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A biotic Stress Induced production of β- carotene, Allophycocyanin and Total Lipids in *Spirulina* sp.

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Abstract

The present study examines the possibility of increasing the levels of some bioactive compounds (β-carotene, phycopiliprotein and total lipids) in *Spirulina* sp. isolated from Wadi El Natron lake (Egypt) and cultivated in modified Zarrouk medium under standard growth condition and abiotic stress including change in culturing condition as (pH, Temperature, light intensity), nutrient limitation (-N, -P, -S) and salinity. The yield production of β-carotene was enhanced with 0.9M NaCl, phosphorus deficiency and at pH 6. Total lipids were also enhanced in all conditions under tested especially with 0.9M NaCl, light intensity 14.52 µmol.m^{-2}.s^{-1}. Phycobiliproteins fractions (phycocyanin, allophycocyanine and phycoerythrine) were elevated with 0.3, 0.6M NaCl as well as phosphorus, nitrogen and sulphur deficiency. Antioxidant enzymatic activities of Spirulina sp. were elevated with all studied a biotic stress factor.

Key words: Allophycocyanin, β-carotene, lipids, *Spiurlina,* Antioxidant enzymes.

Introduction

Cyanobacteria have recently gained increasing interest as microorganisms because they are believed to be an attractive source of various bioactive substances, such as polyunsaturated fatty acids, β-carotene and other phytopigments (carotenoids, chlorophyll and phycocyanin) that function as antioxidants (Wang *et al*., 2007) ,polysulfated polysaccharides as antivirals (Ghosh *et al*., 2004), sterols as antimicrobials (Prakash *et al*. 2010) and mycosporine-like amino acids (MAAs) and scytonemin as photoprotectants (Rastogi & Sinha 2009). Phycobiliproteins (PBPs) are the unique photosynthesis pigments of this group and are water soluble pigments that can be divided into three types according to their maximum absorbance: phycocyanin (PC), phycoerythrin (PE) and allophycocyanin (APC) (Glazer *et al*., 1976; Yamanaka *et al*., 1978).

Cyanobacteria, especially *Spirulina*, a blue green microalga, have been used since ancient times as a source of food because of its high nutritional value (Dillon *et al.* 1995). The cyanobacterium *Spirulina platensis* is rich in nutrients, such as proteins, vitamins, minerals, carbohydrates, and γ-linolenic acid. It is gaining more and more attention, not only for the foods aspects but also for the development of potential pharmaceuticals (Quoc & Pascaud 1996) and medicinal properties; thus, several studies have shown that *Spirulina* or its extracts could prevent or inhibit cancer in humans and animals, and has immuno-promoting effects (Qureshi & Ali 1996; Hirahashi *et al*. 2002; Subhashini *et al*. 2004)

Spirulina is found in soil, marshes, freshwater, brackish water, seawater and thermal springs. Alkaline, saline water (>30 g/l) with high pH (8.5–11.0) favour good production of *spirulina*, especially where there is a high level of solar radiation at altitude in the tropics. *Spirulina platensis* and *Spirulina maxima* thrive in highly alkaline lakes where the cyanobacteria population is practically monospecific (Sasson, 1997).

Spirulina appears to have considerable potential for development, especially as a small-scale crop for nutritional enhancement, livelihood development and environmental mitigation. In particular, the production and use of *spirulina* has the following advantages: it provides an easily digestible high (c. 60 percent) protein product with high levels of β-carotene, vitamin B12, iron and trace minerals (Costa *et al*., 2004) and the rare essential fatty acid γ-linolenic acid (GLA). In addition, it has no obvious negative cultural or religious issues associated with its consumption; its production occupies only a small environmental footprint, with considerable efficiencies in terms of water use, land occupation and energy consumption when compared to traditional terrestrial crops; its production can be conducted at a number of different scales, from household "pot culture" to intensive commercial development over large areas; it has the potential for integration with rural organic waste treatment processes to improve both environmental conditions and improve energy transfer efficiencies (Habib *et al*., 2008).

Phycocyanin is a biliprotein pigments of the blue green algae like *Spirulina* with a variety of pharmacological properties such as antioxidant, anti-inflamatory, neuro and hepatoprotective (Romay *et al*., 2003) and antitumor activities (Liu *et al*., 2000,2005 & Pardhasaradhi *et al*., 2003). Phycocyanin stimulates the antioxidant enzymatic defence systems to modulate

the early radiation response, therefore phycocyanin may be of interest in the radioprotection of subjects exposed to low doses of radiations (Ivanova *et al.*, 2010).

Cyanobacteria may contain significant quantities of lipids with the composition similar to those of vegetable oils (Singh *et al*. 2002). The lipids of some cyanobacterial species are rich in essential fatty acids such as linoleic 18:2n6 and α-linolenic 18:3n3 acids and their C20 derivatives, eicosapentaenoic acids 20:5n3 and arachidonic acids 20:4n6 (Othes & Pire 2001). Some of the filamentous cyanobacteria tend to contain large quantities (20–60% of the total of fatty acids) of polyunsaturated fatty acids (PUFA) (Parker *et al.* 1967; Holton & Blecker 1972; Kyle *et al*. 1992). Where microalgae can be cultured, PUFA in algae have profound benefits and functions in dietetics and therapeutic uses (Carlson & Salem 1991; Innis 1991; Ogata *et al*. 1996; Otheş & Pire 2001). They are believed to have positive effects for the treatment of hypertension, premenstrual tension, various atopic disorders, diabetes and a number of other cases (Roughan 1989; Saito *et al.*, 1992).

The objective of this work is to investigate in more details the effect of abiotic factors as nutrient deficiency (Nitrogen, Phosphorus and Sulphur), change in culture condition as (Light intensity, Temperature and pH) and salinity stress take into our consideration on growth and other metabolic activities of *Spirulina.* To establish a particular cultivation strategy for enrichment of the biomass with some specific added value compounds (phycocyanin, β-carotene and/or fatty acid production) and some antioxidant enzymes from *Spirulina* sp. under testing.

2. Materials and methods

2.1 Microorganism and culture media

In this investigation *Spirulina* sp. was isolated from Wadi El-Natron salt lake (Egypt). *Spirulina* sp. was grown in modified Zarrouk's medium (Zarrouk, 1996) which was used for culture maintenance and to prepare the inoculums as well as to study the growth of *Spirulina* sp. in batch culture.

2.2 Cultivation

Cultivation was done in 500ml Erlenmyer flask and the cultures were gassed with sterile air provided by a small air pump operating at a rate of 0.046 vvm (volumetric flow rate of air per volume of liquid per minute), at 30˚C. The pH of the medium was adjusted to pH 9.00 prior to autoclaving (controlled pH culture). The cultivated flasks were illuminated 24 h with continuous cool white fluorescent lamp at 400 W (philips) (48.4 µ mole. $m^{-2}.s^{-1}$). The concentration of KH_2PO_4 (the phosphorus source), $NaNO_3$ (the nitrogen source), and K_2SO_4, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$ (the sulphur sources) in the medium being modified according to the experimental design explained below in section 2.3.

2.3 Experimental design

Nutrient deficiency: for this study a multilevel factorial design was used in which the concentrations of KH_2PO_4 in Zarrouk's medium were 0.5, 0.25, 0.125, 0.0 g/l for control, -50%, -75%, -100% P respectively, $NaNO_3$ concentrations in were 2.5, 1.25, 0.625 and 0.0 g/l for control, -50%, -75%, -100% N, respectively, the concentrations of K_2SO_4 were 1.0, 0.5, 0.25 and 0.0 g/l, the concentrations of $MgSO_4.7H_2O$ were 0.2, 0.1, 0.05, 0.0 g/l and the concentration of $FeSO_4.7H_2O$ were 0.01, 0.005, 0.0025, 0.0 g/l for control, -50%, -75%, -100% S respectively. In case of -100%S $MgSO_4.7H_2O$ was replaced by $MgCl_2$. Cultures were grown in different concentrations of NaCl (control, 0.3M, 0.6M and 0.9M). The prepared media were adjusted at different pH values at ((control 9), 6, 7, and 11). The temperature was adjusted at ((control 30˚C), 15 ^oC and 40^oC). The flasks were incubated at different light intensity levels ((control 48.4), 27.83 and 14.52 µmol.photon.$m^{-2}.s^{-1}$).

2.4 Growth measurements

The growth of the Spirulina sp. was daily followed by determination of optical density and/or chl. *a* content. In the late of exponential or beginning of the stationary phase according to the method described by Metzner *et a*l. (1965) and Marker (1972). The algal cells were harvested for some metabolic estimation. For determination of dry weight, 10 ml of algal suspension, after filtered through Whatman (GF/F) glass fiber paper, was dried in oven for 24 h at 80˚C, while filtering 20 ml of phosphate buffer (KH_2PO_4 0.01M, pH 7) was added to remove insoluble salts, the filter paper was then put in the oven, after cooling the filter paper was reweighed to evaluate the dry matter (mg/ml algal suspension).

Determination of growth rate by optical density

Optical density of algal suspension were measured at 560nm (Fatma *et al.*,1994) and the growth rate µ (h^{-1}) was determined from the following formula:

$$\mu\ (h^{-1}) = \frac{\ln A_1 - \ln A_0}{t_1 - t_0}$$

Where: A_1 = Optical density at time T_1.

A_0= Optical density at time t_0.

t_1-t_0 = The time elapsed in days between two determinations of optical density.

The generation time (G) can be calculated as follows:

$$G = \frac{\ln 2}{\mu}\ h^{-1}$$

Derermination of phycobiliproteins (phycocyanin, allophycocyanin and phycoerythrin)

Phycobiliproteins contents were determined according to the method described by Bennett and Bogorad (1973). Fifty ml of *Spirulina* culture were centrifuged at 4000 rpm and the pellet was resuspended in 20 ml of sterile distilled water. The quantitative extraction of biliproteins was achieved by combination of prolonged freezing and sonication, followed by centrifugation at 4000 rpm for 20 min. The crude extract was completed to 50 ml distilled water. The concentration of c-phycocyanin, phycoerythrin and Allophycocyanin (mg/g D.wt.) in crude extracts was calculated by measuring the absorbance at 615, 652 and 526 nm, respectively using the following equations:

$PC = (E_{615} - 0.476\ E_{652}) / 5.34$ PC= c-phycocyanin

$APC = (E_{652} - 0.208\ E_{615}) / 5.09$ APC= allophycocyanin

$PE = E_{526} - 2.4\ (PC) - 0.849\ (APC) / 9.62$ PE= phycoerythrin

Total phycobiliproteins = PC+APC+PE

Estimation of Antioxidant enzymes

Preparation of enzyme extract and assay of enzyme activity

Hundred ml of algal culture were centrifuged at 5000 rpm and the pellet was homogenized in 5 ml of 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM ethylenediamine tetraacetic acid (EDTA) and 0.1 g polyvinylpyrrolidone (PVP) by combination of prolonged freezing and sonication. The homogenate was centrifuged at 18000 rpm for 10 min. at 4^{o}C and the supernatants were collected and used for assays of catalase, ascorbate peroxidase and guaiacol peroxidase. All colorimetric measurements (including enzyme activities) were made at 20^{o}C using Unico UV-2100 spectrophotometer. The specific activity was expressed as units/mg protein. Protein concentrations in the enzyme extract were determined by the method of Lowry *et al*. (1951).

Catalase

Catalase (CAT) activity was assayed by following the consumption of H_2O_2 for 1 min according to Aebi (1984) and Matsumura, *et al*. (2002).

Guaiacol peroxidase

Guaiacol peroxidase (POD) activity was measured spectrophotometrically following the method of Tatiana, *et al*., (1999) with some modifications. The reaction mixture (3 ml) consisted of 30 mM potassium phosphate buffer (pH 7), 6.5 mM H_2O_2 and 1.5 mM guaiacol. The reaction was started by the addition of 100 µl enzyme extract. The formation of tetraguaiacol was measured at 470 nm.

Ascorbate peroxidase

Ascorbate peroxidase (APX) activity was determined according to the method of Nakano and Asada (1981) with some modifications. The activity was determined by recording the decrease in A_{290} for 1 min. in 3 ml of reaction mixture containing 50 mM potassium phosphate buffer (pH 7), 0.5 mM ascorbic acid, 0.1 mM EDTA, 100 µl 0.1 mM H_2O_2 and 100 µl enzyme extract. The reaction was started with the addition of H_2O_2. Absorbance was measured at 290 nm in Unico UV-2100 spectrophotometer.

Determination of Total lipids

Total lipid contents were determined by the sulfophosphovanilin method (SPV) Drevon and Schmitt (1964).

Results and discussions

The growth of *spirulina* sp. expressed as daily change in chl.a content (µg/ml) were inhibited by all a biotic factors applied, (temperatures; 15˚C& 40˚C, light intensities; 14.52, 27.83 µmol photon.m^{-2}.s^{-1}, pH values (6,7,11) and with limitation of nitrogen, phosphorus and sulfur in growth medium), as well as high salinity (Fig. 1). The highest inhibitory effect of these a biotic factors were recorded when culture grown without nitrogen and at high level of salinity, while the lowest level was exerted when sulfur from the growth medium was omitted and at pH 6 and 7 in comparison to control cultures. In this respect, Ayachi *et al*. (2007) found that an increase of NaCl concentration caused reduction of growth of *Spirulina* sp. and total inhibition of chlorophyll biosynthesis. Vonshak *et al.* (1988) attributed this growth decrease to an energy shortage caused by pumping out the entering sodium ions and by the synthesis of sugar as osmoticum.

The maximum growth rate and the minimum generation time were recorded at culture grown at pH 6.0 and/ or (-50% P), while the best biomass were recorded when the culture were grown at 30˚C, at high light intensity 48.4 µmol.photon.m^{-2}.s^{-1} and in the presence of (0.05 g/l $MgSO_4$ and 0.0025 $FeSO_4$) as sulfur source (reduction of 75 % of sulfur corresponding to the original concentration of sulfur in Zarrouk's medium). High salinity (0.9M NaCl), completely removed nitrogen source from medium and growth at 15˚C caused severely drop in biomass (dry wt.) of *Spirulina* Table (1). Rafiqul *et al.* (2003) found that maximum specific growth rate of *Spirulina platensis* and *Spirulina fusiformis* was found at 32˚C and 37˚C respectively, both species showed negligible growth at 20˚C and 40˚C.

The yield of phycobiliprotein fractions were recorded in Fig. (3) and it was found that the high yield of phycobiliprotein content were recorded at culture grown in nitrogen free Zarrouk's medium at pH 9.00, under light intensity (48.4 µmol.photon.$m^{-2}s^{-1}$) which incubated at 30˚C. Fatma (2009) found that the optimum temperature for phycobiliproteins was obtained at 30˚C, there was 23.6 % decrease at 20˚C and 38% at 40˚C.Increasing pH (7-9) significantly increased the total phycobiliproteins content in *Nostoc* sp. In *Anabaena* 7120 the amount of phycobiliprotein exceeded in nitrogen-free media than nitrate grown culture (Loreto *et al.*, 2003). UAM206 (Poza-carrion *et al.*, 2001). *Anabaena* NCCU-9 produced highest amount of phycobiliprotein under nitrogen free environment (Hemlata and Fatma 2009).

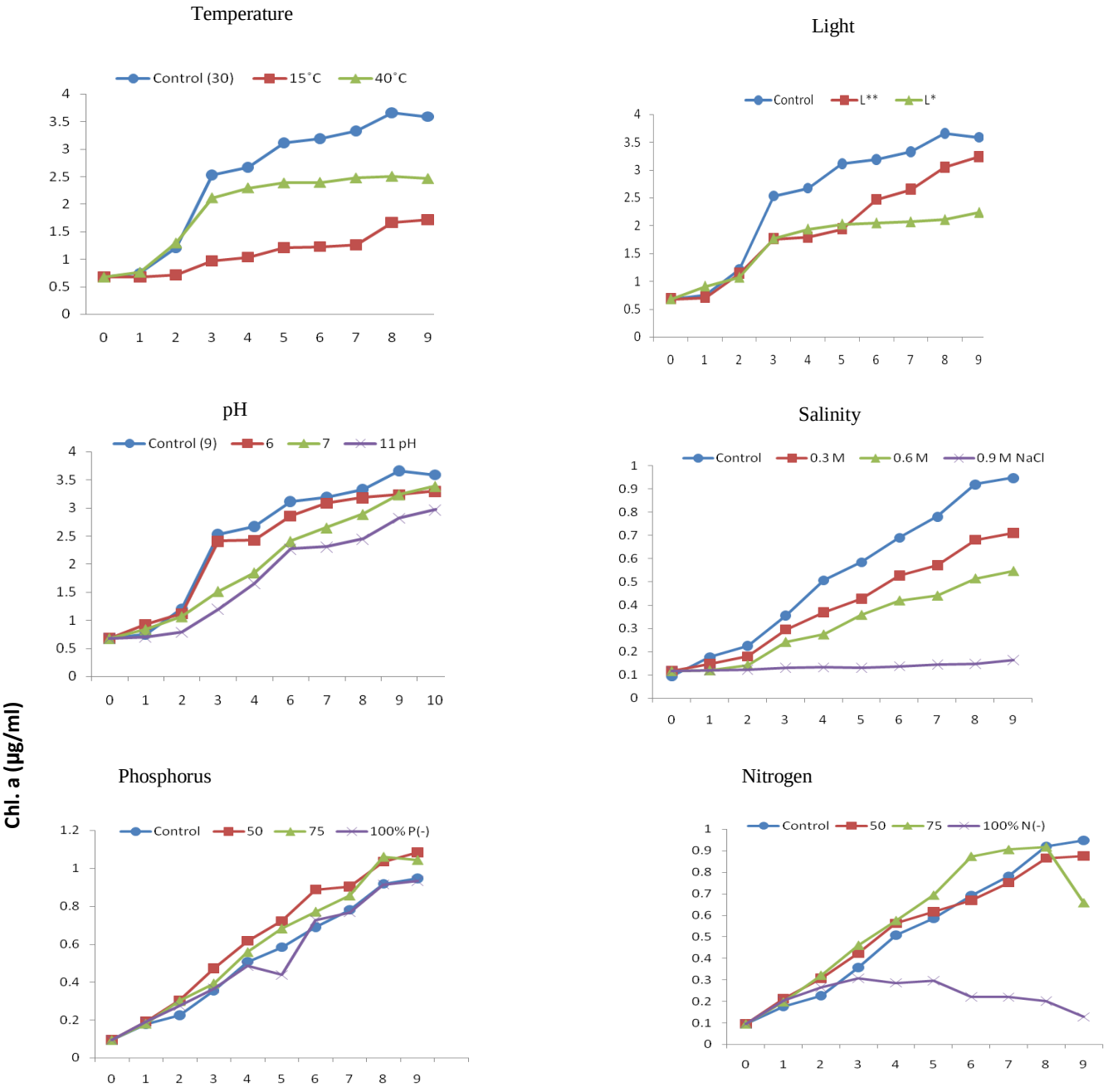
Production of β-carotene of *Spirulina* sp. under testing was stimulated when the culture grown in the presence of 0.9 M NaCl. Absence of phosphorus source from Zarrouk's medium caused significantly increase of β-carotene content while deficiency of nitrogen, decreasing light intensities and temperature or pH caused decrease of β-carotene content (Fig. 2). Incharoensakdi and Phunpruch (1996) found that nitrate deficiency in the growth medium did not cause an increase in β-carotene content, while increasing NaCl in the medium caused an increase in β-carotene content of *Spirulina platensis*.

The effect of 100% nitrogen deficiency and change of light intensities on the lipid content of *Spirulina* sp. under testing were shown in Fig. (2) and found that nitrogen deficiencies and/or lowered light intensity caused accumulation of total lipids contents. Tedesco and Duerr, (1989) indicated that the lack of nitrogen in *S. platensis* culture medium increased total lipid ratio. Olguin *et al.* (2001) cultured *S. platensis* to determine the total content of lipids in two culture media (Zarrouk and complex medium) at different light intensities, the researchers found that total lipid content increased up to 28.6% at low light intensity and to 18% at high light intensity in complex medium which contained 10 times less of nitrogen concentration. Uslu *et al*. (2011) found that nitrogen deficiency (50 and 100% (-N)) in *Spirulina* culture medium increased total lipid ratio (13.60 and 17.05%). Other researchers worked on other algae and found that the reduction of nitrogen content alleviated the lipid content of algae as; Zhila *et al*. (2005) on *Botryococcus braunii*, Pruvost *et al*. (2009) on *Neoochloris oleobudas* and Bulut (2009) on *Chlorella vulgaris.*

The activity of all antioxidant enzymes (catalase, peroxidase, and guiacol peroxidase) were increased under all biotic factors and stress condition in comparison to control (Fig. 2). *Spirulina* a whole spectrum of natural mixed carotene and xanthophyll phytopigments which together with phycocyanin seem to be related to antioxidant activities (Miranda *et al.*, 1998, Bhat and Madyastha, 2000 and Pineiro Estrada *et al.*, 2001). Ramadan *et al*., (2008) said *that S. platensis* gives

considerable yield of lipids which are rich sources of essential fatty acids and lipid soluble antioxidants; moreover the recovered lipid may be suitable for commercial exploitation as a source of lipids for food use and cosmetics production. It has been recorded that polyunsaturated fatty acids, phycobiliproteins, β-crotene and other pigments function as antioxidants (Reddy et al., 2000, Estrada *et al*., 2001). Phycobiliproteins have been described as the strongest antioxidant such as in cases of PC in the studies of Bhat and Madyasth (2000), Benedetti *et al*., (2004) and Huang *et al*. (2007); APC in the studies of Ge *et a*l. (2006) and PE in the studies of Yuan *et al*. (2005), therefore PBPs have gained a somewhat high level of expectation as a potential source of bioactive substances.

Nitrogen deficiencies increased total lipids content; however biomass productivity did not fall significantly. For the feasible biodiesel studies with microalgae, the issue should determine the algal species which are resistant to environmental conditions, high biomass and high lipid productivity. There are some microalgae species having more lipid content but their low biomass productivity will restrict their large scale production for biodiesel. Addition of 0.9 M NaCl stimulate excess production of β-carotene, accumulation of total lipids and caused an increase in the antioxidant enzyme activities such as (Catalase, peroxidase, ascorbate peroxidase) (Fig. 2). In conclusion *Spirulina* can elevate the activity of all the antioxidant enzymes viz., superoxide dismutase, catalase, peroxidases and glutathione reductase , the effect may be due to the high phytopigments (carotenoids , Chlorophyll, phycocyanin). Therefore make *Spirulina* sp. under investigation good candidates for successful cultivation in artificial open ponds and/or in Wadi El Natron lake under different environmental condition as a high value health foods, functional food and as a source of wide spectrum of nutrient "especially these anticancer substances as β-carotene" or can be used as a model for biotechnological production of antioxidant enzymes.



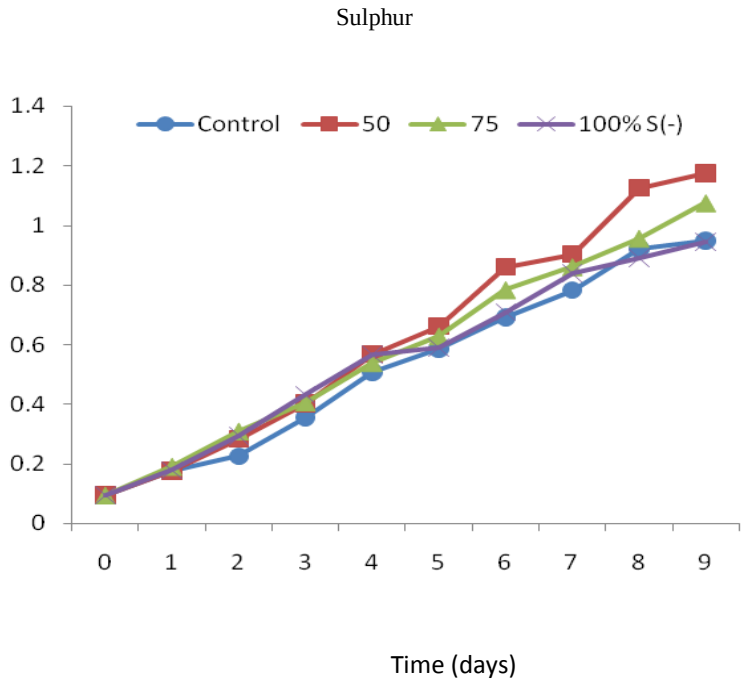


Fig. (1): Growth curves of *Spirulina* sp grown in Zarrouk's medium under a biotic stresses.

Table (1): Effect of a biotic stresses on growth criteria of *Spirulina* sp.

	Temperature				**Light**					**pH**					**Salinity**				
	Chl. a ($\mu g.ml^{-1}$)	**μ_{max} (h^{-1})**	**G (h^{-1})**	**Dry wt. gm/l**		**Chl. a ($\mu g.ml^{-1}$)**	**μ_{max} (h^{-1})**	**G (h^{-1})**	**Dry wt. gm/l**		**Chl. a ($\mu g.ml^{-1}$)**	**μ_{max} (h^{-1})**	**G (h^{-1})**	**Dry wt. gm/l**		**Chl. a ($\mu g.ml^{-1}$)**	**μ_{max} (h^{-1})**	**G (h^{-1})**	**Dry wt. gm/l**
C. (30°C)	3.59 ±.07[c]	0.031	22.4	0.27	**C. (48.8)**	3.59 ±0.07[b]	0.031	22.4	0.27	**C. (9)**	3.59 ±0.07[b]	0.031	22.4	0.27	**C. (0.017 M)**	3.11 ±0.15[c]	0.031	22.2	0.23
15°C	1.72 ±0.01[a]	0.013	55.4	0.105	**L***	3.24 ±0.16[b]	0.02	33.9	0.184	**6**	3.3 ±0.13[ab]	0.032	21.6	0.203	**0.3M**	1.9 ±0.01[b]	0.021	33.2	0.11
40°C	2.47 ±0.08[b]	0.02	33.9	0.264	**L****	2.24 ±0.08[a]	0.021	33.2	0.148	**7**	3.39 ±0.14[b]	0.015	46.7	0.224	**0.6M**	1.89 ±0.14[b]	0.028	24.8	0.09
F	234				**F**	40.8				**11**	2.97 ±0.04[a]	0.018	39.3	0.282	**0.9M**	0.6 ±0.02[a]	0.003	243	0.05
										F	5.9				**F**	98.7			

	Phosphorus				**Nitrogen**					**Sulphur**				
	Chl. a ($\mu g.ml^{-1}$)	**μ_{max} (h^{-1})**	**G (h^{-1})**	**Dry wt. gm/l**		**Chl. a ($\mu g.ml^{-1}$)**	**μ_{max} (h^{-1})**	**G (h^{-1})**	**Dry wt. gm/l**		**Chl. a ($\mu g.ml^{-1}$)**	**μ_{max} (h^{-1})**	**G (h^{-1})**	**Dry wt. gm/l**
C.	3.11 ±0.15[a]	0.031	22.2	0.23	**C.**	3.11 ±0.15[c]	0.031	22.2	0.23	**C.**	3.11 ±0.15[b]	0.031	22.2	0.23
50%	3.13 ±0.14[a]	0.033	20.9	0.263	**50%**	1.76 ±0.01[b]	0.014	50.1	0.212	**50%**	3.17 ±0.06[b]	0.021	33.4	0.255
75%	2.68 ±0.14[a]	0.018	39.3	0.254	**75%**	1.59 ±0.19[b]	0.025	27.4	0.159	**75%**	3.02 ±0.2[b]	0.014	50	0.261
100%	2.77 ±0.18[a]	0.025	27.7	0.227	**100%**	0.29 ±0.03[a]	0.017	41.6	0.11	**100%**	2.03 ±0.07[a]	0.014	50	0.229
F	2.27				**F**	88.2				**F**	15.4			

ISSN ophycocyanin

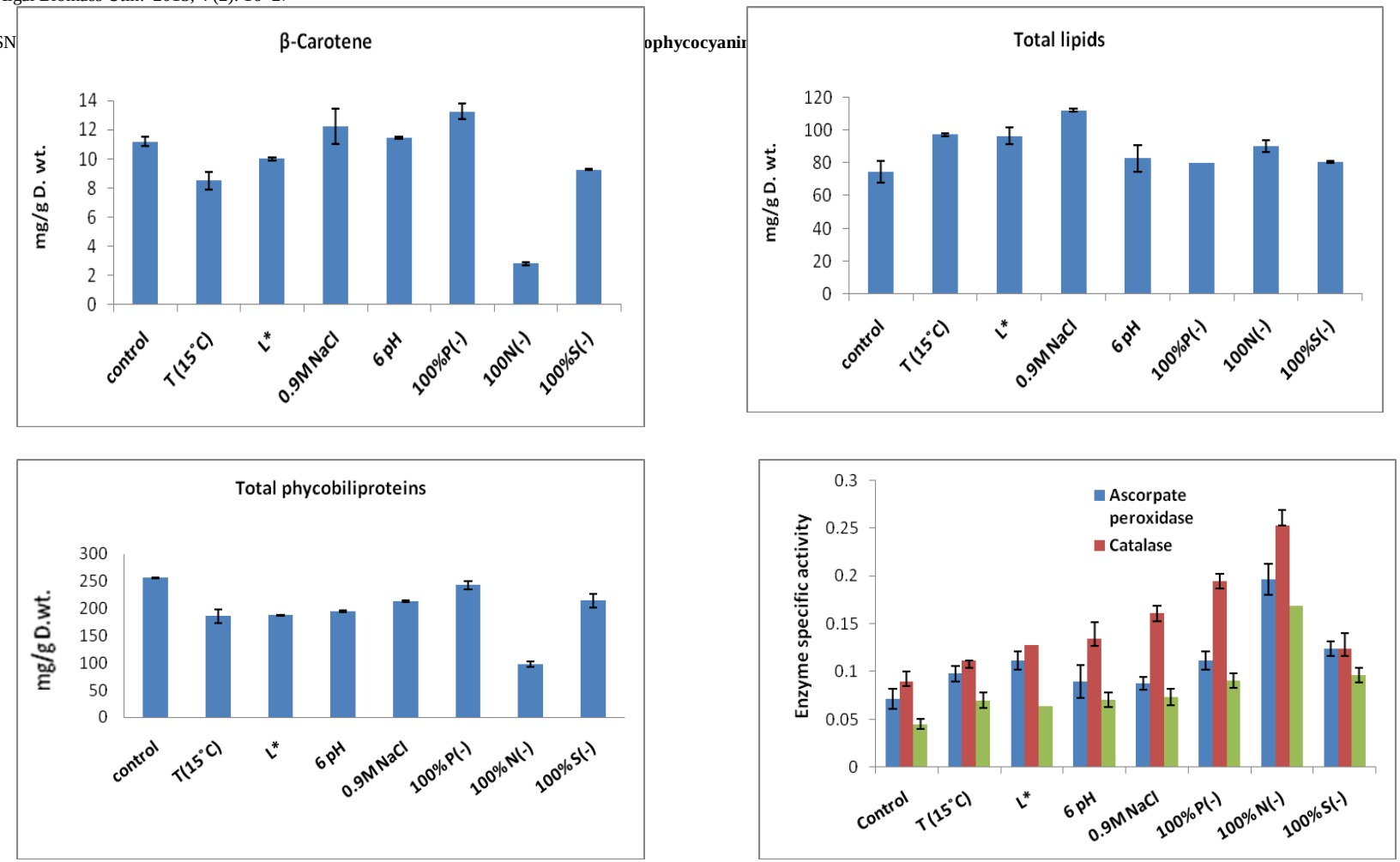
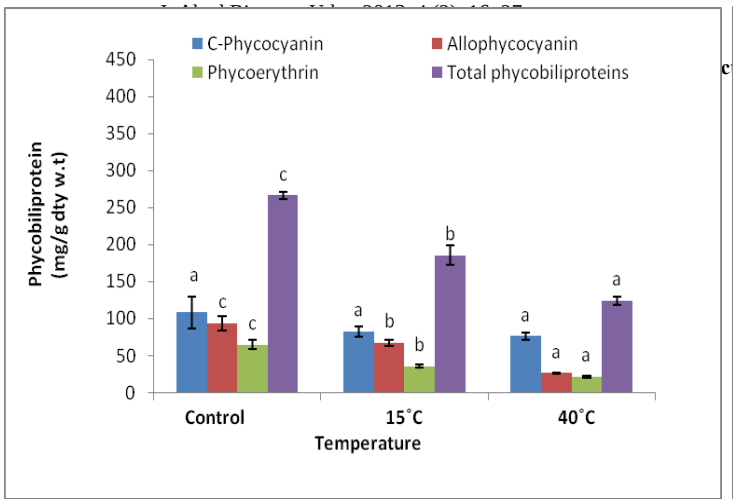
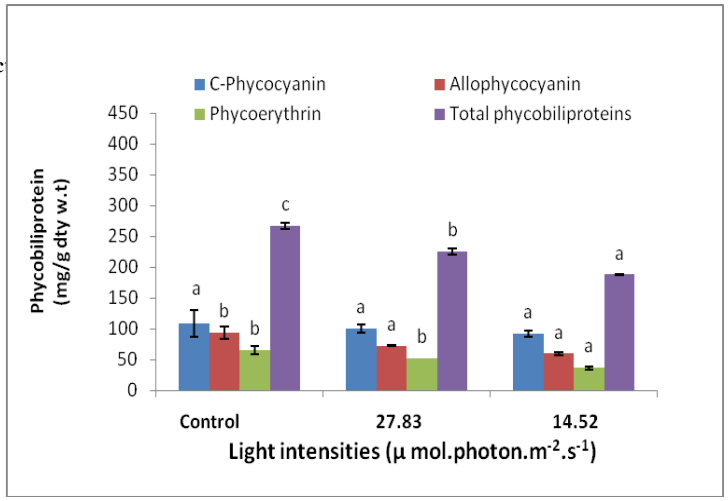
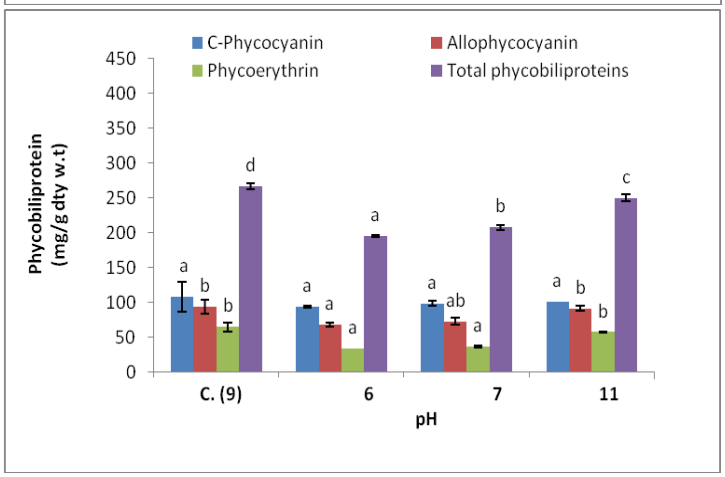
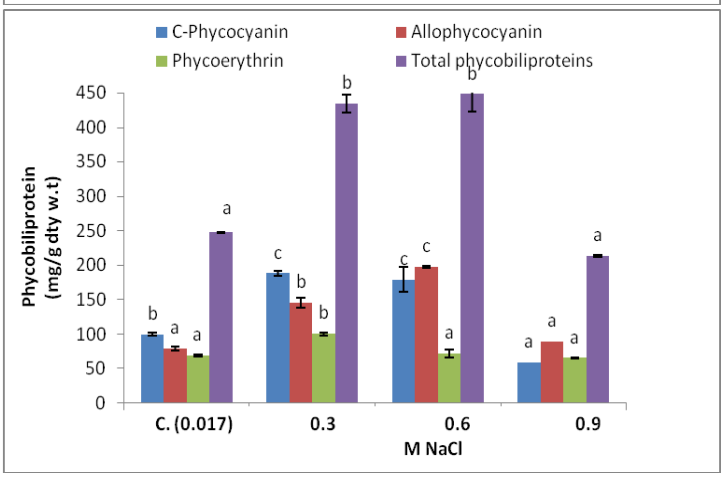


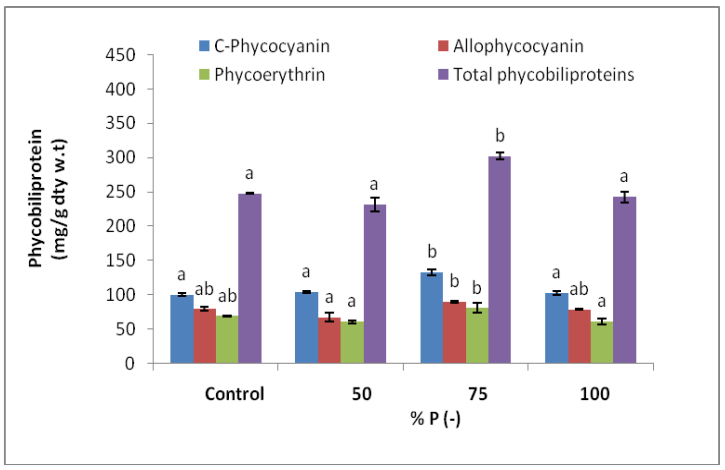
Fig. (2): Production of total lipids, β-carotene, total phycobiliprotein and antioxidant enzymes from *Spirulina* sp. under some a biotic stress.

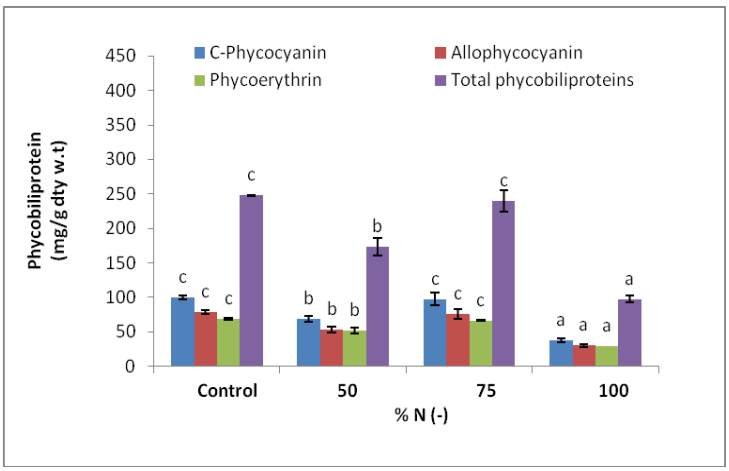












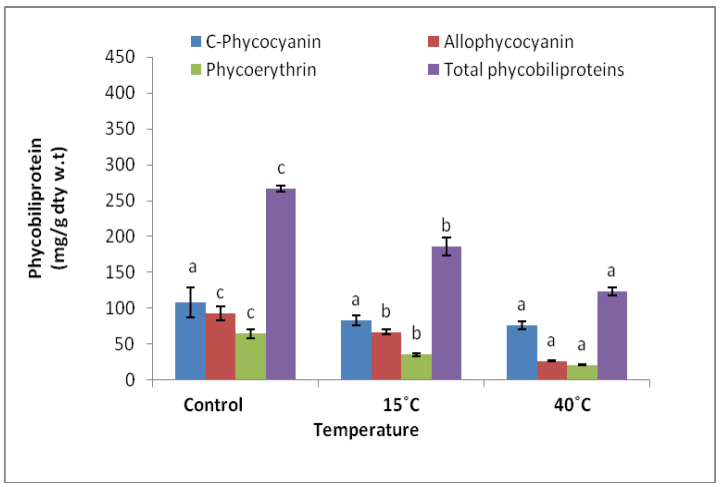


Fig. (3): Effect of a biotic stress on phycobiliprotein fractions of *Spirulina sp.*

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