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ISOLATION, IDENTIFICATION AND BIOCHEMICAL COMPOUNDS OF CYANOBACTERIA ISOLATES FROM SALINE SOILS IN KAFR EL-SHEIKH GOVERNORATE

El Sheekh, M.M.[1];M.A.Zayed[2];Faiza K. A. Elmossel[3]and Reham.S.A.Hasan[2]

[1] **Faculty of Sciences, Tanta University.**

[2] **Faculty of Sciences, Menoufia University.**

[3] **Soils, Water and Environment research institute-Sakha Agriculture Research station,**

ABSTRACT

Using of mineral fertilizers in agricultural production has resulted in serious problems in the soil. Many solutions were suggested such as using blue green algae (cyanobacteria). Nitrogen fixing cyanobacteria one of bacterial communities which play a vital role to fix nitrogen and increase the plant's ability to tolerate salinity. In this work 12 soils samples were collected from different salty sites in (Baltim, Elhamoul, El-Ryad and Seidy-Salem) at Kafr El-Sheikh Governorate. Selected soil samples were analysed physiochemically, Cyanobacterial samples were isolated, purified and identified microscopically based on morphological observation. The propagated cyanobacteria isolates were also tested for their biomass accumulation, nitrogen content and their biochemical composition, i.e., carbohydrates, polysaccharides, protein and pigments content. Four nitrogen fixing cyanobacteria isolated from different saline soil, namely *Nostoc calcicola, Anabaena variabilis, Nostoc linkia*, and *Nodularia herveyana*. *Nostoc calcicola* produce the highest metabolic components, dry weight, chlorophyll a, carotene, intracellular nitrogen, carbohydrates, exopolysaccharides, and proteins (0.949 gL^{-1}, 3.083 mg mL^{-1}, 0.192 mg mL^{-1}, 3.363 mg g^{-1}, 29.7%, 0.198 mg ml^{-1}, and 25.85 mg g^{-1}) respectively, as compared with other isolates. These results deduced using *Nostoc calcicola* in many applications as fertilization, food, pharmaceutical and cosmetics industries.

INTRODUCTION

Cyanobacteria are a group of extraordinarily diverse Gram-negative prokaryotes that originated 3.5 billion years ago. Their diversity ranges from unicellular to multicellular, coccoid to branched filaments, nearly colourless to intensely pigmented, autotrophic to heterotrophic, psychrophilic to thermophilic, acidophilic to alkylophilic, planktonic to barophilic, fresh water to marine including hypersaline (salt pans). They are found both free living and as endosymbionts. (Thajuddin and Subramanian, 2005). Using of N_2-fixing cyanobacteria is the ultimate goal of N_2 fixation research which aims to decrease the dependence on chemical N fertilizers for food production. This is due to indiscriminate use of chemical fertilizers for a longer period drastically disturbed the natural ecological balance (Jha *et al.*, 2001). Cyanobacteria in recent years have application in various fields like biotechnology, pharmacology, agriculture etc. Due to presence of wide spectrum of bioactive compounds cyanobacteria has possesses antiviral,

antibacterial, antifungal and anticancer activities. Recent studies have also shown that cyanobacteria have capability to degrade environmental pollutants and are also being used as a promising source of alternative energy (Ananya *et al*., 2014). The cyanobacteria showed high rates of nitrogen fixation at 10–20% NaCl (Fernandes *et al.*, 1993). This work aimed to obtain cyanobacterial isolates from saline soil, which have the ability to fix nitrogen, increase soil fertility and produce the metabolites such as proteins, exopolysaccharides, pigments and carbohydrates which are valuable substances with potential applications in the food, pharmaceutical and cosmetics industries.

MATERIALS AND METHODS

Soil samples

The experiments of this work were carried out at Sakha Agricultural Research Station, Kafr El-Sheikh. In this study, soil samples were collected from 12 sites in four different regions in Seidy-salem, El hamoul, El-Ryad and Baltim at Kafr El-Sheikh Governorate Table (1), during summer 2012.The samples were collected from the upper surface layer of the soil (0-15 cm).

Enrichment and isolation of cyanobacteria

Cyanobacterial isolates were grown in conical flasks containing sterilized $BG11_0$ medium with pH 7.2 and incubated under illumination light at 28 ± 2°C. Cyanobacterial colonies were isolated by streaking the enriched sample on respective growth medium-agar plates. The unialgal cultures were purified as described by (Pringsheim, 1949).

Identification of cyanobacterial isolates

Identification of cyanobacterial cultures were done microscopically based on morphological observation, the length and the width of the vegetative cells also the width of the sheath, type of spores, presence or absence of hormogonia, presence or absence of spores and its position, number of heterocysts and its repetition, presence of akintes and its type, the nature of cell wall, presence or absence gas vacuoles, as well as pigment color was taken in consideration according to (Desikachary, 1959).The photomicrographs were taken using a BEL® Photonics biological microscope (Italy) fitted with a Canon Powershot G12 digital camera.

Cyanobacterial Biomass (Dry Weight): Dry weight was measured according to (Rafiqul *et al*., 2005).

Nitrogen Measurement: Total nitrogen in the algal filaments and filterate was measured by micro-kjeldahl according to (Page *et al*., 1982) and (Jackson 1958).

Determination of Chlorophyll a and Carotenoid: The photosynthetic pigments, chlorophyll a, and carotenoid, were determined using the spectrophotometric method adopted by (Mackinny, 1941).

Determination of Phycobillins: Phycobillins content was measured according to the method adopted by (Bennett and Bogorad, 1973).

Estimation of Total Carbohydrate: After pigment extraction, the algae cells were extracted with 1N NaOH in a boiling water bath for 2 hours as described by (Payne and Stewart, 1988). Total soluble carbohydrates were

quantitatively determined by the method of phenol-sulphoric acid described by (Kochert, 1973b).

Table (1): - Some physical and chemical characteristics of soil samples collected from different sits at Kafr El-Sheikh Governorate.

Variables		Baltim		El-Hamoul			Seidy Salem					El-Ryad	
		Site 1	Site ١٢	Site 2	Site 4	Site ٩	Site 6	Site 7	Site 8	Site 10	Site ١٣	Site ٣	Site ١١
Physical analysis of the soil	Sand %	11.41	11.21	6.76	7.23	6.65	8.34	7.58	9.53	٧.٢٦	9.53	6.75	6.75
	Silt %	32.87	32.67	32.02	32.43	٣٤.٩٥	32.67	٣١.٨٨	٣١.٧٢	٣١.٧٦	٣١.٨٨	34.80	٣٢.٢٥
	Clay %	55.72	56.12	61.22	60.34	٥٨.٤٠	58.99	٦٠.٥٤	٥٨.٧٥	٦٠.٩٨	٥٨.٥٩	58.45	٦١.٠٠
	Texture class	Clay	Clay	Clay	Clay	Clay	Clay	Clay	Clay	Clay	Clay	Clay	Clay
Soil PH		٧.٨٠	٧.٨٩	٧.٧٣	٧.٩٣	٧.٨٧	٧.٩٨	٨.٠١	٨.١١	٧.٨٢	٧.٩٣	٧.٩٢	٨.٠٢
EC, dS/m		٧٣.٧٠	٧٠.٤١	٤٠.٧٠	١٥.٩٣	٧.٧٥	١٣.٩٤	٦.٣٢	١٠.٢٧	١٠.٨٧	١٥.٩٣	٤.٢٣	١٠.١١
Soluble cations, meq/ 100g	Mg^{++}	٣٩٩.٥	١٠٢.٨٩	٨٦.٦	٣٤.٤٣	٢٣.٩٢	٢٢.٠٧	١٥.٠٤	٢٠.٥	٢٠.٩٥	٣٤.٤٣	١٢.٤٣	٢١.١٣
	Ca^{++}	١٣٤.٠	١٠٥.١٦	١٣٧.٨٥	٣٣.٥٠	٢١.٤٤	٢١.٣٣	١٢.٠٦	١٥.١٥	١٧.٢٠	٣٣.٥	١٢.٠٥	٢١.٤٦
	Na^{+}	١٩٥.٦	٤٩٢.٢٥	١٧٧.٩٥	٩٠.٥٠	٣٠.٦٠	٩٥.٥٠	٣٤.٩٠	٦٥.٧٥	٦٩.٢٥	٩٠.٥٠	١٧.٠٠	٥٦.٨٧
	K+	٨.٠٠	٤.٥٥	٥.٠٠	١.٥٠	١.٥٤	٠.٥٠	١.٦٨	١.٥٠	١.٥٠	١.٥٠	٠.٧٧	١.٦٥
Soluble anions, meq/ 100g	So_4^{--}	٥٤٧.٧٤	١٥٦.٦٦	٢٢٨.١٤	٦٩.١٤	٤٣.٥٨	٤٣.٠٧	٣١.٠٣	٣٢.٥٢	٣٤.٠٢	٦٩.١٤	٢٢.٤٥	٥٢.٦٦
	HCo_3	٤.٠٦	٥.٧٧	٤.٠٦	٤.٦٩	٣.١٢	٣.١٣	٣.٧٦	٦.٢٥	٦.٨٨	٤.٦٩	٣.٥٦	٢.٦١
	CO_2^{--}	--	--	--	--	--	--	--	--	--	--	--	--
	CL^{-}	١٨٥.٣	٥٤٢.٤٢	١٧٥.٢٠	٨٦.١٠	٣١.٨٠	٩٣.٢٠	٢٨.٨٤	٦٤.١٣	٦٨.٠٠	٨٦.١٠	١٦.٢٤	٤٥.٨٤

Estimation of Cyanobacterial Exopolysaccharides (EPS): The cyanobacterial EPS were extracted according to the method adopted by (Seifter *et al*., 1959).

Estimation of Total Protein Contents: After pigment extraction, the algae cells were extracted with 1N NaOH in a boiling water bath for 2 hours as described by Payne and Stewart (1988). Total soluble proteins were quantitatively determined using the method described by Bradford, (1976).

Statistical Analysis: The obtained collected data were subjected to the statistical analysis, using the analysis of variance (ANOVA). The LSD range tests were used to compare between the means (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

In this study, soil samples were collected from 12 sites in four different regions at Kafr El-Sheikh Governorate Table (1). The results showed that these sites differ in physiochemical properties. The large variation between these samples in electric conductivity (E.C), which ranged from 4.23 to 73.70 dSm^{-1}, depending on the distance from the sea. Also, pH was varied among Balteim, Elhamoul, El-Ryad and Seidy Salem. Thus, it is worthly to mention that soils of Balteim are the lower quality and higher salinity that those obtained of the other sites. It appears that many cyanobacteria isolated from coastal sites tolerate saline environments (i.e. are halotolerant) rather than require salinity (i.e. are halophilic). As frequent colonisers of euryhaline (very saline) environments, cyanobacteria are found in salt works and salt marshes, and are capable of growth at combined salt concentrations as high as 2-3 (%) (Reed *et al*., 1984)

Isolation of cyanobacteria:-

Isolation and purification program of cyanobacteria dominant in soil samples collected from different sites at Kafr El-Sheikh Governorate revealed that 4 out 12 isolates were successfully obtained as bacterial -free as possible isolate number (1, 2 and 3) in addition to one isolate was toxic (No.4) as described by Mondo *et al.,* (2012), and Naeem,(2012).

Identification of cyanobacterial isolates:-

The names and locations of the isolated cyanobacteria strains are shown in Table (2) the description of these cyanobacteria isolates is as the following:

Table (2): Isolated cyanobacteria from different location at Kafr El-Sheikh Governorate :

Cyanobacteria Isolate number	Isolate name	Location
Isolate 1	*Nostoc calcicola*	Baltim site (1), Seidy Salem site (6, 7, 8, 13), El-Hamoul Site (9)
Isolate 2	*Anabaena variabilis*	El-Hamoul Site (2, 4), El-Ryad Site (11),
Isolate 3	*Nostoc linkia*	El-Ryad Site (3), Seidy Salem Site (10)
Isolate 4	*Nodularia herveyana*	Baltim Site (12)

1. *Nostoc calcicola*

This isolate was described as the thullus is mucilaginous, slightly diffluent, expanded, olive, grey or blue green, often up to 5cm in diameter., filamently loosely; sheath mostly indistinct, or indistinct only at the periphery of the thullus, colorless or yellowish brown; trichome 2.5 µ broad, pale blue-green; cells barrel-shaped, subspherical, rarely longer than broad; heterocyst subspherical, 4-5µ broad; spores subspherical, 4-5 µ broad, with smooth yellowish membrane. The isolate identified as *Nostoc linkia,* which belongs to order *Nostocales,* family *Nostocaceae* and genus *Nostoc,* shown in Fig (1).

2. *Anabaena variabilis*

This isolates was described as the thullus dense, soft, mucilaginous, deep green; trichomes, 3.1 – 4.2 µ broad, blue-green, often irregularly curved and more or less entangled with each other, slightly constricted at the joints, attenuated at ends, the terminal cell being conical with a sharp or rounded apex, without mucilaginous sheath; cells cylindrical, up twice as long as broad, rarely barrel-shaped, and almost as long as broad; heterocysts single intercalary, and distributed at regular intervals throughout the length of the trichome, cylindrical, 4.2 – 5.2 µ broad and 8.4 – 12.6 µ long. Spores in short or long chain, ellipsoidal or barrel-shaped, remote from heterocyst, 4.2-6.3 µ broad and 6.3-10.5 µ long, with a thick, smooth and colorless outer wall. The isolate identified as *Anabaena variabilis*, which belongs to order *Nostocales*, family *Nostocaceae* and genus *Anabaena*, shown in Fig (2)

3. *Nostoc linkia*

This isolate was described as the thallus of various size, tuberculate, mucilaginous, irregularly expanded when old, blue-green, later blakish green; filaments densely entangled, much contorted; trichome deeply constricted, non-motile, appear loosely connected; cells more or less barrel shaped, 4.0-6.5 µ long, 4.0-5.0 µ diameter.; heterocyst sub-spherical, 4.0-6.5 µ diameter.,

6.5 µ long. The isolate identified as *Nostoc **c**alcicola,* which belongs to order *Nostocales,* family *Nostocaceae and* genus *Nostoc,* shown in Fig (٣)**.**

4- *Nodularia herveyana*

Filaments were straight and composed of discoid vegetative cells 7-12 µ wide (mean 8.1 µ) and 2.7-3.6 µ long (mean 3.4 µ), with gas vesicles. Filaments usually had an evident colorless, transparent sheath. Heterocytes were 6.1-10.9 µ wide (mean 10 µ) and 4.5-6.4 µ long (mean 5.6 µ). They were present after every 12 to 16 vegetative cells (mean 13 cells), the isolate identified as *Nodularia herveyana,* which belongs to order *Nostocales,* family *Nostocaceae and* genus *Nodularia,* shown in Fig (4).

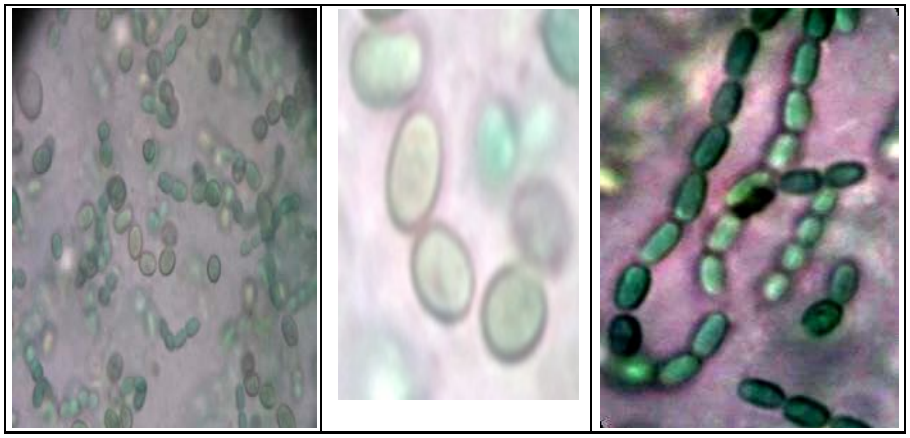

Fig (1): *Nostoc calcicola* under microscope showing vegetative cells and heterocyst.

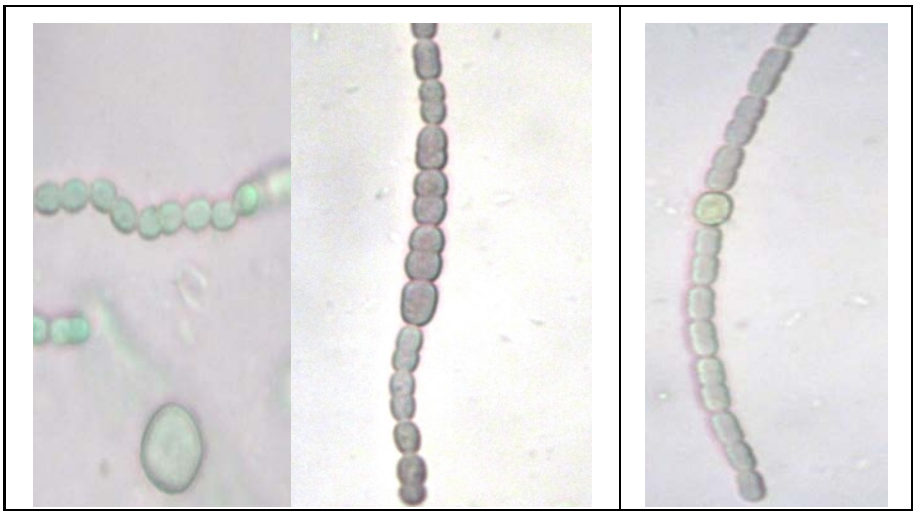

Fig (2): *Anabaena variabilis* under microscope showing vegetative cells and heterocyst .

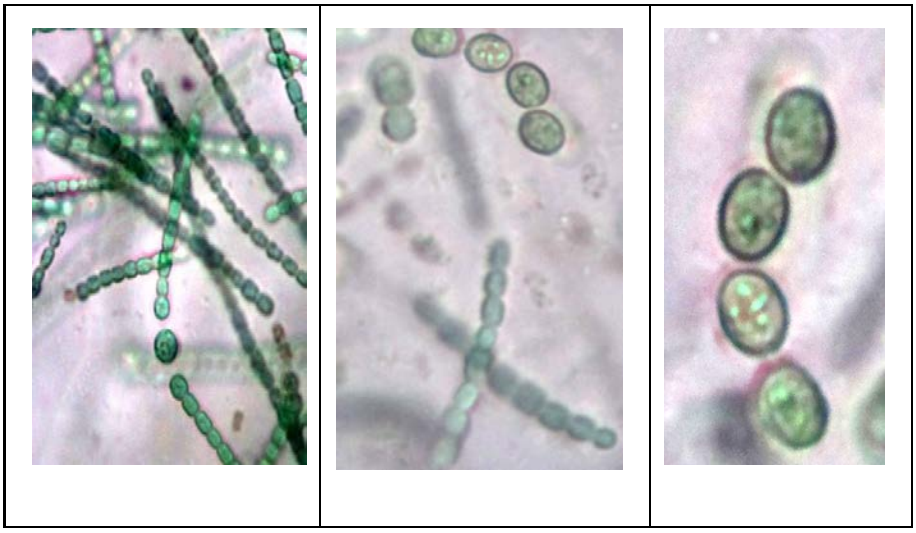

Fig (3): *Nostoc linkia* under microscope showing vegetative cells and heterocyst.

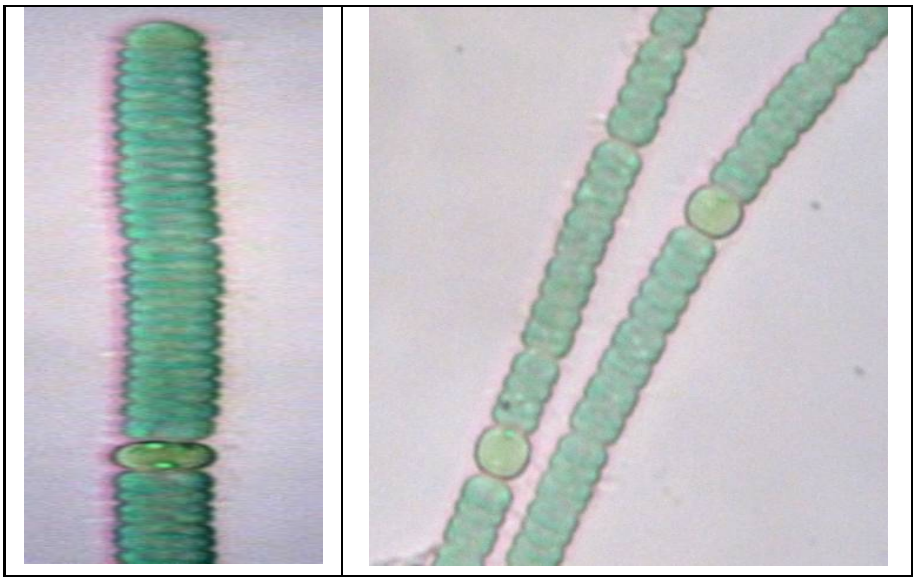

Fig (4): *Nodularia herveyana* under microscope showing vegetative cells and heterocyst .

Biochemical analyses of cyanobacterial isolates:

Cyanobacterial Biomass (Dry Weight):

Data in Fig. (5) show that dry weight of the tested cyanobacteria strains increased with increasing the incubation period till the fifth week for *N. calcicola*, and *N. linkia*, and fourth week for *A. variabilis,* then the biomass

decreased with increasing the incubation period. The highest mean value was 0.949 g L^{-1} recorded at the fifth week, while the lowest value was 0.164 g L^{-1} recorded at the first week. These results are in agreement with those obtained by Adly (2011) who found that the biomass production of the cyanobacteria strains isolated from Egyptian rice soils, had progressively increased with increasing the incubation period from one to 4 weeks.

Fig (5): Dry weight (g L^{-1}) of cyanobacteria strains grown on BG11 medium at different incubation periods.

Pigments Determination:

Chlorophyll a content of cyanobacteria:

Data in Fig. (6) show the chlorophyll a content of the isolated cyanobacteria. i.e*. N. calcicola, A. variabilis and, N. linkia* under different incubation periods. Results indicated that chlorophyll a content increased with increasing incubatin period. The highest mean value was 2.508 mg.ml^{-1} medium which recorded at the fifth week. Our results are in agreement with that obtained by Somchanh *et al*. (2010).

Fig (6): Chlorophyll a contents of the isolated cyanobacteria strains at different incubation periods.

Carotenoid content of cyanobacteria:

Carotenoid content (µg ml^{-1}) in cyanobacterial isolates, under different incubation periods is illustrated in Fig. (7). The carotenoid content of cyanobacteria isolates increase by increasing the incubation periods. Results revealed that the highest Carotenoid Pigments content by *N. calcicola* followed by *Nostoc linkia,* and *Anabaena variabilis* which give (0.192, 0.084, and 0.080 µg.mL^{-1}) respectively in the fifth week. The results are in agreement with Bakiyaraj *et al*. (2014) who stated that the maximum carotenoid content (0.078µg/ml) was noticed in 12th day and minimum (0.049 µg/ml) on 24th day. Under all other concentrations there was a gradual increase in carotenoid pigment upto 12th day followed by a gradual decline up to 24th day.

Fig (7): Carotenoid content of cyanobacteria strains at different incubation periods.

Phycobillins (Allophycocyanine and Phycoerthrine) content of cyanobacterial strains

Data in Fig. (8 and 9) show the Allophycocyanine and Phycoerthrine content (µg.L^{-1}) of *N. calcicola, A. variabilis* and, *N. linkia* under different incubation periods. *N. calcicola* showed the highest Phycoerthrine content (7.160 µg L^{-1}) in the fifth week, followed by 6.820 and 6.814 µg L^{-1} in the fifth weeks for *N. linkia and A. variabilis* respectively. These results are in agreement with those obtained by (Becker, 1994).

Fig (8) : Allophycocyanine contents of cyanobacteria strains measured at different incubation periods.

Fig (9): Phycoerthrine contents of cyanobacteria strains at different incubation periods.

Nitrogen content:

Data presented in Fig. (10 and 11) showed that the extracellular N of cyanobacterial isolates increased as incubation periods increased upto the fifth week. The highest Extracellular- nitrogen recorded with *N. calcicola* followed by *Anabaena variabilis,* and *Nostoc linkia* was (0.076, 0.055 and 0.055 mg ml^{-1}) respectively in the fifth week.

Results presented in Fig. (11) revealed that the highest Intracellular-N content of *N. calcicola* recorded 3.363 mg g^{-1} dry weight followed by *Nostoc linkia,* which recorded 2.240 mg g^{-1} dry weight, followed by *Anabaena variabilis,* which give 2.117 mg g^{-1} dry weight in the fifth week. These results are in harmony with those obtained by El-Gaml (2006).

Fig (10): Extracellular- nitrogen contents of cyanobacteria strains at different incubation periods.

Fig (11) : Intracellular- nitrogen contents of cyanobacteria strains at different incubation periods.

Total Carbohydrate of cyanobacterial strains:

Data in Fig. (12) show the total carbohydates content of *N. calcicola, A. variabilis* and*, N. linkia* under different incubation periods, The highest total carbohydrate content (TCC) of 29.7 % was attained by *Nostoc calcicola* followed by *Nostoc linkia,* which give 23.1 % in the sixth week, then *Anabaena variabilis,* which give 17.83 % in the fifth week. These results are in agree with Brian Robert Jordan (٢٠٠٤)

Fig (12): Total carbohydrate contents of cyanobacteria strains measured at different incubation periods.

Exopolysaccharides (EPS) of cyanobacteria strains:

Exopolysaccharides contents (mg ml^{-1}) of cyanobacterial strains measured at different incubation periods are presented in Fig. (13). Exopolysaccharides content of cyanobacterial strains increase by increasing the incubation periods, The highest EPS content recorded by *N. calcicola* which give 0.198 mg ml^{-1} in the sixth week followed by (0.183, 0.090 mg ml^{-1})

which recorded by *Anabaena variabilis,*and *Nostoc linkia* respectively in the fifth week. These results are in agreement with Chakraborty *et al*. (2012).

Fig (13): Exopolysaccharides (EPS) contents of cyanobacteria strains at different incubation periods.

Total Protein Contents (TPC) of cyanobacteria strains:

Data illustrated by Fig. (14) show the total protein content of *N. calcicola, A. variabilis* and, *N. linkia*. The total protein content (TPC) increase with increasing the time of incubation then decreased in the sixth week. However, the highest TPC were (25.850, 17.687, 17.190 mg g^{-1}) which recorded by *Nostoc calcicla, Anabaena variabilis* and *Nostoc linkia* respectively in the fifth week. These results are in agreement with (Gonzalez et al. 2010) who revealed that dry biomass of cyanobacteria exhibited total protein content ranged from 30- 55%.

Fig (14): Total Protein contents of cyanobacteria strains at different incubation periods.

CONCLUSION

The cyanobacterial strains (*Nostoc calcicla, Anabaena variabilis* and *Nostoc linkia* and *Nodularia herveyana*) were isolated from different salty regions at Kafr El-Sheikh Governorate, The highest biomass was recorded by *N. calcicola* followed by *Nostoc linkia,and A. variabilis*. Data revealed a significant difference between chlorophyll a content, carotenoid, phycopilin pigments, proteins, exopolysaccharides and carbohydrates of all tested cyanobacteria strains, Among the tested strains *N. calcicola* showed the highest average followed by *A. variabilis and N. linkia* respectively. From this work we conclude that the cyanobacterial strains *Nostoc calcicla, Anabaena variabilis* and *Nostoc linkia* have the ability to fix nitrogen, and produce proteins, exopolysaccharides, pigments and carbohydrates which are valuable substances with potential applications in the food, pharmaceutical and cosmetics industries, Also they can increase soil fertility, and decrease soil and water pollution.

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عزل وتعريف والمحتوى البيوكيميائى لعزلات السيانوبكتريا من اراضى ملحية فى محافظة كفر الشيخ

مصطفى محمد الشيخ[١] ، محمد أحمد زايد[٢]، فايزة كمال عبد الفتاح الموصل[٣]و ريهام صلاح عبد الوهاب حسن [٢]

[١] كلية العلوم . جامعة طنطا.

[٢] كلية العلوم.جامعة المنوفية.

[٣] معهد بحوث الاراضى والمياه والبيئة - محطة بحوث سخا.

إن زيادة استخدام الاسمدة المعدنية فى الانتاج الزراعى يؤدى إلى مشاكل عديدة فى التربة، وتعتبر السيانوبكتريا المثبتة للأزوت واحدة من المجاميع الميكروبية التى تلعب دورا حيويا فى زيادة قدرة النبات على تحمل الملوحة. وفى هذا البحث تم تجميع ١٢عينة تربة من مواقع مختلفة فى محافظة كفر الشيخ (بلطيم - الحامول- الرياض- سيدى سالم) لعزل السيانوبكتريا .وقد تم عزل وتنقية١٢ عزلة وتعرفيها ميكروسكوبيا على أساس الشكل المورفولجى. تم إختبارانتاجها للكتلة الحيوية وبعض المركبات البيوكيميائية مثل انتاج الكربوهيدرات والبروتين والسكريات العديدة ومحتوى الصبغات. تم الحصول على أربع عزلات وهى نوستوك كالسيكولا، أنابينا فاريابيليس،نوستوك لينكيا،نوديولاريا هيرفيانا كأكفأ العزلات. وكانت اعلى العزلات إنتاجا للقياسات البيوكيميائية هى للنوستوك كالسيكولا كالتالى الكتلة الحيوية (0.949جرام / لتر) كلوروفيل أ (3.083ملجرام/ مل)، كاروتين(0.192ملجرام /مل)، النيتروجين (3.363ملجرام / جرام)، الكربوهيدرات (29.7%)،السكريات العديدة (١٩٨.٠ ملجرام/مل)،البروتين (25.85 ملجرام/جرام) . والنتائج تنصح باستخدام هذه العزلات السابقة فى كثير من التطبيقات مثل التسميد الحيوى ،صناعة الغذاء وصناعة الادوية وخاصة نوستوك كالسيكولا.