000; Mallick, 2002; De-Bashan *et al*., 2002; Tanwar *et al*., 2007). Microalgae offer a low cost and effective approach to remove the excess nutrients and other contaminants because of their high capacity for inorganic nutrient uptake for tertiary wastewater treatment, while producing potentially valuable biomass (Martínez *et al*., 2000; Muñoz and Guieyssea, 2000; Chevalier *et al*., 2000). However, one of the major drawbacks of using microalgae in wastewater purification is the harvesting of biomass from the treated effluent (Mallick, 2002; Aslan and Kapdan, 2000).

Recently, research efforts have increasingly focused on the use of non-suspended algae; either attached or immobilized, as a valid method that avoids the harvesting problem (Vílchez *et al*., 2001; Jiménez-Pérez *et al*., 2004).Earlier studies have consistently indicated efficient and rapid removal of nitrogen and phosphorus from wastewater by immobilized algae. Carrageenan, chitosan and alginate are the polymers often used in these algal systems (James, 1998), with alginate beads being used most frequently (Tam and Wang, 2000; De-Bashan and Bashan, 1997-2003; Moreira *et al*., 2006).

As a novel ‘‘green technology,” microalgae have many advantages in the removal of nitrogen and phosphorus; these include: (1) low cost due to sufficient solar energy, (2) simultaneous fixation of CO_2, (3) lack of extraorganic carbon requirement (as compared to biological nitrification–denitrification), (4) discharge of oxygenated effluents into water bodies, (5) avoidance of sludge handling problems and (6) high economic potential of harvested algal biomass (for feedstock, fertilizers, biogas, biofuels, and so on) (Aslan and Kapdan, 2000).

A.chroococcum and *B.megatherium* where considered as microalgae growth-promoting bacteria (MGPB), these bacteria are capable of fixing atmospheric nitrogen and solubilizing phosphorus; it demonstrated

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that the observed growth promotion might improve the capabilities of microalgae to remove nutrients from natural wastewater (Ali *et al*., 2012; Hernandez *et al*., 2009).

Calcium alginate represents the most commonly employed system for its easiness in gel formation. Once liquid alginate solutions are contacted with polycation (Ca^{2+}), they immediately transformed into gel by binding between guluronic acid blocks in alginate and Ca^{2+} (Jiménez-Pérez *et al*., 2004). The major advantage of alginate gel entrapment is that immobilized cells do not suffer extreme physical-chemical condition changes during the immobilization process. Permeability, null toxicity and transparency of formed matrix imply a very gentle environment for immobilized cells (Smidsrød and Skjåk- Braek, 1990). Smaller beads/capsules have the advantage of a higher surface to volume ratio allowing good transport of essential nutrients and are less fragile. Diffusion limitations within larger beads may limit cellular metabolism as the lack of essential substances like oxygen supply to the interior of the beads may lead to cell death as a result of consumption from the surrounding cells, better dispersion, better mechanical strength, easier implantation and potential access to new implantation sites. Therefore, a good control of bead size and shape is crucial and should be carefully controlled (Melvik and Dornish, 2004).

The aim of this investigation is to evaluate the use of microalga *Scenedesmus quadricauda* in free, immobilized and co-immobilized forms in the removal of ammonia and phosphate from polluted water resources.

Materials and Methods

Analytical methods

Physico-chemical parameters, biological and microbiological examinations were carried out according to Standard Methods for Examination of Water and Wastewater 21th Edition (2005). Chlorophyll (a) was determined using (Jenway, Model 6715 UV/VIS) spectrophotometer (APHA, 2005).

Microalga and culture medium

Microalga *Scenedesmus quadricauda* was identified up to the species level according to the key of freshwater algae (Hustedt, 1976; Streble and krauter, 1978; Komárek and Fott, 1983; Komárek and Anagnostidis, 1989). *S. quadricauda,* was isolated from Rosetta branch, River Nile, Egypt (Fig. 1). Strain was isolated by spreading 0.1 ml of water sample into petri-dish containing artificial medium Bold's basal medium (BBM) plus 1% agar. Single colonies of alga were then re-cultivated in BBM (50 ml) and aerated by filtered air pumped at a rate of 4 L min^{-1}, maintained at temperature of 25 ± 2ºC, fluorescent lamp with light intensity 3100 lux and 16/8 h light/dark cycle (Bischoff and Bold, 1963; Nichols and Bold, 1965).

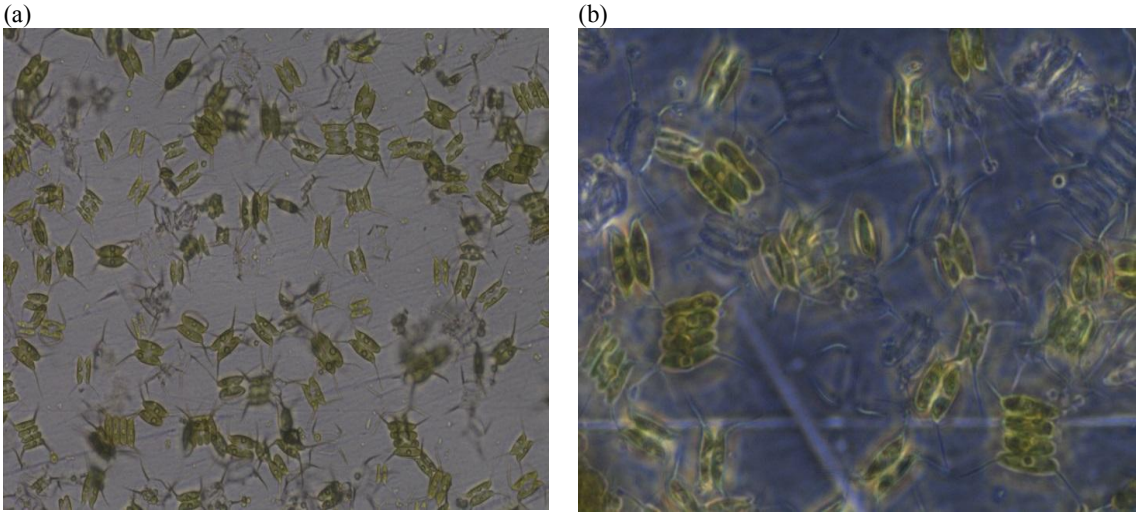

Fig. 1: *Scenedesmus quadricauda* at magnification power 200X (a) and 400X (b).

Preparation of different concentration ratios of free microalga S. quadricauda

After two weeks of cultivation, algal cells were harvested by filtration through manifold membrane with pore size 0.45 µm; the algal cells washed several times with saline solution (0.85 % NaCl) afterwards re-suspended in saline solution which gave a solution with cell count = 6.8*10^5 cell/ml. The free microalga became ready to be used in treating the inorganic pollutants; the algal suspension was used to remove ammonia and

phosphate from artificial samples. Four different concentration ratios 35%, 55%, 75% and 95% of Microalga *S. quadricauda* were prepared by addition of 35, 55, 75 and 95 ml of algal suspension, had algal count =6.8×10^5 cell/ml, and completed to 100 ml artificial sample, respectively. All experiments were performed at pH 7.0

Bacterial culture preparation:

The source of *A. chroococcum and B. megatherium* media suspension was obtained from soil fertility and microbiology department, Desert Research Center, Cairo, Egypt. The suspension was centrifuged at 2000 rpm and the residue washed twice by sterile saline solution and re-suspended in sterile saline solution. The systematic biotechnology was used taking fresh liquid cultures for 48 hours old from pure local strains of *A. chroococcum* and *B. megatherium* at the rate of ~10^8 cfu/ml (Hill and Sawers, 2000; Nautiyal, 1999).

Immobilization procedures

Low, medium and high concentrations of microalga were prepared on stocking beads by mixing sodium alginate (5%) and polyvinyl alcohol (5%) (ratio 5:1) with different volume (15, 10, 5) ml of algal suspension ($4.3*10^6$cell/ml). The mixture was then titrated with 2% $CaCl_2$ and the beads were allowed to harden in the $CaCl_2$ solution for 1 hr. After that, the beads were washed several times in distilled water to remove any residual $CaCl_2$. Then, the prepared beads were used to remove ammonia and phosphate from artificial solutions. Neat polymer were prepared without any algal cells as a control (Fig. 2)

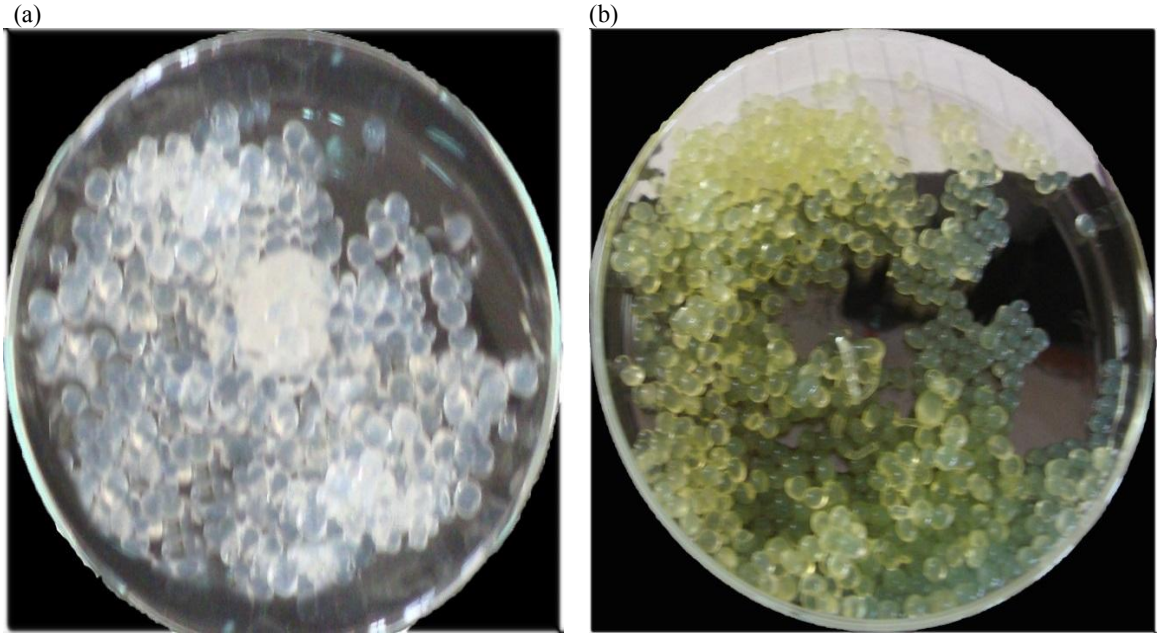

Fig. 2: Simple picture by digital camera of neat polymer (a) and immobilized microalga beads (b).

Co-immobilization of microalga and bacteria in alginate beads:

The co-immobilization step was done by the addition of 2.5 ml of *A.chroococcum* and 2.5 ml of *B.megatherium* during the preparation of the three different concentrations of microalga immobilized beads (low, medium and high stocking beads). MGPB control was prepared without any algal cells (bacteria only) as a control were routinely used

Results and Discussion

The removal of ammonia and phosphate were studied using free, immobilized and co-immobilized microalga.

Free microalga

The free microalga *S. quadricauda* culture was used to remove ammonia and phosphate from artificial samples, and the effect of initial concentration of ammonia, phosphate, time and concentration ratio of microalgae, were studied.

Removal of ammonia

(A) Effect of time

Artificial samples with 1 and 5 mg/l ammonia concentrations were treated with constant concentration ratio of microalga 95%; the removal of ammonia was measured against time from zero to 5 days. The results show that at 1 mg/l initial concentration, ammonia has been completely removed after 3 days. For 5 mg/l ammonia, the maximum removal percentage reached up to 91.8% after 4 days, and then nearly remains constant. Since uptake is the main mechanism of nutrient removal by microalgae, the microalgal population growth rate directly affects the nutrient removal rate (Xin *et al*., 2010). It was noticed that chlorophyll (a) concentration increased with time that reflects the growth of microalga using ammonia as a source of nutrient with insignificant increment (Fig. 3).

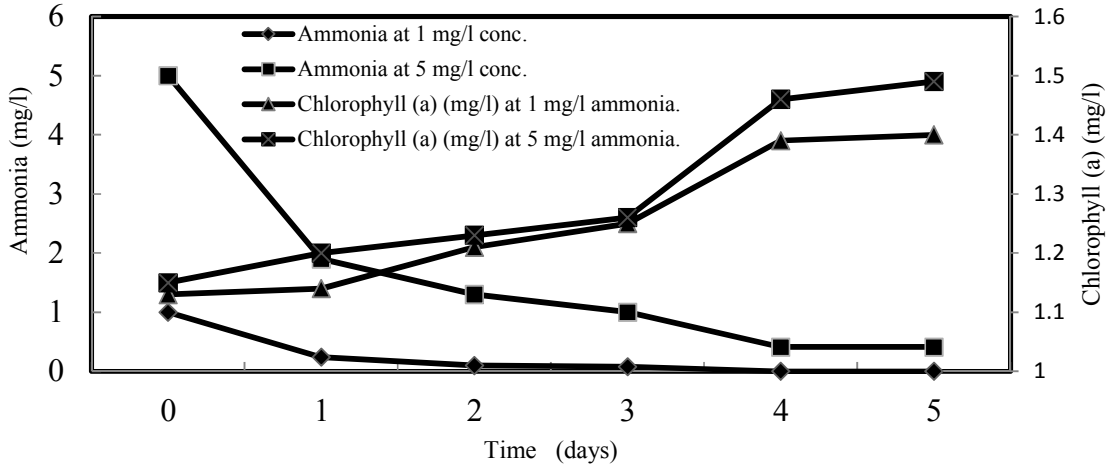

Fig. 3: Time course of ammonia removal and development of chlorophyll (a) at concentration ratio (95%). ([NH_3] = 1 mg/l & [NH_3] = 5 mg/l)

Heidari *et al*., 2011 mentioned that there was no significant correlation between chlorophyll a and NH_3 (and NO_2) removal rate. However, there is a significant correlation between NH_3 (and NO_2) concentrations and biomass. Meanwhile, Singh and Dhar (2007) reported a significant correlation co-efficient between available nitrogen removal with dry weight and chlorophyll content; in *Nostoc muscorum* and *Anabaena variabilis* has indicated usefulness of sewage effluent for cultivation of microalgae with efficient nutrient scavenging ability. However, the correlation coefficient between these parameters in *Oscillatoria princeps*, *Plectonema* sp. and *Chlorella vulgaris* was insignificant.

The same increment in chlorophyll (a) was occurred in 1 and 5 mg/l ammonia concentrations due to the difference in concentration are not very high. Aslan and Kapdan 2010 mentioned that the different low concentrations of ammonia did not reflect significant change in chlorophyll (a) but with increase ammonia concentration from 13.2 mg/l to 410 mg/l the final chlorophyll (a) content was significantly increased from10.7 mg/l to 27.3 mg/l (Jiménez-Pérez *et al*., 2004).

(B) Effect of algal concentration ratio

The initial cell concentration of microalga is critical for removing ammonia and phosphate. Higher removal efficiency can be achieved by properly increasing algal cell concentration, while lower cell concentrations would reduce the nutrient removal efficiency (Zhang *et al*., 2012).

The artificial samples with 1 and 5 mg/l concentration of ammonia were treated with different concentration ratios of microalga (35%, 55%, 95% and 95%) and the residual concentration of ammonia was measured after 4 days to find out the most efficient algal concentration ratio. The results show that for 1 mg/l concentration, ammonia was completely removed using *S. quadricauda* microalga with concentration ratios of 75% and 95%, while in case of 5 mg/l, the removal percentage reaches up to the maximum 94% when using microalga with concentration 95%, (Fig. 4). These results are in agreement with a previous investigation which indicated that the nutrient removal rate was dependent on algal concentration which determines the substrate diffusion (Zhang *et al*., 2012).

The chlorophyll (a) concentrate ion is an indication for algal growth rate which increased with time by the increase in ammonia removal percent and algal concentration ratio (Fig. 5).

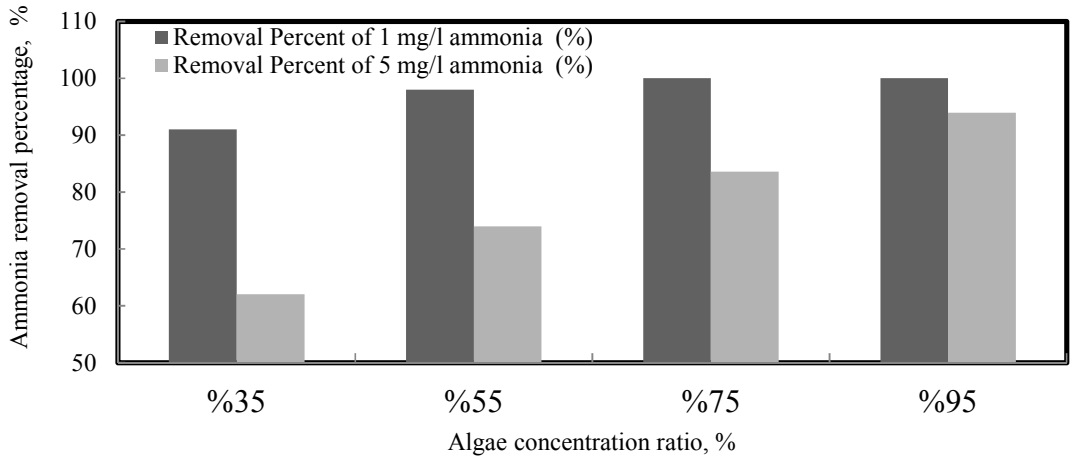

Fig. 4: Effect of algal concentration ratio on the removal percent of ammonia. ([NH3] = 1 mg/l & [NH3] = 5mg/l).

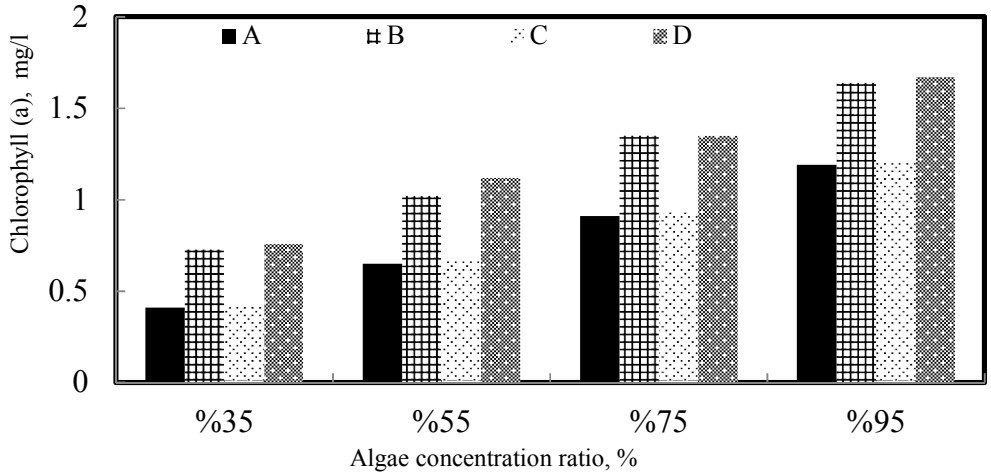

Fig. 5: Effect of algal concentration ratio on the developed concentration of chlorophyll (a). ([NH_3] = 1 mg/l and [NH_3] = 5 mg/l)

(A: initial conc. of chlorophyll (a) (mg/l) at 1 mg/l ammonia, B: conc. of chlorophyll a (mg/l) after 4 days at 1 mg/l ammonia, C: initial conc. of chlorophyll (a) (mg/l) at 5 mg/l ammonia, D: conc. of chlorophyll (a) (mg/l) after 4 days at 5 mg/l ammonia)

Removal of phosphate

(A) Effect of time

The artificial samples with 1 and 5 mg/l phosphate concentrations were treated with constant concentration ratio of algae (95%). The removal of phosphate was measured against time up to 6 days. The results showed that the residual phosphate concentration decreases with time and reaching maximum percentage of removal 86% after 4 days and 61% after 6 days for 1 and 5 mg/l phosphate, respectively; this is in agreement with a previous study which reported a significant decrease in wastewater phosphate content during the cultivation of three different species of algae; *Scenedesmus abundanse*, *S. quadricauda* and *Chlorella vulgaris* (Kassim, 2002). This phenomenon was also found in another green alga *Botryococcus braunii* when it was cultivated in secondarily treated sewage (Wang *et al*., 2013; sawayama *et al*., 1992). Also, Sreesai and Pakpain (2007) reported that approximately 55% of total phosphorous removal was achieved by *Chlorella vulgaris* in

septage effluent wastewater. Lee and Lee (2001) mentioned that microalgae *Chlorealla kessleri* was able to uptake only 8–20% phosphorus under the light/dark cycle for $(PO_4\text{-}P)_o$ = 10mg/l. The results revealed also that the growth of microalga using phosphate as a source of nutrient is illustrated by increasing of chlorophyll (a) concentration, (Fig. 6), (Fried *et al*., 2003; Ryan *et al*., 1972; Frink and Machlis, 1968; Sikka and Pramer,1968 and Tubea *et al*., 1981).

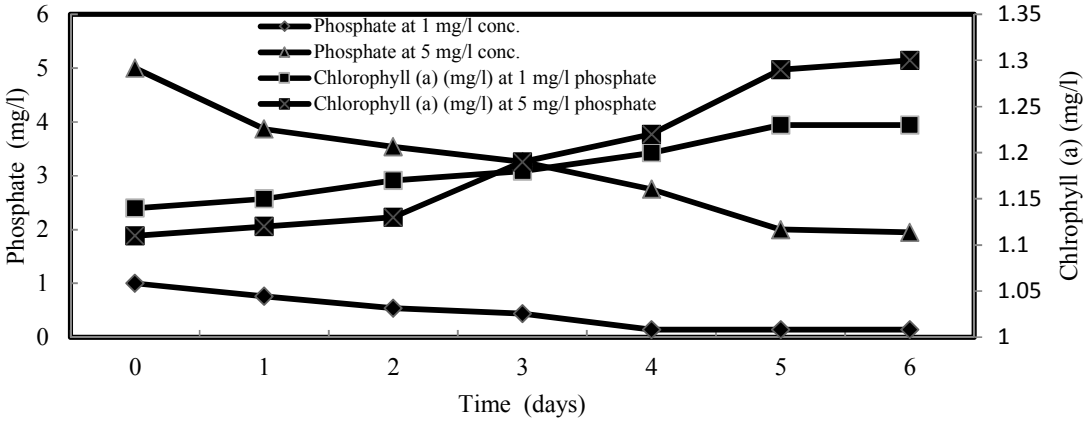

Fig. 6: Time course of phosphate removal and development of chlorophyll (a) at concentration ratio (95%). ($[PO_4^{-3}]$ =1 mg/l & $[PO_4^{-3}]$ =5 mg/l)

(B) Effect of algal concentration ratio

The artificial samples with 1 and 5 mg/l concentrations of phosphate were treated with different concentrations ratios of microalga (35%, 55%, 75% and 95%) and the removal of phosphate was measured after the 4 days for 1 mg/l phosphate artificial sample and after 6 days for the 5 mg/l phosphate artificial sample, to find out the most efficient algal concentration ratio for each concentration individually. The results showed that as the concentration ratio of microalga increases the percentage removal of phosphate increases. Maximum phosphate removal percentage was 89 % after 4 days and 63.4% after 6 days at 95% algal concentration for 1 and 5 mg/l phosphate concentrations, respectively, (Fig. 7). The different efficiencies of phosphate removal by *S. quadricauda* indicated that the phosphate removal was dependent on algal cell concentration (Zhang *et al*., 2012). As expected by increasing algal cell concentration more nutrients uptake occurs and more removal of phosphate is achieved from the media. Again, the chlorophyll (a) concentration is an indication of algal growth rate, which increased with time by increasing the phosphate removal percent and algal concentration ratio, Figure (8).

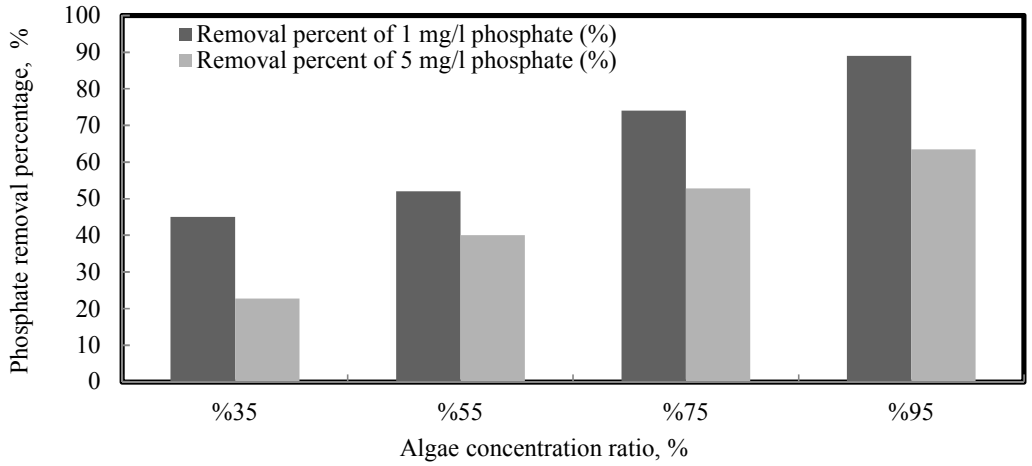

Fig. 7: Effect of algal concentration ratio on the removal percent of phosphate. ($[PO_4^{-3}]$ = 1 mg/l & $[PO_4^{-3}]$ = 5 mg/l)

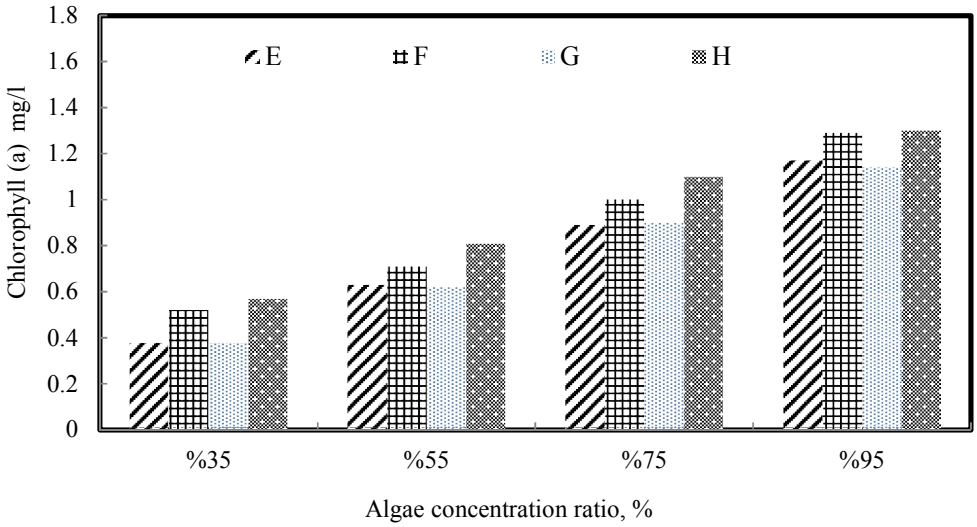

Fig. 8: Effect of algal concentration ratio on the developed concentration of chlorophyll (a). ($[PO_4^{-3}]$ = 1 mg/l, $[PO_4^{-3}]$ = 5 mg/l)
(E: initial conc. of chlorophyll (a) (mg/l) at 1 mg/l phosphate, F: conc. of chlorophyll (a) (mg/l) after 4 days at 1 mg/l phosphate, G: Initial conc. of chlorophyll (a) (mg/l) at 5 mg/l phosphate, H: conc. of chlorophyll (a) (mg/l) after 6 days at 5 mg/l phosphate).

Immobilization of microalga in alginate beads

The produced beads by three different concentrations of microalga (5, 10, and 15) which were, classified as low, medium and high stocking beads, respectively, were used to remove ammonia and phosphate from artificial solutions of 5 mg/l ammonia and phosphate.

Removal of ammonia.

The artificial samples with a constant concentration of ammonia (5 mg/l) were treated with both neat polymer and low, medium and high stocking beads, and the removal of ammonia was measured daily up to 5 days.

The results showed that ammonia removal increases by increasing the population of microalga in the beads. Ammonium would be freely available to the immobilized algae as their free counter parts (Lau *et al*., 1997). The initial cell concentration of *S. quadricauda* is critical for removing ammonia. Higher removal efficiency can be achieved by properly increasing cell density in gel, while lower cell concentrations would reduce the nutrient removal efficiency. On the other hand, higher cell concentrations in gel were not effective or necessary because it may reduce light penetration. Meanwhile, it resulted in self-shading and limits the growth and activities of algal cells (Zhang *et al*., 2012). This can be explained based on the results obtained in this study which indicated that the medium and high stocking beads achieved about 100% ammonia removal at the 5 days (Chevalier *et al*., 2000; Tubea *et al*., 1981).

Nearly no removal was observed by the use of neat polymer. Therefore, the role of polymer is just a carrier tool making all the microalga exposed to the solution and to solve the common harvesting problem (De-Bashan *et al*., 2004), Figure (9).

Removal of phosphate.

The artificial samples with a constant concentration of phosphate (5 mg/l) were treated with both neat polymer and low, medium and high stocking beads, and the removal extent of phosphate were measured daily for 6 days.

The results showed that the phosphate removal increases by increasing population of microalga in the beads, but the differences among the three stocking beads were insignificant. Meanwhile, nearly no removal was observed by the use of neat polymer. Therefore, the role of polymer is just a carrier tool making all the microalga exposed to the solution and to solve the common harvesting problem, Figure (10).

Too low cell concentrations of algal cell in stocking beads would reduce the nutrient removal efficiency. On the other hand, too high cell concentrations in gel and thickness were also not effective, which would reduce the amount of light penetrating through the algal beads, and enhance the self-shading effects which then limit the growth and activities of the algal cells (Zhang *et al*., 2012).

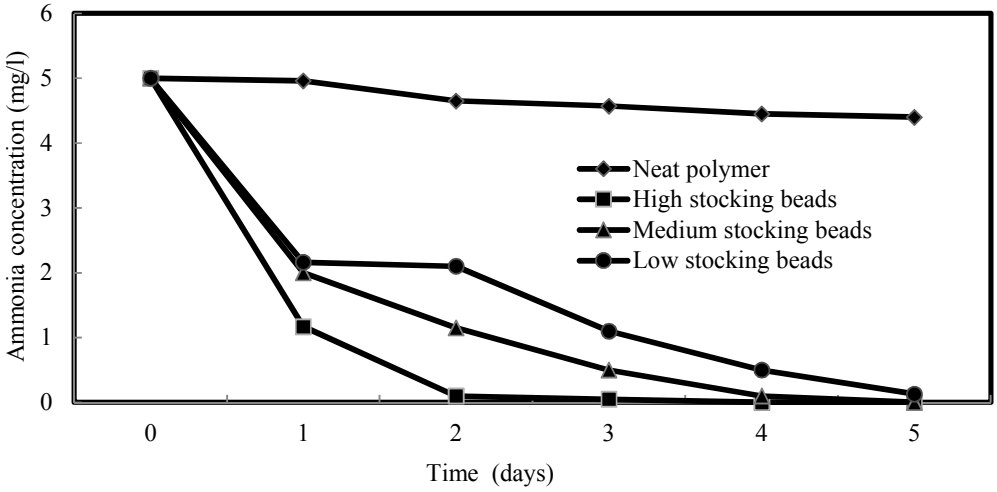

Fig. 9: Effect of time on the removal of ammonia, using low, medium and high stocking beads. ([NH_3] = 5 mg/l)

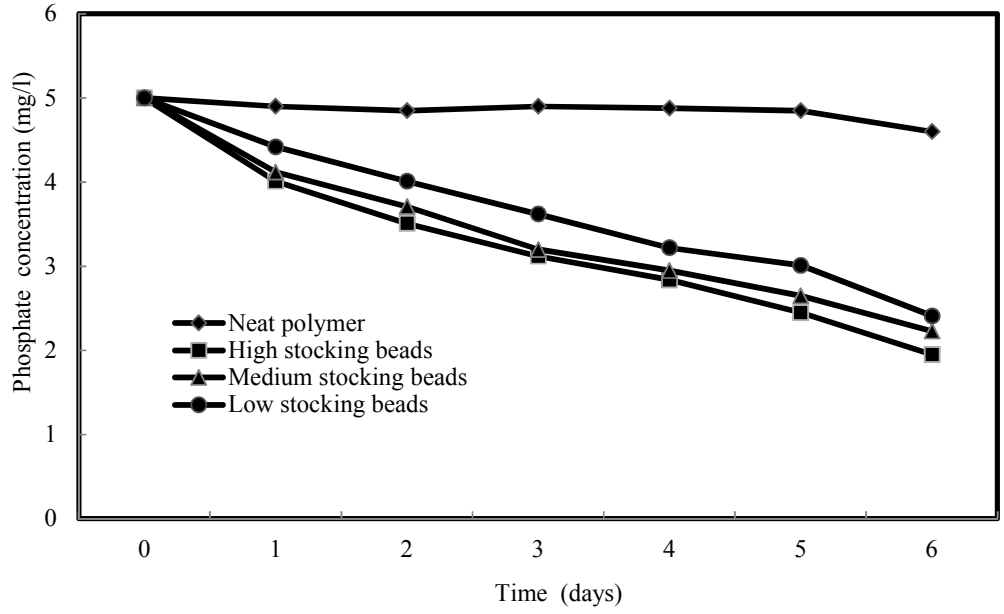

Fig. 10: Effect of time on the removal of phosphate, using low, medium and high stocking beads. ([PO_4^{-3}] = 5 mg/l)

Co-immobilization of microalga and bacteria in alginate beads

The produced beads by three different concentrations of microalga (5, 10, and 15) with *A. chroococcum* and *B. megatherium* classified to co-immobilized low, medium and high stocking beads, respectively, were used to remove ammonia and phosphate from artificial solutions of 5 mg/l ammonia and phosphate.

Removal of ammonia.

The artificial samples with a constant concentration of ammonia (5 mg/l were treated with MGPB control and different concentrations of co-immobilized microalga (low, medium and high stocking beads) and the removal of ammonia were measured daily for five days. The percentages of ammonia removal after 1 day were 85.8, 82 and 81.6% for high, medium, low stocking beads, respectively. The behavior of the high, medium and low stocking beads was nearly similar, although the high stocking recorded higher ammonia removal but the difference was insignificant. The high and medium stocking beads achieved 100% removal after 3 and 4 days, Figure (11).

Microalga growth promoting bacteria alone did not remove measurable quantities of ammonium or phosphorus. However, co-immobilization of *S. quadricauda* with *A. chroococcum* significantly enhanced ammonium removal, although *S. quadricauda* were capable of eliminating most of the ammonium when immobilized alone (Melvik and Dornish, 2004; Lau *et al*., 1997).

Removal of phosphate.

The artificial samples with a constant concentration of phosphate (5 mg/l) were treated with MGPB control and different concentrations of co-immobilized microalga (low, medium and high stocking beads) and the removal of phosphate was measured against time daily for 6 days, Again the results showed that the percentage of phosphate removal increases by increasing population of microalga in the beads, Figure (12).

Removal of phosphate from the artificial samples was always better when microalga were co-immobilized with *A. chroococcum* and *B. megatherium*. Controls of MGPB were incapable of removing any phosphate (Melvik and Dornish, 2004).

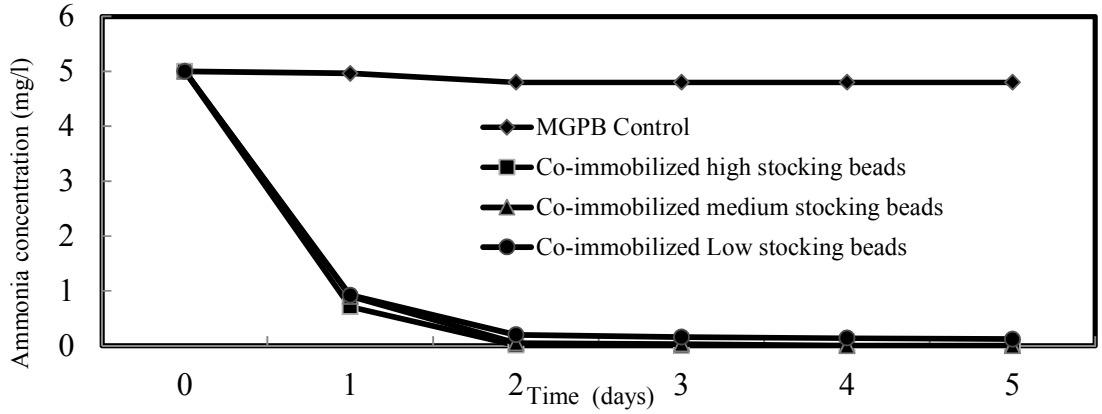

Fig. 11: Effect of time on the removal of ammonia, using co-immobilized low, medium and high stocking beads. ($[NH_3]$ =5 mg/l)

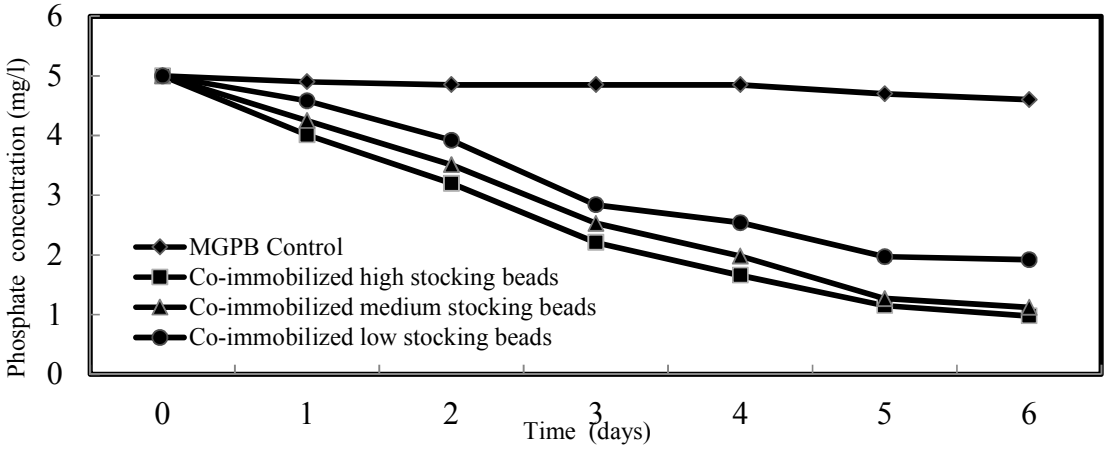

Fig. 12: Effect of time on the removal of phosphate, using co-immobilized low, medium and high stocking beads. ($[PO_4^{-3}]$ = 5 mg/l

Summary

The present study demonstrated that the use of microalga S.*quadricauda* is capable of reducing ammonia and phosphate from water resources and in order to overcome the harvesting problem, immobilization of microalga on small alginate/ polyvinyl alcohol beads was carried out. Co-immobilization of S.*quadricauda* with *A.chroococcum* and *B.megatherium* in small alginate beads enhanced the ability of microalga S.*quadricauda* in ammonia and phosphate removal from water resources. As a green technology, these represent new approaches to biological removal ammonia and phosphate.

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