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PIGMENTS PRODUCTION FROM CYANOBACTERIA *Spirulina platensis* .

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ABSTRACT

A local strain of *Spirulina platensis* isolated from El-Khadra lake – at Wadi El-Natroun, Egypt - was used to study the effect of increasing dose of salinity in the growth medium (from 250 to 500 mM) and pH from 9.5 to 11,0 on its efficiency for producing carotenoid pigments.

The obtanied results showed that increasing pH value of *Spirulina platensis* growth medium from 9.5 to 11.0 increased β-carotene pigment production. The highest concentration of this pigment was obtained at pH range between 9.8 to 10.10. Increasing salinity level from 250 to 500 mM increased β-carotene content. The highest yeild of the pigment was obtained at 450mM salt concentration. HPLC analysis showed a new pigment obtained at pH value of 9.8 and salinity of 450 mM which identified as lutein.

Keywords : *Spirulina platensis*, Carotene production, salinity stress, Lutein.

INTRODUCTION

In recent years, considerable interest has been expressed in the outdoor cultivation of *Spirulina* for commercial purposes (Vonshak and Richmond 1981; Richmond 1988). Due to its pigment composition, *Spirulina* is used as a feed ingredient for pigmentation of ornamental fish, especially gold fish and fancy red carp also it was used for improvement of the integumentary color of cultured fish such as tilopia and sweet melt (Miki, *et al.*, 1986)

The filamentous cyanobacterium *Spirulina plantensis* can be isolated form a wide range of habitats, which differ in their quality (Cifferi 1983). In some salty and highly alkaline aquatic environments, *Spirulina spp.* strains may form, a bloom representing more than 90% of the total phytoplankton biomass (Rechmond 1988). In such habitats it can grow under arid and semi-arid conditions, at daily evaporation rate of 1-2 cm which leads to a constant increase of salt concentration (Vonshak 1987).

The pigment composition of *Spirulina* is typical of cyanobacteria. The only chlorophyll present is chlorophyll-a, its content varying from 0.8 to 1.5 percent of dry weight (Paoletti *et al*, 1980). The xanthophylls content of freeze-dried *Spirulina* is considerable, reaching 6.9 g/kg. The other major carotenoids are myxoxanthophyll (37%), a monocyclic carotenoid attached to rhamnose, β-carotene,28% and zeaxanthin, 17%(Paoletti, *et al.*, 1971).

In a stability study of β-carotene in *Spirulina* , (Seshadri *et al.*, 1999) found that lower drying temperature reduced decomposition of β-carotene. Addition of antioxidants and elimination of air were also found to contribute to the preservation of β-carotene.

Spirulina biomass cultivated outdoors demonstrated an increase in the content of myxoxanthophyll and asullaxanthin (Vincenzini *et al* 1986).

The high intracellular sodium concentration is usually toxic to most biological systems (Wyn-Jones and Gorham 1983). Adaptation to salinity stress requires the development of mechanisms, that limit salts accumulation inside the cell. These mechanisms may consist of low permeability of the plasma membrane to sodium, combined with energy-consuming extrusion of entering sodium ions. Such mechanism is the Na+/H+ antiporter in which the extrusion of sodium form cell is coupled to the inwardly movement of protons (Blumwald *et al.*, 1984b; Krulwich 1986). Another aspect of adaptation to salinity is a build up of internal organic osmosis in order to cope with unbalanced osmotic pressure (Mckay *et al.*, 1984; Reed 1986; Hageman *et al.*, 1987). Warr *et al.*, 1985 showed that Cyanobacteria are charcteristic to extreme environment such as instance in deserts, and alkaline systems (Ward *et al.*, 1989). Two greater lakes in Ethiopia, lake Kilotes and Aranguadi, both characterized by their high salt content and alkalin pH, which support a dense population of *Spirulina*. In the lake Aragnuadi, (characterized by high alkalinity pH of 10.3), the only present microorganism is *S. Platensis* and its abundance is very high, turning the water in deep green. The high biomass of *S. platensis* was responsible for the extremely high photosynthetic rate of (1.2 to 2.4 g of oxygen produced/m2) (Witton; ,1992;Comaa *et al.*,2000). In Egypt, (Aly 2000) discovered the dominance of an algae in El-Khadra lake in Wadi-El-Natrun which live under extreme condition of pH 10.5 and salt concentration of 0.55 M, which identified as S. platensis.

The aim of this work is to quantify the effect of pH and salinity on the production of caroteniod pigments by local *Spirulina* strain.

MATERIALS AND METHODS

Organism and growth conditions:

The cyanobacterium *Spirulina platensis used in this study* originally isolated from El-Khadra lake at Wadi-El-Natrum, El-Behera Governorate this algae has been grown in batch culture under sterile conditions in modified. (Aiba and ogama medium 1977). The sodium salts content of the medium was 250mM; most of it as sodium bicarbonate. The inoculated flasks and tubes were incubated on a rotary shaker (at 25°C and 400 rpm) illuminated with white fuorescent lamps.

NaCl stress; Exponentially growing cells were harvested and re-suspended at a concentrations of chlorophylls 3 mg; in fresh medium containing NaCl at the indicated concentrations (250mM, 300mM, 350mM, 400mM, 450mM and 500mM).

Different levels of pH values of the growth medium were experimented. Strile NaOH solution was used for adjustment of the pH levels of the growth medium. The increase in chlorophyll content of the tested cultures was taken as indicator for *Spirulina* growth. Chlorophyll-b was assayed as described by (Bennet and Gogorad, 1973). Photosynthesis was assayed as the light-dependent oxygen evolution by means of a clarktype oxygen electrode (Yellow Spring. USA).

Determination of chlorophyll (a and b) and total carotenoids content of the tested cultures were determined in acetone extract from dried products by spectro- photometer at 450, 647 and 750 nm respectively against methanol according to (Jeffrey and Humphrey,1975).

Pigment preparation and chromatography:

Pigment extraction: The algae were sedimented at room temperature by a gentle centrifugation at 400rpm for 5min. For pigment extraction, an equivalent of 10 mg dr.wt. of algae was disrupted by grinding in a mortar and with a pestel in the presence of sea sand and small amounts of 90% acetone or tetrahydrofuran solvent for control and stressed algae respectively. The extract was dried under vacuum under a nitrogen stream and redissolved in 90% aqueous acetone for analysis . All the operations were performed under dim light as recommended by (Jeffery and Humphrey 1975) and was measured after freeze-drying.

The thin layer-chromatography was performed on 0.25 mm thick silica plates (60F254.Merck) and eluted with 70% Acetonitril + 20% Dichloro-methane + 10% Methanol solvent and measured at wave length 450A^0.

Fractionation of β-carotene was performed by HPLC – Dionex Chromeleon, (at IGV lab., Grermany). Using Zorbox column, ODS (250 x 4.6 mm,). The mobile. phase was mixture of 70% Acetonitril, 20% Dichloro-methane and 10% methanol, the flow rate of the gas was m^3/min.

RESULTS AND DISCUSSION

Cyanobacteria inhabit environments which vary drastically in their saline levels. Recently, many studies were published on the response of cyanobacteria to different saline environments. Different *Spirulina* species have been isolated from a variety of saline environments (Gabbay and Tel-Or., 1985)

Exposure of *Spirulina* cultures to high NaCl concentrations results in an immediate cessation of growth. After a lag period, a new of steady state is established. Not only in growth inhibited for at least 24h after the exposure to the high NaCl concentration, but a decrease in biomass is observed after which a new steady-state exponential growth rate is established. The new growth rates after adaptation are slower and inversely correlated to the increased NaCl concentration in the medium (Vonshak *et al.,* 1988b). A decrease in the growth rate due to salt stress has also been demonstrated in other cyanobacteria (Vonshak and Richmond 1981 and Blumald and Tel-Or 1982). It is worth to mention that the length of the time lag is exponentially correlated to the degree of stress imposed on the cells. This lag period in many cases is associated with a decline in chlorophyll and biomass concentrations in the culture (Vonshak *et al.*, 1988b).

The response of *Spirulina* to salinity with regard to the degree of growth inhibition, adaptability to salt levels and the rate of adaptation varies widely depending on the strain used in the study.

It has been suggested that exposure of *Spirulina* to high salinity is accompanied by a higher demand for energy by the stressed cells (Blumwald and Tel-Or, 1982). They compared the changes in the photosynthetic and respiratory activity of *Spirulina* over a period of 30min to 48h. after exposure to 0.5 and 1.0M NaCl.

Biomass concentration was taken as an indicator of growth. They noticed marked decrease in the photosynthetic oxygen evolution rate 30min after exposure to both salt concentrations. This decline was followed by a recovery period characterized by a lower steady -state rate photosynthesis which was faster at 0.5M than at 1.0M NaCl concentration(pulz and Scheibenbogen,1998).

The pH of the medium is one of the most important factors in *culturing Spirulina*. Maintaining a pH over 9.5 is mandatory in *Spirulina* cultures in order to avoid contamination by other algae.

Results in Table (1) show that the optimum pH value which yielded the highest chlorophyll-a amount (1.89 mg/L) was 10.7 after 14 days of incubation compared to the control (pH 9.5) which gave only 0.15 mg/L. Increasing incubation time decreased chlorophyll-a amount may be due to the harmful effect of the alkaline pH on the growing organism.

Table 1: Effect of pH on Chlorophyll-a [mg/l]

Incubation Time (days)	pH of the growth media					
	9.5 (Control)	9.8	10.10	10.40	10.70	11.00
0	0.20	0.20	0.20	0.20	0.20	0.20
7	0.18	0.29	0.68	0.38	0.43	0.10
14	0.15	0.76	0.54	1.22	1.89	0.72
21	0.47	0.74	0.76	0.81	0.71	0.99
28	1.28	1.77	1.80	0.80	0.91	0.46

In Table (2) the highest β-carotene and yield (1.82 mg/L) was recorded at pH. 10.4 after 14 days of incubation. From tables (1&2) the amount of carotene grows parallel with the amount of chlorophyll-a. Increasing or decreasing growth medium pH or incubation time induced the same effect on both tested parameters.

Table 2: Effect of pH on β-Carotene [mg/l]

Incubation Time (days)	PH of the growth media					
	9.5 (Control)	9.8	10.10	10.40	10.70	11.00
0	0.57	0.57	0.57	0.57	0.57	0.57
7	0.57	0.68	1.01	0.27	0.40	0.19
14	1.48	1.28	0.76	1.82	1.46	0.74
21	0.79	1.37	1.07	0.98	0.93	1.20
28	0.98	1.93	1.54	0.72	0.97	0.93

These results show also that the decrease in the amount of β-carotene and chlorophyll-a after 14 days at pH 11.0 and 10.7 may be on the expense of the increase in the amounts of xanthophylls and zeaxanthin as reported by (Mervgn, 1983).

The effect of salinity of the growth medium on the *spirulina* production of chlorophyll-a and β-carotene (Tables 3,4) showed that the increase of Salinity from 250 to 500 mM Nacl, decreased chlorophyll-a content in the growth medium as the incubation time extended up to 28 days compared to control (250 m M Nacl) . On the other hand β-carotene increased in the growth medium at the same incubation time when the salinity levels reached 400 and 450 m M Nacl / L. In this regard there are some fluctuations in the β-carotene content within the different tested salinity levels (Table 4).

The results in fige. 1,2,3,4,5 illustrate the effects of PH and salinity on chlorophyll-a and β-carotene pigment production as well as there constituents. From these results, variations in the composition of *Spirulina* chlorophyll-a and its carotinoed pigments composition could be observed.

The results in Fig. (1) of thin layer-chromatography clearly shown that pH 10.4 affect the pigments produced by the algae higher In β- carotene , while pH-10.7 showed higher contents of β-carotene as well as the appearance of new pigments namely, Cantaxanthin, Axanthin.

Table 3: Effect of salinity stress on Chlorophyl- a [mg/l]

Incubation Time (days)	Salt *Concentration in (m.M).*					
	250 (Control)	**300**	**350**	**400**	**450**	**500**
0	0.20	0.20	0.20	0.20	0.20	0.20
7	0.18	0.17	0.32	0.58	0.48	0.25
14	0.15	0.46	0.29	0.44	0.14	1.27
21	0.47	0.70	0.72	0.51	0.87	0.81
23	1.28	0.55	0.45	0.93	0.67	0.66

Table 4: Effect of salinity stress on Carotene [mg/l]

Incubation Time (days)	NaCl *Concentration in m.M.*					
	250 (Control)	**300**	**350**	**400**	**450**	**500**
0	0.57	0.57	0.57	0.57	0.57	0.57
7	0.57	0.49	0.76	0.94	0.96	0.69
14	1.48	0.90	0.74	0.91	1.21	0.32
21	0.79	1.02	1.14	0.86	0.72	0.67
28	0.98	0.91	0.74	1.47	1.24	0.82

By increasing the salinity from 350 to 400 and 450 m/M Nacl., the highest peak was obtained at concentration of the salt 400mM.(1.47 mg/L) for β-caroteine and at 250 m.M. for chlorophyll-a 1 28 (mg/L).

It is clear from the paper chromatogram (Fig 1) that the pH values of 9.8 , 10.1 and 10.4 resulted in the highest concentration of β-carotene.

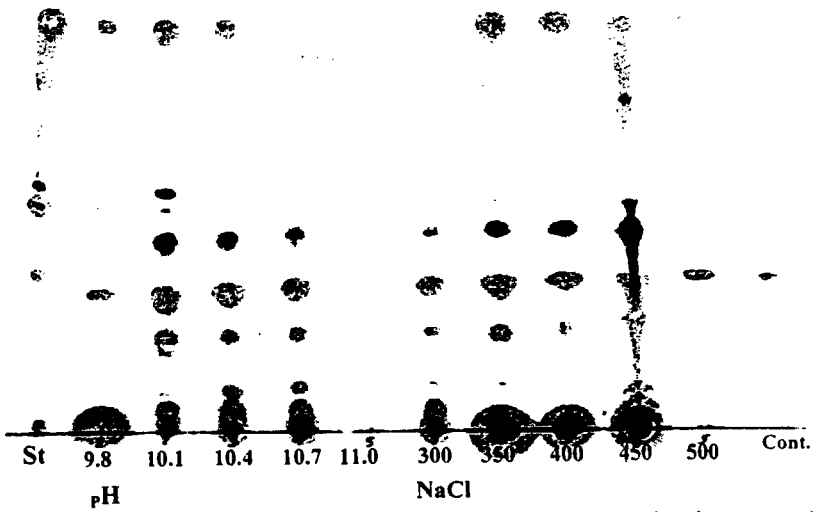


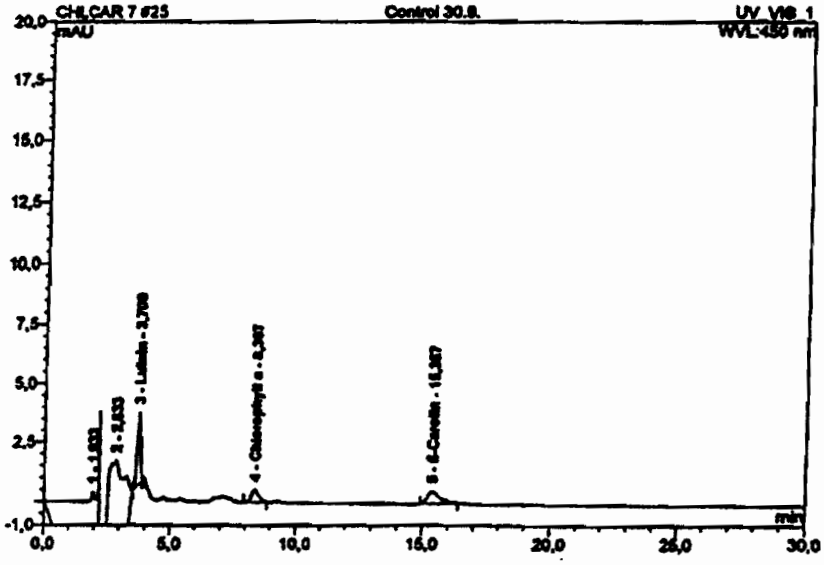
Fig :1 : The effect of pH and NaCl concentration in the growth medium on the production of pigments.

Increasing salinity of the growth medium up to 450 mM Nacl induced high concentrations of pigments which were unknown compared with the standard. s.

These unknown compounds were extracted and studied by means of HPLC. The obtained results illustrated Figs. (2,3,4,5,) showed two main pigments lutein and β-carotene amounted 4.054 and 0.801 height (mAU mille Absorbance Unit), respectively. These results are in harmony with the findings of (Palla and Busson, (1969), and Miki *et al.*, 1986), who stated that β-carotene is found in cyanobacteria that do not carry out crystallization which found in *S. plantensis* isolated from lake Chad by Palla and Busson (1969). They have detected small amounts of echinenone and of β-cryptoxanthin compounds.

It is clear from fig (3) which showed the effect of salinity and the pigment production of the *Spirulina plantinsis.* Including β-carotene and lutein. The obtained results show that salinity (400 mM) induced the highest amount of β-carotene which reached 1.24 mAU as compared to only 0.57 mAU in the control after 28 days . The lutein content reached 5.338 mAU ,while in the control was only 3.82 mAU . These results were previously obtained by Miki etal (1986) who found that β-carotene is known as the most important vitamin (A) precursor in human nutrition besides β-carotene of theoritical vitamin A activity 1.667.000 i.u.g (cited by Bauernfend *et al*. 1971). On protein basis. *S. platensis* appears to have a higher provitamin A content .

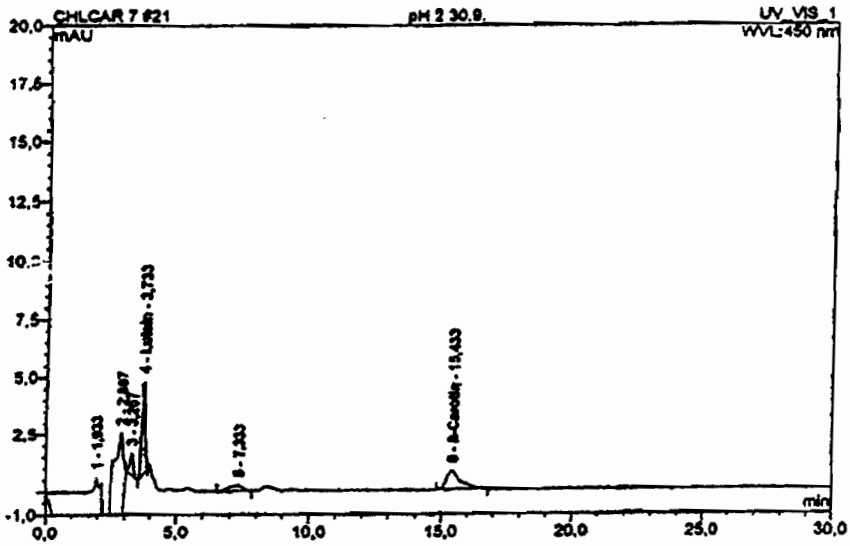
Fig – 2: Effect of standard medium of *Spirulina plantinsis* (control) on the pigments production after 28 days incubation.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount µg/l	Type
1	1,93	n.a.	5,912	6,987	48,14	n.a.	BMB
2	2,83	n.a.	5,601	6,618	45,60	n.a.	BMB
3	3,70	Lutein	3,062	0,449	3,09	0,556	bMB
n.a.	n.a.	Canthaxanthin	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	n.a.	Chlorophyll b	n.a.	n.a.	n.a.	n.a.	n.a.
4	8,37	Chlorophyll a	0,528	0,185	1,28	5,250	BMB
5	15,37	ß-Carotin	0,491	0,276	1,90	1,627	BMB
Total:			18,614	14,514	100,00	7,433	

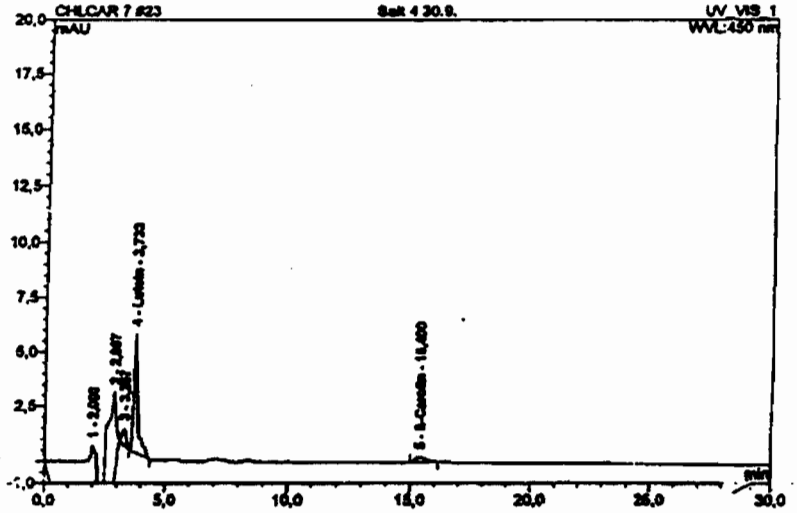
mAU min : milli Absorbance Unit.
WVL : Wave Value Length.
n.a : name absent.

Fig – 3 : Effect of PH 10.10 on the content of pigments produced by *Spirulina platensis*



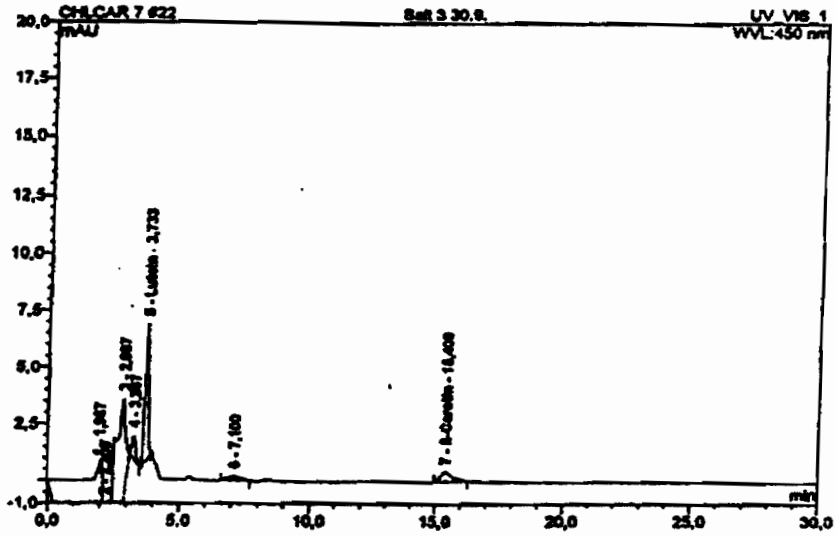
No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount mg/l	Type
1	1,93	n.a.	7,775	8,631	65,30	n.a.	BMB
2	2,87	n.a.	4,597	3,180	24,06	n.a.	BMb
3	3,27	n.a.	0,922	0,145	1,10	n.a.	bMB
4	3,73	Lutein	4,054	0,594	4,49	0,736	BMB
5	7,33	n.a.	0,228	0,157	1,19	n.a.	BMB
n.a.	n.a.	Canteaxanthin	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	n.a.	Chlorophyll b	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	n.a.	Chlorophyll a	n.a.	n.a.	n.a.	n.a.	n.a.
6	15,43	ß-Carotin	0,801	0,511	3,87	3,019	BMB
Total:			18,377	13,219	100,00	3,754	

Fig – 4 : Effect of salinity (450mM) on the constituents of pigments produced by *Spirulina platensis*



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount mg/l	Type
1	2,00	n.a.	8,868	9,355	66,95	n.a.	BMb
2	2,67	n.a.	4,320	3,307	23,67	n.a.	bMb
3	3,27	n.a.	0,740	0,135	0,97	n.a.	bMB
4	3,73	Lutein	5,436	1,053	7,53	1,304	BMB
n.a.	n.a.	Cantaxanthin	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	n.a.	Chlorophyll b	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	n.a.	Chlorophyll a	n.a.	n.a.	n.a.	n.a.	n.a.
5	15,40	B-Carotin	0,228	0,122	0,88	0,723	BMB
Total:			19,414	13,973	100,00	2,027	

Fig – 5 : Effect of salinity (400mM) on the constituents of pigments produced by *Spirulina platensis*



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount mg/l	Type
1	1,97	n.a.	9,019	9,578	69,00	n.a.	BMb
2	2,20	n.a.	3,324	0,305	2,19	n.a.	bMB
3	2,67	n.a.	4,722	2,590	18,66	n.a.	BMb
4	3,27	n.a.	1,020	0,167	1,20	n.a.	bMB
5	3,73	Lutein	6,041	0,894	6,44	1,107	BMB
6	7,10	n.a.	0,207	0,118	0,85	n.a.	BMB
n.a.	n.a.	Centaxanthin	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	n.a.	Chlorophyll b	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	n.a.	Chlorophyll a	n.a.	n.a.	n.a.	n.a.	n.a.
7	15,40	ß-Carotin	0,414	0,230	1,66	1,360	BMB
Total:			24,748	13,881	100,00	2,467	

It could be concluded that the increasing of pH value of the growth medium of *Spirulina plantinsis* form 9.5 to 11.4 has led to the increase in the β-carotene pigment. The highest concentration of this pigment was obtained at pH range between 9.8 to 10.1. Concerning the increase of salinity of growth medium from 50-500 mM an increase of β-carotene was found. The highest content of the pigment was obtained at salt concentration of 450 mM. At high pH and salinity values, new pigments were obtained identified by HPLC as lutein. The highest concentration was obtained at pH value of 9.8 and salinity of 450 mM.

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إنتاج بعض الصبغات من الطحلب الأخضر المزرق "سبيرولينا بلاتنسيس"

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أجريت تجربة معملية لدراسة تأثير التركيزات المتزايدة من الملوحة فـــى بيئـــة النمو (٢٥٠-٥٠٠ مللمول ص كل) والقلوية ما بين رقمى الأس الأيدروجينـــى ٩,٥-١١- على نمو الطحلب "سبيرولينا بلاتنسيس" وإنتاج بعض الصبغات النباتية الهامة من الطحلـب مثل الكلوروفيل أ ، ب والكاروتينات.

وأوضحت نتائج الدراسة أن الزيادة فى رقم الأس الأيدروجينى لبيئة النمو مـــن ٥-١١-أدى إلى زيادة إنتاج صبغة البيتاكاروتين وأن أعلى كمية تم الحصول عليها مـــن تلك الصبغة كانت عند رقم pH ; ١٠,١ ثم إنخفضت بعد ذلك ، كذلك أدت زيادة الملوحـــة إلى زيادة إنتاج صبغة البيتاكاروتين ووصلت أقصاها عند تركيز ملوحة ٤٥٠ مللمول.

هذا ومن جهة أخرى لوحظ أن التركيزات الأعلى من الملوحة والقلوية تؤدى إلى إنتاج صبغات جديدة أمكن التعرف على واحدة منها وهـــى "ليوتيـــن" بإســـتخدام جـــهاز الكروماتوجراف فائق الأداء HPLC عند رقم pH – ٩,٨ وتركيز أملاح ٤٥٠ مللمول.