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Enhancement of *Chlorella vulgaris* Growth and Bioremediation Ability of Aquarium Wastewater Using Diazotrophs

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Abstract: Treatment of aquarium wastewater represents an important process to clean and recycle wastewater to be safely returned to the environment, used for cultivation or to minimize the multiple renewal of water. *Chlorella vulgaris* was an important freshwater microalgae which used in wastewater treatment, and increasing its potential of treatment can be achieved with existence of N_2-fixing bacteria. Co-culturing of *Chlorella vulgaris* with the diazotrophs, *Azospirillum brasilense* or *Azotobacter chroococcum* in three different media; aquarium wastewater (AWW), sterile enriched natural aquarium wastewater (GPM) and synthetic wastewater media (SWW) were studied. Biomass yield of the microalgae was estimated by determination of chlorophylls (a and b), total carotenoid and the dry weight of *C. vulgaris*. Also determination of ammonia, nitrite, phosphate and nitrate in the culture were done. The presence of diazotrophs significantly increased the biomass of *C. vulgaris* by increasing its microalgae pigments (chlorophylls a and b, and total carotenoids). The highest pigments percentage was reported due to addition of *A. brasilense* to *C. vulgaris* (18.3-133.5%) compared to *A. chroococcum* (23.9-56.9%). As well as increased dry weight from 12 to 50%. There was also improved removal of nitrate, nitrite, ammonia and phosphate; where, the highest removal percentage was reported due to addition of *A. chroococcum* to *C. vulgaris* (0.0-52%) compared to *A. brasilense* (0.6-16.4%). *A. brasilense* and *A. chroococcum* can support *C. vulgaris* biomass production and bioremediation activity in the aquarium to minimize the periodical water renewal.

Key words: *Chlorella vulgaris*, diazotrophs, biomass production, bioremediation, aquarium wastewater

INTRODUCTION

Shortage of fresh water represents a problem due to many reasons such as climatic changes and bad use of water. Treatment and recycle of water can maintain the sustainable supplies of water for future generations. Nitrogen and phosphorous discharged through agricultural, sewage and industrial effluent are the major constituents of the wastewater. Treated wastewater still include some nitrogen and phosphorous in the form of nitrate, nitrite, ammonia and phosphorus. Aquarium wastewater also contains high levels of nitrogen and phosphorus due to fish feeding and excretion so; fish developers need to change aquarium water at least every three days. Microalgae play an important role in wastewater treatment since it requires nitrogen, phosphorous, CO_2 and light for their autotrophic growth (Abe *et al*., 2002; Ma *et al*., 2004).

Microalgae are unicellular microscopic algae which live in freshwater and marine ecosystems. Microalgae are capable to perform photosynthesis so they are very important for life on earth; they can use carbon dioxide gas and produce high percent of oxygen to grow photoautotrophically. Moreover, they are used in water bioremediation (Oswald, 1992; Wilde and Benemann, 1993), feeding humans and animals (Becker, 1988; De Pauw and Persoone, 1988), pigment production (Johnson and An, 1991) and in agriculture (Metting, 1992).

Chlorella vulgaris is a green freshwater unicellular microalga and known as one of the fastest growing microalgae. Many authors have reported that *C. vulgaris* is used for removal of nitrogen and phosphorus compounds as well as heavy metals from wastewater (Oh-Hama and Miyachi, 1992; Tam *et al*., 1994, 1998).

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Plant Growth-promoting Bacteria (PGPB) used in the agriculture to enhance the growth of numerous agricultural crops (Bashan, 1998; Ali *et al*., 2005). Such bacteria are also used as "Microalgae-growth Promoting Bacteria" (MGPB) to enhance growth of the unicellular microalgae. For example *Flavobacterium* sp. can promote the growth of marine microalgae, *Chaetoceros gracilis* (Suminto and Hirayama, 1996). Also, *Bacillus pumilus* (Hernandez *et al*., 2009), *Phyllobacterium myrsinacearum* (Gonzalez-Bashan *et al*., 2000) and *A. brasilense* (Gonzalez and Bashan, 2000) enhanced growth of the freshwater microalga *C. vulgaris*.

The microalgae are always associated with bacteria in natural and artificial aquatic environments (Mouget *et al*., 1995). Microalgae growth-promoting bacteria, *Azotobacter* and *Azospirillum* were used as biofertilizers in aquaculture (Puente *et al*., 1999; Garg and Bhatnagar, 1999; Tripathy and Ayyappan, 2005; Ali *et al*., 2011) and significantly increased the phytoplankton population and consequently the yield of fish.

The aim of this study is to minimize the withdrawal of aquarium wastewater and maintain the same water for longer time through supporting the growth of the freshwater microalga *C. vulgaris* by Co-culturing with *A. brasilense* or *A. chroococcum*. This enhancement of *C. vulgaris* growth in aquarium wastewater increases fish natural food and improves chemical properties of aquariums.

MATERIALS AND METHODS

Microorganisms: The freshwater microalgae *C. vulgaris* was isolated from assembler wastewater pond of fish farm Research Station, El-Qanater Elkhayrea, Egypt and purified as recommended by Pringshiem (1946). Two strains of Plant Growth-promoting Bacteria (PGPB) were used; *A. brasilense* (Azos. R7) was isolated from *Ricinus communis* and *A. chroococcum* (Azt) was isolated from *Hordeum vulgare* (Hamza *et al*., 1994). They were grown on N-deficient combined carbon sources medium, CCM (Hegazi *et al*., 1998) at 32°C in a rotary shaker for 3 days for *A. brasilense* and 5 days for *A. chroococcum*. The PGPB cells were harvested by centrifugation at 7000 rpm for 15 min and washed twice with sterile solution (0.85% NaCl).

Growth medium: Three different media were separately used:

- Synthetic wastewater growth medium (SWW) of De-Bashan *et al.* (2005) which consists of (mg L^{-1}): NaCl: 7, $CaCl_2$: 4, $MgSO_4.7H_2O$: 2, K_2HPO_4: 21.7, KH_2PO_4: 8.5, Na_2HPO_4: 33.4 and NH_4Cl: 3
- Aquarium wastewater medium (AWW) was prepared by filtered aquarium wastewater through membrane filter paper (47 mm prefilter pad and 0.45 μm porosity) under vacuum to remove indigenous planktonic organisms, then it was pasteurized (EPA, 1971)
- The sterile enriched natural aquarium wastewater (GPM medium of Loeblich, 1975), it comprises of; 750 mL from latter filtered aquarium wastewater, 204 mL distilled water, KNO_3: 0.238 g L^{-1}, soil extract: 15 mL L^{-1}, KH_2PO_4: 0.0397 g L^{-1}, vitamin B_{12}: 1 μg L^{-1} and 30 mL L^{-1} trace elements solution (mg L^{-1}): $(NaH_2O)_2$: 1.27, H_3PO_3: 1.14, $FeCl_3.4H_2O$: 0.0484, $MnCl_2.4H_2O$: 0.144, $ZnCl_2$: 0.0104 and $CoCl_2.6H_2O$: 0.004) pH of the three media was adjusted at 7.0

Experimental design: Two experiments were prepared individually, the first used *A. brasilense* and the second used *A. chroococcum*. Each experiment contained nine treatments; three culture media, AWW, SWW, GPM and each culture media received three status of inoculation, the first was received bacteria without microalgae, the second was received microalgae without bacteria and the third was received microalgae with bacteria.

Growth conditions: Approximately 108 bacterial cells of diazotrophs and 5000 cells mL^{-1} of *C. vulgaris* were used. Cultures were grown in batch cultures (200 mL medium in 500 mL Erlenmeyer flask) and incubated in a locally made controlled incubator at temperature 25±1°C and light intensity of 4000 lux under a day/night program of 14 h. light followed by 10 h darkness for 7 days. Samples for analysis were taken after 7 days of incubation.

Determination of biomass yield of the microalgae

Determination of the microalgae pigments: Spectrophotometric measurements of absorbance, at different wave-lengths, were adopted for estimating the chlorophylls (a, b) and total carotenoid contents of *C. vulgaris* alone or co-cultured with *A. brasilense* or *A. chroococcum* (APHA, 1992). Chlorophyll (a) and (b) concentrations were calculated (Jeffrey and Humphrey, 1975) and total carotenoids content was also determined (Timothy *et al*., 1985).

Determination of the microalgae dry weight: Dry weight of the microalgae was scored, the samples were filtered in filter paper (0.45 μm pore size) and the filter was dried at 105°C for 24 h (APHA, 1992).

Determination of nutrient in the culture: Colorimetric methods were used to determine ammonia, nitrite and phosphate (APHA, 1995) and nitrate (Mullin and Riley, 1955).

Statistical analysis: Data were statistically analyzed using analysis of variance, ANOVA using the STATISTICA 6.0 software (Stat soft, Tulsa, USA). The differences between groups were considered significant at $p<0.05$.

RESULTS

The biomass yield and cell pigment content of the freshwater microalgae *C. vulgaris* were positive affected by the addition of either *A. brasilense* or *A. chroococcum*. Addition of *A. brasilense* to the microalgae raised chlorophylls a content from 17.8 to 23.4 $\mu g\ L^{-1}$, chlorophylls b from 12.8 to 22.8 $\mu g\ L^{-1}$ and total carotenoids from 15.9 to 24.1 $\mu g\ L^{-1}$ in AWW media. The addition of *A. chroococcum* was also raised chlorophylls a content from 14.8 to 18.5 $\mu g\ L^{-1}$, chlorophylls b from 12.4 to 16.6 $\mu g\ L^{-1}$ and total carotenoids from 10.2 to 15 $\mu g\ L^{-1}$ in AWW media (Fig. 1). Moreover GPM and SWW media exhibited the same increase in cell pigments content after

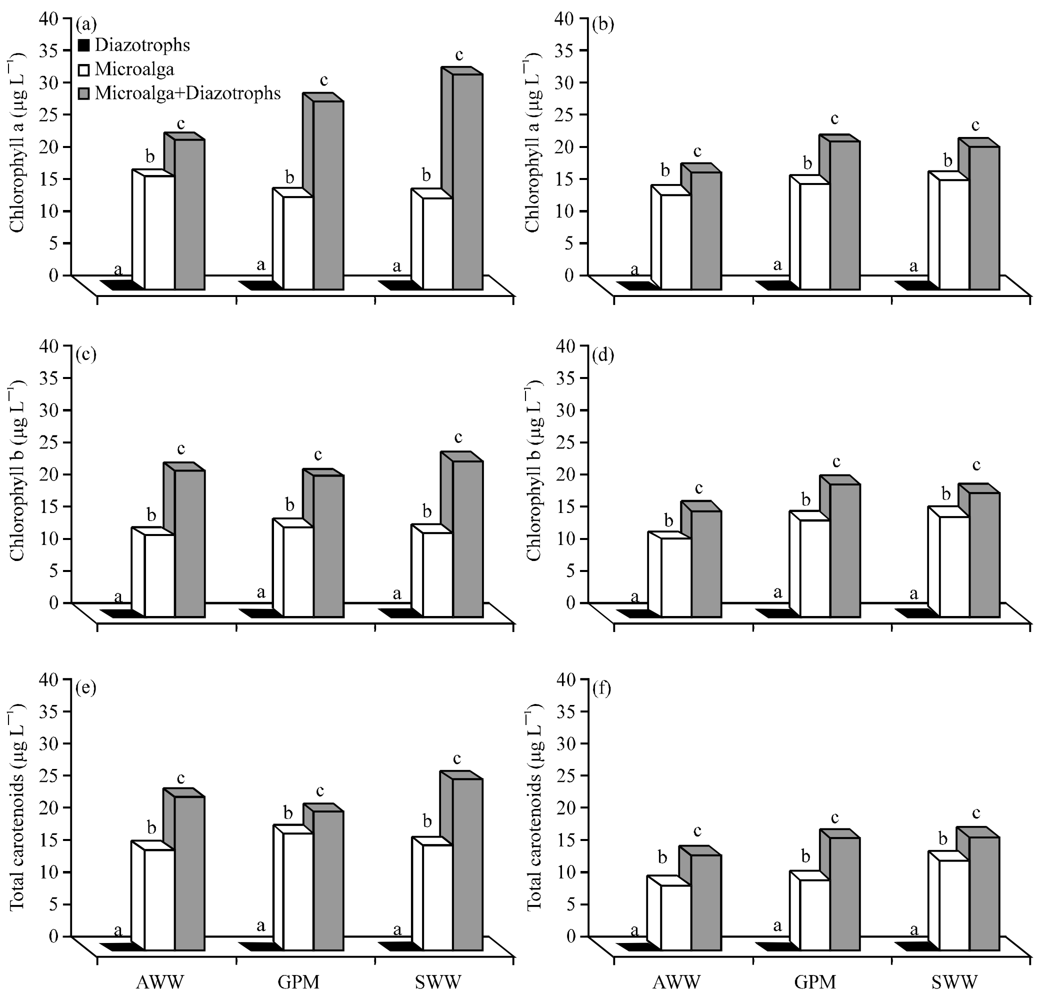

Fig. 1(a-f): Pigment production by the microalga *C. vulgaris* (a, c, e) With *A. brasilense* and (b, d, f) With *A. chroococcum* after 7 days of incubation in batch cultures, SWW: Synthetic wastewater medium, AWW: Aquarium wastewater, GPM: Sterile enriched natural aquarium wastewater, Different letters differ significantly at $p\leq 0.05$

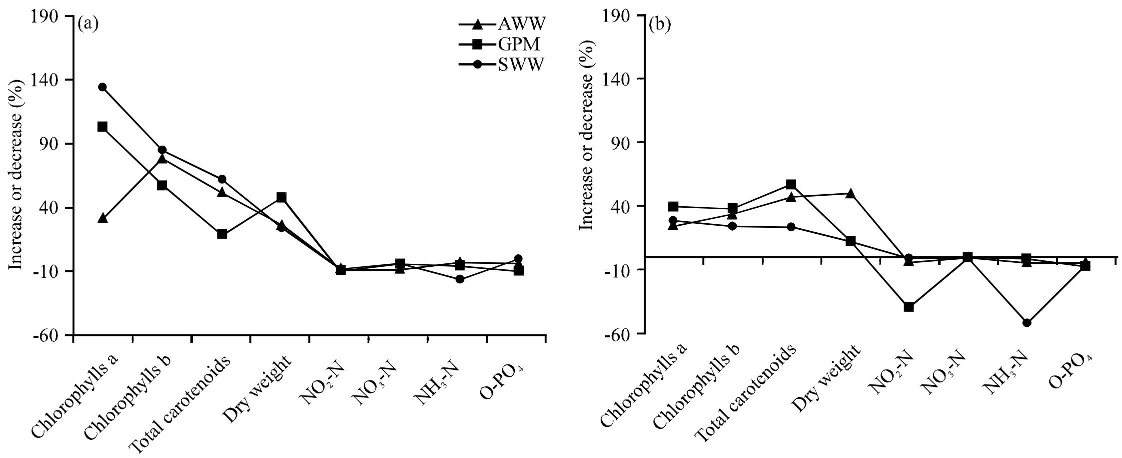

Fig. 2(a-b): Change percentages in biomass of *C. vulgaris* and nutrient contents in culture media due to inoculation with (a) *A. brasilense* and (b) *A. chroococcum* after 7 days of incubation in batch cultures, SWW: Synthetic wastewater, AWW: Aquarium wastewater medium, GPM: Sterile enriched natural aquarium wastewater, % increase or decrease = {(microalgae with bacteria-microalgae alone)/microalgae alone}×100.0

Table 1: Statistical analysis of the effect of diazotroph strains and type of culture media on *C. vulgaris* biomass and the residual of nutrient

Parameter	Chlorophyll a	Chlorophyll b	Total carotenoids	Dry weight	NO_2-N	NO_3-N	NH_3-N	O-PO_4
	(μg L^{-1})			(mg L^{-1})				
Effect of diazotrophs								
Diazotrophs[1]	0.00^c	0.00^c	0.00^c	0.0047^c	0.330^a	1.509^a	1.961^a	41.14^a
Microalga[2]	15.91^b	13.89^b	14.46^b	0.0081^b	0.235^b	1.309^b	0.167^b	38.53^b
Microalga+Diazotrophs[3]	25.05^a	21.04^a	20.53^a	0.0105^a	0.187^c	1.252^c	0.136^b	36.45^c
Effect of strains								
A. brasilense[4]	14.77^a	12.11^a	13.71^a	0.0098^a	0.235^b	2.358^a	0.704^b	38.40^a
A. chroococcum[5]	12.53^b	11.17^b	9.62^b	0.0058^b	0.266^a	0.354^b	0.806^a	39.02^a
Effect of media								
AWW[6]	12.43^b	10.77^b	10.87^b	0.0080^a	0.112^c	0.280^c	0.526^c	39.89^a
GPM[7]	13.97^a	12.00^a	11.53^{ab}	0.0081^a	0.521^a	3.237^a	0.794^b	39.72^a
SWW[8]	14.56^a	12.16^a	12.59^a	0.0073^b	0.118^b	0.552^b	0.944^a	36.52^b

AWW: Aquarium wastewater, GPM: Sterile enriched natural aquarium wastewater, SWW: Synthetic wastewater, Means followed by the same letter are not significantly different at $p \leq 0.05$, [1]Average value of treatments of diazotrophs (*A. brasilense* or *A. chroococcum*) without microalga irrespective of culture media, [2]Average value of treatments of microalga without diazotrophs irrespective of culture media, [3]Average value of treatments of microalga with diazotrophs irrespective of culture media, [4]Average value of treatments of *A. brasilense* with or without microalga irrespective of culture media, [5]Average value of treatments of *A. chroococcum* with or without microalga irrespective of culture media, [6]Average value of treatments using AWW culture medium with or without microalga or diazotrophs, [7]Average value of treatments using GPM culture medium with or without microalga or diazotrophs, [8]Average value of treatments using SWW culture medium with or without microalga or diazotrophs

diazotrophs addition as shown in Fig. 1. Percentage increases ranged from 25 to 78%, 18.3 to102.3% and 23.9 to 133.5% for the culture media AWW, GPM and SWW respectively (Fig. 2). In general, the AWW culture medium recorded the lowest pigments amount compared to SWW and GPM (Table 1). Also, the highest increase percentage was reported due to addition of *A. brasilense* to *C. vulgaris* (18.3-133.5%) compared to *A. chroococcum* (23.9-56.9%).

Dry weight of the freshwater microalgae *C. vulgaris* confirmed the positive effect of addition N_2-fixing bacteria (Fig. 3), where, increases in dry weight ranged from 12 to 49% (Fig. 2).

Removal of nutrients (NO_2-N, NO_3-N, NH_3-N and O-PO_4) from the SWW, AWW and GPM culture media was always better with addition of diazotrophs to *C. vulgaris* (Fig. 4), the removal reached up to 42% nitrite, 100% nitrate, 91% ammonia and 17% phosphate; compared to 40% nitrite, 100% nitrate, 88% ammonia and 14% phosphate by the microalgae alone. The highest removal percentage was reported due to addition of *A. chroococcum* to *C. vulgaris* (0.0% to 52) compared to *A. brasilense* (0.6-16%) (Fig. 2). Also, the significantly maximum removal was recorded in AWW culture medium (Table 1).

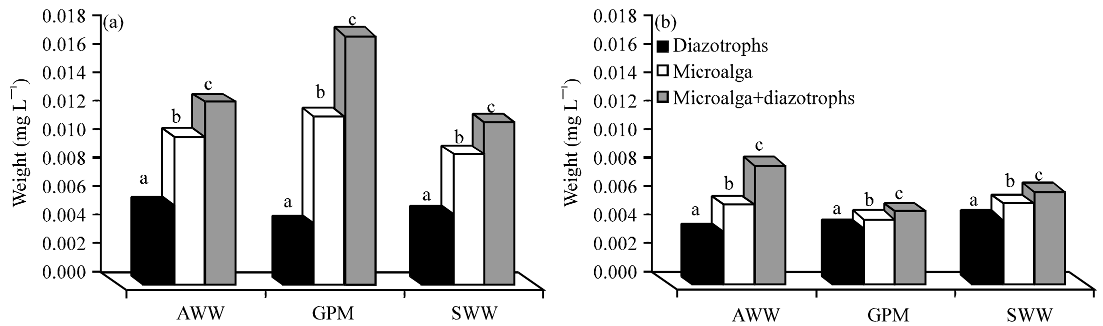

Fig. 3(a-b): Dry weight of *C. vulgaris* culture media due to (a) *A. brasilense* and (b) *A. chroococcum* inoculation after 7 days of incubation in batch cultures, SWW: Synthetic wastewater, AWW: Aquarium wastewater medium, GPM: Sterile enriched natural aquarium wastewater, Different letters differ significantly at $p \leq 0.05$

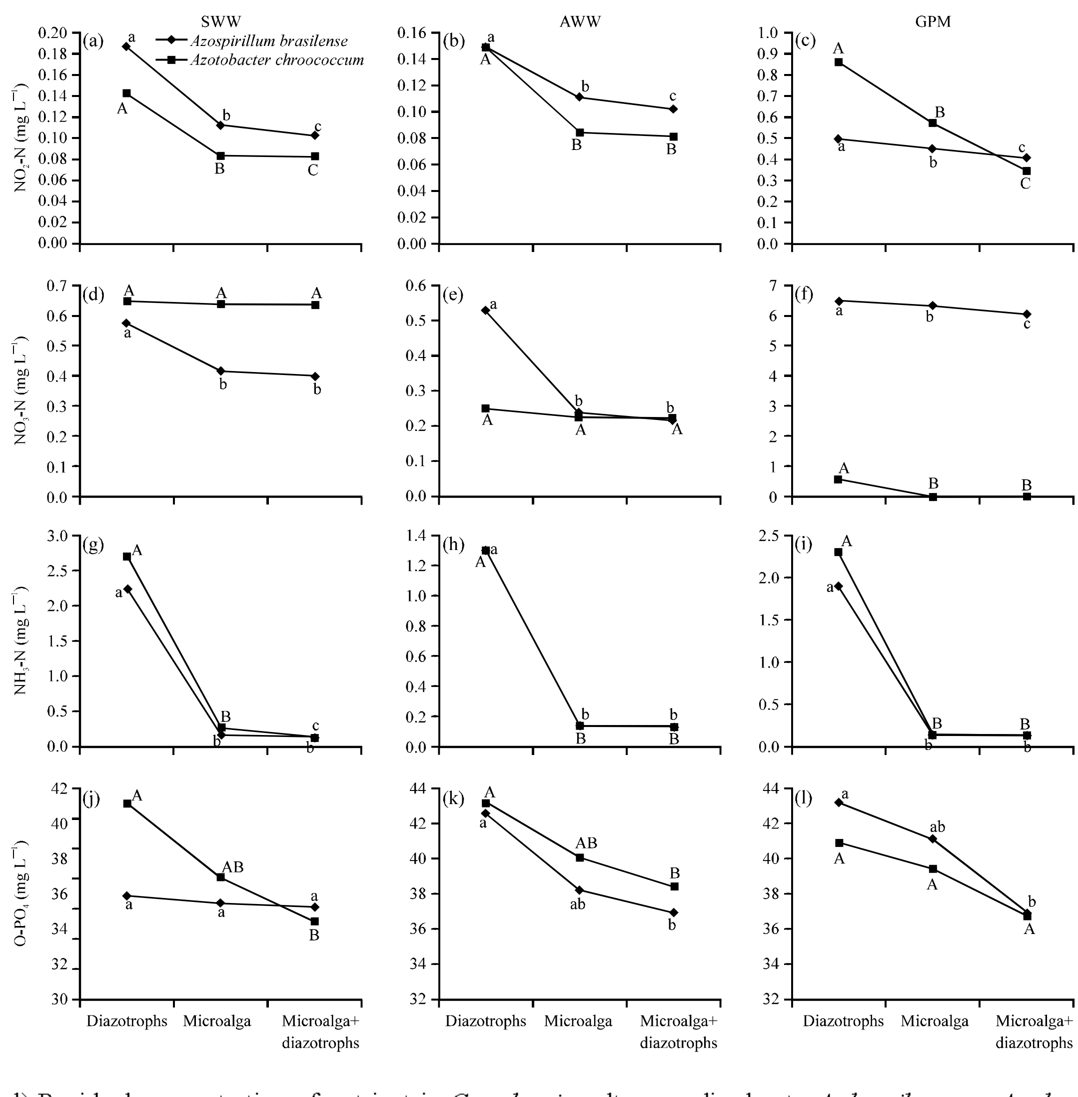

Fig. 4(a-l): Residual concentration of nutrient in *C. vulgaris* culture media due to *A. brasilense* or *A. chroococcum* inoculation after 7 days of incubation in the three batch cultures, SWW: Synthetic wastewater, AWW: Aquarium wastewater medium, GPM: Sterile enriched natural aquarium wastewater, Different letters differ significantly at $p \leq 0.05$

DISCUSSION

Bacteria and microalgae are ubiquitous and abundant microorganisms in aquatic environments; with both stimulative and inhibitory effects (Fukami *et al*., 1997). *C. vulgaris* is closely associated with the terrestrial plant-associative N_2-fixing bacteria (Gonzalez-Bashan *et al*., 2000). It is clearly noticed from the present study that the combination of the freshwater microalga *C. vulgaris* and a MGPB, *A. brasilense* or *A. chroococcum*, significantly increased microalgal pigments, thus reflecting the effect of MGPB on *Chlorella* population, this is in agreement with the results of Gonzalez-Bashan *et al*. (2000) and De-Bashan *et al*. (2002a), where bacteria often supply growth-promoting substances such as vitamins (Fukami *et al*., 1997) for phytoplankton. Also, *Azospirillum* species produce plant hormones, mainly auxins (indole-3-acetic acid), as do many other PGPB (Gonzalez-Bashan *et al*., 2000). In addition, the increase in pigment production, biomass, dry weight and bioremediation ability of microalgae when combined with diazotrophs in the present study can be explained by the fact that plant growth-promoting bacteria enhance bioremediation of wastewater treated with microalgae by increasing microalgal populations and metabolism (Patten and Glick, 1996; Costacurta and Vanderleyden, 1995). Concerning diazotrophs, *A. brasilense* recorded higher levels of microalgae pigments and lower removal of N-forms compared to *A. chroococcum* (Table 1), this reflects the higher ability of *A. chroococcum* in bioremediation, where it was used in industrial wastewater treatments e.g., alpechin (Hegazi *et al*., 2009) and baker's yeast effluent (Ali *et al*., 2005). Also, *Azotobacter* spp. is able to form capsular polysaccharides to entrap heavy metals (Pasetti *et al*., 1996) and have a mechanism for protecting their nitrogenase against high O_2 concentrations (Hegazi *et al*., 1984). Regarding the different wastewater media used in the present study, AWW recorded lower levels of microalgae pigments and higher removal of N-forms compared to other media (Table 1), this is because, the role of diazotrophs appear in the media of low combined nitrogen (Hernandez *et al*., 2009). Besides, the necessary nutrients existing in the GPM and SWW media did successfully support pigment production of the microalga but these increases in biomass of chlorella were not related to the decreases in nutrients, this reflects the role of diazotrophs in supporting both assimilation and uptake of nutrients of the microalga (De-Bashan *et al*., 2002b, 2004), this indicate that, AWW did not require additives to increase microalgae biomass then nutrient removal. On the other hand, the removal of phosphorus was poorer compared to N-forms in all treatments, this is in agreement with the results obtained by Hernandez *et al*. (2006). Also the increased phosphate and N-forms in the media containing diazotrophs is due to the ability of *A. brasilense* and *A. chroococcum* to fix nitrogen as well as the dead bacteria cells may leak phosphorus to the environment (Bashan, 1998).

CONCLUSION

The present findings strongly support the necessity of diazotrophs occurrence in the aquarium to secure the proper activity and ability of the freshwater microalgae *C. vulgaris* in bioremediation of the existing wastes. Also it increases the growth and biomass yield of *C. vulgaris* which represents a good food for fishes. This may minimize the periodical renewal of aquarium water and maintain the same water for longer time with available food and lower nitrogen and phosphorus. While this work needs more researches to be applied in the field to maintain water and face the water shortage.

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