Using diazotrophic bacteria for biomass production of microalgae

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ABSTRACT

Microalgae are necessary for aquaculture ponds, where they represent the natural nutritional base and the primary source of the food chain. They are also stabilizers of pond water quality. Therefore, in this study different concentration of *Azospirillum brasilense* or *Azotobacter chroococcum* were used as growth-promoting bacteria for biomass production of *Chlorella vulgaris* in the Bold's basal medium and in the ground water supplemented with various concentrations of aquaculture water. The obtained results indicated that, bacterial inoculation significantly increased chlorophyll a (>227 – 385%) in the Bold's basal medium for *Chlorella vulgaris*. Also in the ground water experiment inoculation did significantly increase net primary productivity, chlorophyll a and dry weight. Dissolved oxygen, chemical oxygen demand, nitrite, nitrate, ammonia and total dissolved solids were also increased as well. The obtained results support using diazotrophic bacteria as a growth promoter for increasing natural food. More studies should be done to be used to evaluate the use of such bacteria on a large-scale and decide how much can be included in the fish cultures.

Keywords: growth-promoting bacteria, *Azospirillum brasilense*, *Azotobacter chroococcum*, *Chlorella vulgaris*, net primary productivity, chlorophyll a

INTRODUCTION

Microalgae have an important role in aquaculture and play a vital role in stabilizing pond water quality via either ammonia uptake or oxygen production. Also, microalgae have an important role in fish feed. Microalgae are the bottom of the food chain in all aquatic ecosystems. They provide nutrients, essential amino acids, vitamins and energy, which are transferred through the food chain. Therefore microalgae are the main cereal food for the zooplankton and consequently for fish and other larvae.

There are some microalgae growth-promoting bacteria which are capable of generating and releasing some beneficial biofactors (De-Bashan *et al.*, 2004). Examples include *Phyllobacterium myrsinacearum*, *Azospirillum brasilense* and *Bacillus pumilus* (Gonzalez-

Bashan *et al*., 2000; Gonzalez and Bashan, 2000 and Hernandez *et al*., 2009). Therefore, researchers recommended the use of microalgae growth-promoting bacteria (*Azotobacter* and *Azospirillum*) as biofertilizers in aquaculture (Garg and Bhatnagar, 1999; Puente *et al.*, 1999; Tripathy and Ayyappan, 2005 and Ali *et al.*, 2011). The application of bio-fertilizers has become a necessary in the aquacultures because of the ever-increasing demand of the health conscious population of the world and the high cost of chemical fertilizers. Besides, bio-fertilization is alleviate deterioration of natural and environmental pollution.

Thus, this study was conducted to use *Azospirillum brasilense* or *Azotobacter chroococcum* bacteria to increase the growth of *Chlorella vulgaris*, and increase of productivity of ground water supplemented with various concentrations of aquaculture water.

MATERIALS AND METHODS

Microorganisms

The freshwater microalgae *Chlorella 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 diazotrophs were used; *Azospirillum brasilense* isolated from *Ricinus communis* and *Azotobacter chroococcum* 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^{o}C in a rotary shaker for 3 days for *Azospirillum brasilense* and 5 days for *Azotobacter chroococcum*. Bacterial cells were harvested by centrifugation at 7000 rpm for 15 min and washed twice with sterile solution (0.85% NaCl).

Growth medium

Two different media were used: 1) Bold's basal Medium (BBM) (Nichols, 1973) as a growth medium of *Chlorella vulgaris*.2) Ground water was collected from Faculty of Agriculture, Cairo University supplemented with various concentrations of aquaculture water (collected from aquaculture pond of Fish Farm Research Station, El-Qanater Elkhayrea) to increase nutrients as well as increase fauna and flora for ground water.

Growth conditions

Approximately 10^{8} bacterial cells of diazotrophs and batch culture technique (two hundred ml medium in 500 ml Erlenmeyer flasks) were used. In the first experiment standard growth medium of *Chlorella vulgaris* (BBM) was inoculated with the individual diazotroph strains with percentage additives (0,1,5 and 10% v/v). Samples for analysis were taken after 3, 5, 7 days of incubation. For the second experiment ground water was supplemented with aquarium wastewater by five concentrations (0,5,10,20 and 50 %v/v), each concentration was inoculated with *A. brasilense* or *A. chroococcum* in four concentrations (0,5,10 and 20%v/v). Incubation was done in a locally made controlled incubator at temperature of 25±1ºC and light intensity of 4000 lux under a day/night program of 14 h light followed by 10 h darkness for 7 days.

Determinations

1. Determination of biomass yield of the microalgae

Biomass yield of the microalgae was determined by measuring the microalgae pigment (chlorophyll a) and microalgae dry weight; spectrophotometric measurements of absorbance, at different wavelengths, were adopted for estimating the chlorophyll a (APHA, 1995). Chlorophyll a concentrations were calculated according Jeffrey and Humphrey (1975). 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 ºC for 24 h (APHA, 1995).

2. Determination of Physicochemical analyses and nutrient

Electrical conductivity (EC), pH and total dissolved solids (TDS) were measured using pH meter model JENWAY (4330). Dissolved oxygen (DO) was measured using the modified Winkler method (APHA, 1995). Chemical oxygen demand (COD) was carried out using the potassium permanganate method (Golterman and Clymo, 1971). Colorimetric methods were used to determine ammonia and nitrite (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).

RESULTS

Biomass production of *Chlorella vulgaris* on BBM media as affected by diazotrophic bacteria

Biomass yield (chlorophyll a and dry weight) of *Chlorella vulgaris* in the BBM medium was positively affected by the addition of various concentrations of diazotrophic bacteria (*A. brasilense* or *A. chroococcum*) (Fig. 1). Addition of *A. brasilense* to the culture medium of *Chlorella vulgaris* (BBM) significantly increased chlorophyll a content from 19 to 299 µgl^{-1}, 10 to 165 µgl^{-1}, and 13 to 169 µgl^{-1} in 1%, 5% and 10% v/v *Azospirillum* additives respectively. Also, addition of the *A. chroococcum* increased chlorophyll a content from 13 to 368 µgl^{-1}, 11 to 178 µgl^{-1}, and 16 to 168 µgl^{-1} in 1%, 5% and 10% v/v *Azotobacter* additives respectively,

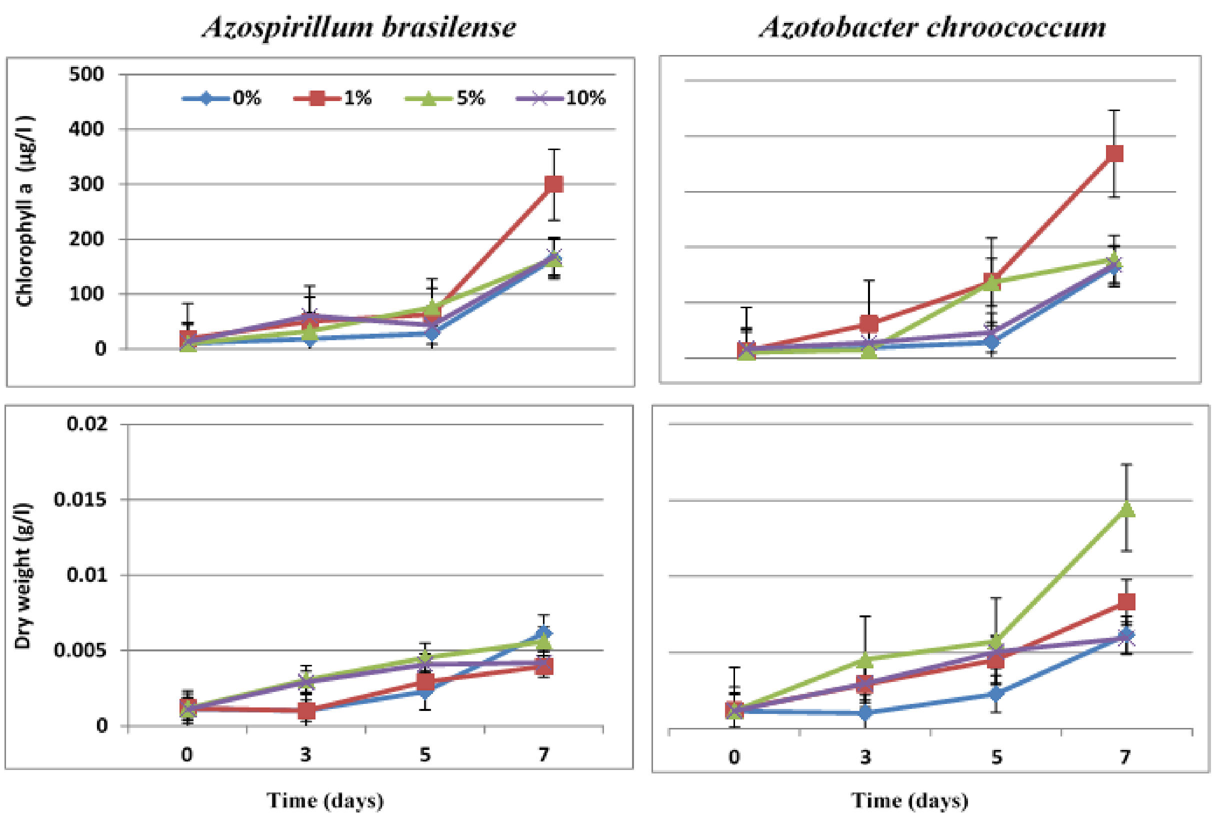

Fig. 1: The biomass yield (chlorophyll a and dry weight) of *Chlorella vulgaris* culture in the Bold's Basal medium as affected by *A. brasilense* or *A. chroococcum*. Bar whiskers represent SE.

compared to 11 to 165 µgl^{-1} in the culture without diazotrophic bacteria. In general, addition of diazotrophic bacterial dramatically improved biomass production of *Chlorella vulgaris*, especially the bacterial concentration of 1% which yielded the highest chlorophyll a 299 and 368 µgl^{-1} for *A. brasilense* and *A. chroococcum* respectively after 7 days incubation. Also, the results revealed that, there are significant differences between control and all other bacterial concentrations at the beginning of growth period (three and five days), while at the end (seven days) there are no significant differences between control and only 5% and 10%v/v bacterial concentrations (Fig. 1). The highest increase in percentage was recorded due to addition of *A. chroococcum* to the culture medium (385% on adding 1% after 5 days incubation) compared to *A. brasilense* (227% on adding 10% after 3 days incubation).

Dry weight of the microalgae *Chlorella vulgaris* confirmed the positive effect of addition of diazotrophic bacteria to the microalgae culture medium (Fig. 1). Where increases in dry weight ranged from 0.001 gl^{-1} at the beginning of experiment to 0.0039, 0.0056 and 0.0042 gl^{-1}

at the end of the experiment for 1%,5% and 10% v/v respectively in the case of adding *A. brasilense* and to 0.0083, 0.0145 and 0.0059 gl^{-1} in the case of addition *A. chroococcum* compared to 0.0062 gl^{-1} in the control. The highest percentage of increase in dry weight was recorded in the 5% v/v bacterial additives after 3 days incubation (204% and 353% for *A. brasilense* and *A. chroococcum* respectively).

In general, the obtained results supported the positive value of using diazotrophic bacteria as a growth factor for biomass production of *Chlorella vulgaris.* In addition, the results showed that, *A. chroococcum* recorded high biomass than *A. brasilense*. Economically, bacterial additives should not exceed 5%.

Effect of inoculation with diazotrophs on net primary productivity, chlorophyll a, dry weight and chemical properties of ground water supplemented with various concentrations of aquaculture water.

Changes in chlorophyll a, net primary productivity (NPP), dry weight and chemical properties of ground water supplemented with various concentrations of aquaculture water after being inoculated with diazotrophs were illustrated in Figures (2, 3). Generally, the various concentrations of diazotrophs increased chlorophyll a. Where, chlorophyll a ranged from 7.1 to 175.0 μgl^{-1} and 4.7 to 158.1 μgl^{-1} for *A. brasilense* and *A. chroococcum* treatments respectively, compared to 3.7 to 88.2 μgl^{-1} in the control. The highest significance ($p<0.05$) for chlorophyll a was reported with 20% inclusion in both microbial treatment. Also, there are no significant difference ($p>0.05$) in chlorophyll a in both microbial treatment (Table 1).

Net primary productivity (NPP) values recorded significant increase ($p<0.05$) in *A. brasilense* (53.8-426.3 $mgC/m^3/hr$) and in *A. chroococcum* (62.5-191.0 $mgC/m^3/hr$) compared to control (36.3-119.1 $mgC/m^3/hr$). Moreover, *A. brasilense* treatment recorded higher significant value ($p<0.05$) than *A. chroococcum* (Table 1).

Dry weight of samples was significantly affected by diazotrophs treatment ($p<0.05$). The increasing in dry weight ranged from 15 to 428% and 7 to 287% with *A. brasilense* and *A. chroococcum* treatments respectively. Dry weight increased with increasing bacterial concentrations, where the highest value was recorded in 20% v/v *A. brasilense* or *A. chroococcum* treatments. In addition, *A. brasilense* treatment recorded higher dry weight value ($p<0.05$) than *A. chroococcum* (Table 1).

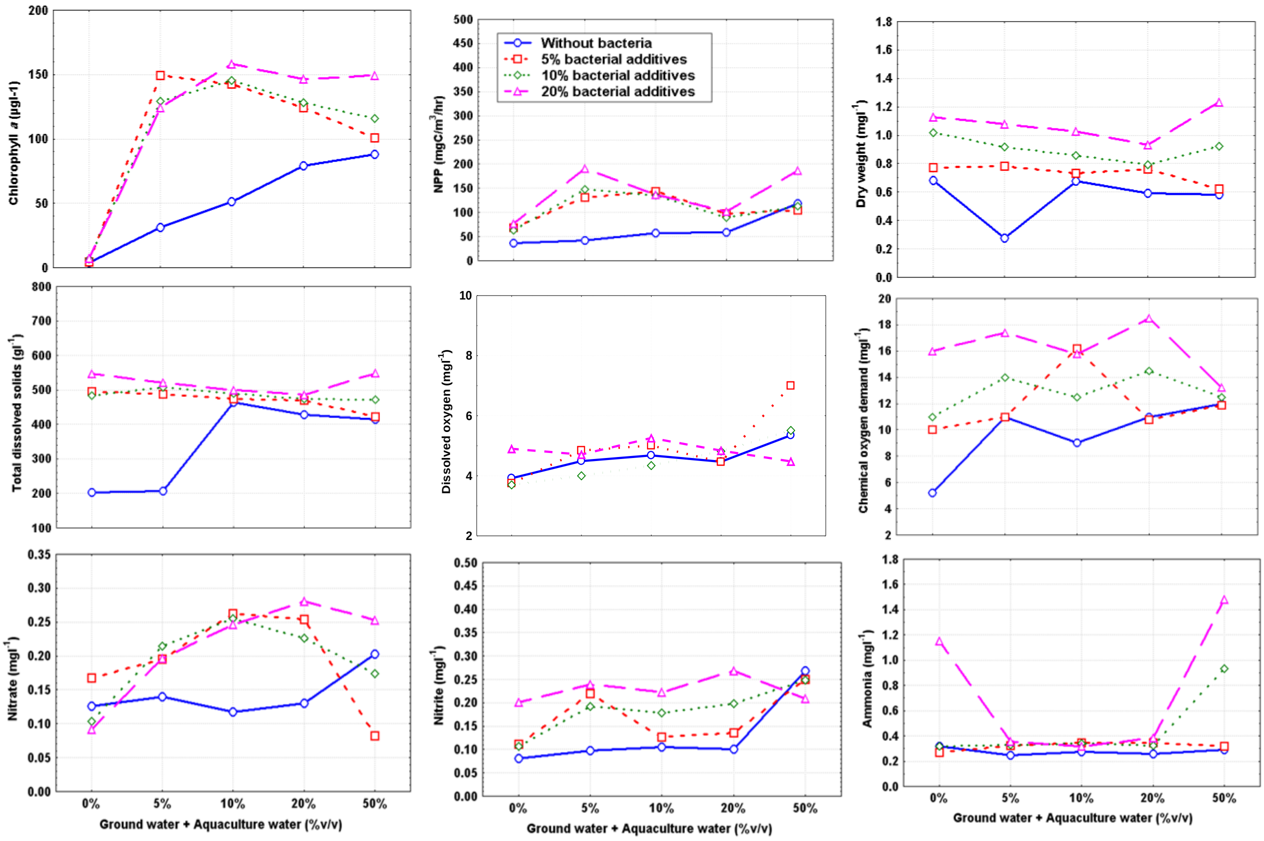

Fig. 2: Effect of inoculation of various concentrations of *Azotobacter chroococcum* on net primary productivity, chlorophyll a, dry weight and chemical properties of ground water supplemented with various concentrations of aquaculture water.

Inoculation of various concentrations of *A. brasilense*or *A. chroococcum* in water significantly increased total dissolved solids (TDS) and electric conductivity (EC). The increasing in TDS reached 226% and 170 % with *A. brasilense* and *A. chroococcum* treatments respectively. Also, the increasing in EC reached to 226% and 170% with *A. brasilense* and *A. chroococcum* treatments respectively. In general, TDS increased with increasing bacterial concentrations and the highest value was recorded in 20% v/v *A. brasilense* or *A. chroococcum* treatments. Moreover, *A. brasilense* treatment recorded higher TDS and EC value (p<0.05) than *A. chroococcum* (Table 1).

DO recorded significant increase (p<0.05) in *A. brasilense* treatments compared to control while there were no significant difference (p>0.05) among *A. chroococcum* treatments and control (Table 1). The highest DO values were recorded in 20% of *A. brasilense* (8.91 mgl^{1}).

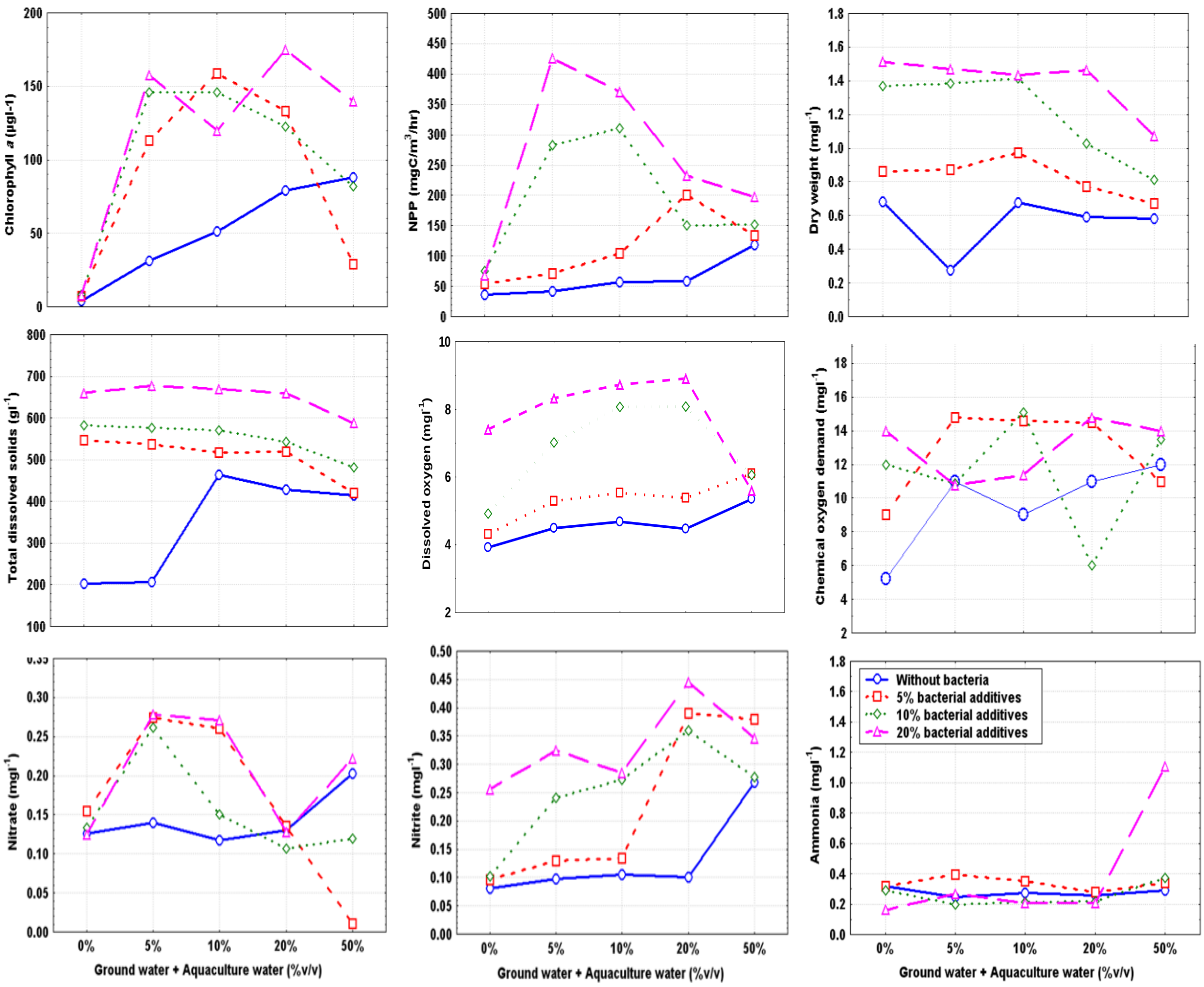

Fig. 3: Effect of inoculation of various concentrations of *Azospirillum brasilense* on net primary productivity, chlorophyll a, dry weight and chemical properties of ground water supplemented with various concentrations of aquaculture water.

Diazotrophic bacteria inoculation treatments recorded significant increase in COD (Table 1), where the increase in COD reached 169% and 208% with *A. brasilense* and *A. chroococcum* treatments respectively. In general, *A. chroococcum* treatments recorded the highest significant increase in COD (Table 1).

Both bacterial treatment showed significant increase ($p<0.05$) in nitrite compared to control. The highest bacteria concentration recorded higher percentage increase of nitrite, which reached 345% and 168% with *A. brasilense* and *A. chroococcum* treatments respectively. In general, *A. brasilense* treatments recorded the highest increase in nitrite compared to *A. chroococcum* treatments (Table 1).

Table 1: Statistical analysis of the effect of strains and bacterial additives (percentage v/v) on dry weight, chlorophyll a, net primary productivity and chemical analyses of ground water supplements with aquaculture water.

Parameters	Dry weight mgl-1	Chlo[1] µgl-1	NPP[2] mgC/m3/hr	COD[3]	DO[4]	NO_2-N	NO_3-N	NH_3-N	EC[5] dSm-1	TDS[6] gl-1
				mgl-1						
Effect of strains (Rao R (240,589)=20.77; *p*<0.000)										
Without bacteria	0.5611 c	50.71 b	62.57 c	9.64 c	4.58 b	0.130 c	0.143 b	0.277 c	0.572 c	343.3 c
A. brasilense	1.1406 a	103.04 a	188.67 a	12.43 b	7.87 a	0.269 a	0.175 b	0.330 b	0.928 a	569.9 a
A. chroococcum	0.9052 b	108.86 a	118.86 b	13.69 a	4.77 b	0.194 b	0.200 a	0.503 a	0.819 b	491.6 b
Effect of bacterial additives (Rao R (240,589)=20.77; *p*<0.000)										
0%	0.5611 d	50.7 c	62.6 d	9.64 c	4.58 d	0.130 c	0.143 b	0.277 c	0.572 c	343.3 d
5%	0.7815 c	96.3 b	111.0 c	12.38 b	5.17 c	0.197 b	0.180 ab	0.329 b	0.815 b	488.8 c
10%	1.0513 b	103.0 b	151.6 b	12.20 b	6.52 b	0.218 b	0.174 ab	0.355 b	0.864 b	518.0 b
20%	1.2359 a	118.6 a	198.7 a	14.60 a	7.28 a	0.280 a	0.209 a	0.565 a	0.942 a	585.5 a

Means at the same column with the same alphabetical letters are not significantly different (*P*>0.05);[1] chlorophyll a; [2]net primary productivity; [3]chemical oxygen demand; [4]dissolved oxygen; [5]electrical conductivity; [6]total dissolved solids.

On the contrary, *A. chroococcum* treatments recorded higher nitrate than *A. brasilense* treatments, and *A. brasilense* treatments showed no significant differences compared to control (Table 1).

In respect to ammonia, all bacterial treatments showed significant increase ($p<0.05$) in ammonia compared to control. In addition, *A. chroococcum* treatments recorded higher ammonia values than *A. brasilense* treatments (Table 1). In general, the highest ammonia value was recorded with the highest percentage bacterial additives (20% v/v), while there are no significant differences between 5% and 10% v/v bacterial concentrations (Table 1).

DISCUSSION

At present, the application of biofertilizers is very important for the safety of the environment against environmental damage that occurs during the use of chemical fertilizers, in addition to the high economic value of being of a low-cost. Application of N_2-fixing bacteria as a biofertilizers in soil (Hamza *et al*., 1994 and Ali *et al*., 2005) and in aquaculture (Tripathy and Ayyappan, 1998 and 2005; Ali *et al*., 2011) is efficient and generates value-added products. In addition N_2-fixing bacteria used as microalgae-growth promoting bacteria, MGPB, (Gonzalez-Bashan *et al*., 2000). In this study addition of *A. chroococcum* or *A. brasilense* to culture medium (BBM) of *Chlorella vulgaris* has significantly increased growth and biomass of *Chlorella vulgaris*, while addition of diazotrophs increased chlorophyll a and dry weight of *Chlorella vulgaris*, especially in the beginning of the *Chlorella vulgaris* growth. This reflects the ability of these bacteria to produce various phytohormones and vitamins (Fukami *et al*., 1997). Our results agree with Gonzalez and Bashan (2000) who used *A. brasilense* for enhancement of the growth of *Chlorella vulgaris*. Also, in this study using N_2-fixing bacteria with ground water supplemented with different concentrations of aquaculture water increased chlorophyll a, net primary productivity and dry weight of the water samples, this is reflects the role of diazotrophic bacteria for increasing productivity of aquaculture. This comes in accordance with Tripathy and Ayyappan (1998) who used N_2-fixing bacteria in aquaculture. The results of this study indicated that, irrespective of bacterial additives, *A. brasilense* recorded higher significant values for dry weight, chlorophyll a, NPP, DO, nitrite, EC and TDS compared with *A. chroococcum*. This may be because *Azotobacter* spp. has the highest rate of respiration and efficient oxidation of organic compounds (Hegazi *et al*., 1984), there is also demonstrations of increased ammonia in *A. chroococcum* treatments. Moreover, *Azotobacter* spp. have the ability to biodegrade materials, this is explains highest increase in COD for *A. chroococcum* treatments. In addition, increasing bacterial concentration increased productivity of water. This may be because low content of nutrients with high concentration of diazotrophic bacteria promotes growth of existent microalgae and consequently growth of existent fauna, this is caused rise of organic carbon and increased productivity of water. It is clear that increasing supplementation of ground water with high concentration of aquaculture water causes high amount of total organic carbon and soluble nitrogen which are inappropriate environmental conditions for diazotrophic bacteria (Hernandez *et al*., 2009).

CONCLUSION

Results indicated that *A. chroococcum* and *A. brasilense* were successfully used as growth factors for increasing *Chlorella vulgaris* biomass as well as increased natural foods for fish production in the safety and healthy scale. Besides, *A. chroococcum* and *A. brasilense* successfully increased productivity in the ground water supplemented with low amount of aquaculture water. Further investigations are needed for evaluation of using diazotrophic bacteria on a large scale especially in fish ponds running by ground water and deciding how much can contribute in the fish culture.

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