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Thioflavin T Production in *Coelastrella saipanensis* LC752948.1: Impact of Sodium Chloride, growth phases, and their effect on growth parameters

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Abstract

The present study aimed to investigate the possible production of Thioflavin T and the effect of NaCl concentrations and growth phases on the growth rate, doubling time and proline of *C. saipanensis* N. Hanagata (Scenedesmaceae, Shaerophleales). The alga was cultured in BG 11 medium and six NaCl concentrations were used in the experiments during different growth phases. The results have unveiled the presence of Triflavin T in the alga. The study results showed a growth rate decrease at all NaCl concentrations except in control treatment, while the doubling time, was recorded highest value (14 days) at the NaCl concentration of 0.08 M. The highest value of Proline (0.509 mg. L^{-1}) was recorded at the treatment of 0.08 M of NaCl and recorded 0.3 mg. L^{-1} in the second phase of alga growth, and increased up to 0.695 mg. L^{-1}in the interaction between NaCl concentrations and growth phases (0.08 M of NaCl and the second phase). The highest value of Thioflavin T (25.386 mg. L^{-1}) was recorded at 0.005 M of NaCl and 21.937 mg. L^{-1} at the second phase of growth. While its concentration reaches 27. 335mg.L^{-} in combined experiments (0.005 M of NaCl and the second phase of growth). Therefore, the results of this study unveiled the presence of Thioflavin T pigment in C. *saipanensis*. The NaCl concentrations and growth phases have an impact on the pigment and proline concentrations. This finding highlights the possible use of the alga to produce the Thioflavin T pigment for different purposes.

Keywords: *C. saipanensis.* , Growth rate, NaCl, Thioflavin T., Proline.

Introduction

Microalgae is considered a potential source of bioactive compounds and nutraceuticals compounds[1]. Algae represent a very diverse and wide group of living organisms. They are found in freshwater, salty and brackish environments either singly or in colonies[2]. Many studies have focused on the compounds of biologically active microalgae[3-5], such as anti-cancer compounds anti-inflammatory anti-viral, anti-immunity, anti-bacterial, and anti-cancer compounds[6,7].

Coelastrella was first identified by the scientist Chodat in 1922 as a unicellular alga or group of small-sized, spherical to oval cells; their diameter is 6-15 µm. Plastids are cup-shaped and wall-positioned[8]. mentioned that *Coelastrella* is important as oil-riched source and as a tool for wastewater treatment[7]. A few studies in Iraq investigated alga *Coelastrella*, including its identification[9,10], while other studies were conducted on its antibacterial activity[11] and for bioremediation treatment[12].

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Coelastrella saipanensis has been nominated to produce active compounds. In addition, it has been proven that the extract of this alga shows an activity which is anti- cancer tumor of hepatic cells[13]. Report the strategy called elicitation to trigger the production of phytochemical compounds[14]. It is one of the most effective means used in the biotechnology field to increase the production of secondary metabolic compounds[14,15].

Exposure to different environmental stresses is accompanied by the accumulation of secondary metabolic compounds and amino acids like proline. They are considered initiators of proteins and play an important role in plant metabolism[16]. Algal extracts have a great role in cosmetics, medicines, and agricultural industries. Plus, they can be valuable ingredients in many products that protect against the attack of free radicals, and they prevent diseases that are mainly related to oxidative stress[17]. Flavonoids or polyphenols are regarded as essential compounds, and they are one of the largest taxonomic categories of phenolic compounds[18]. These compounds are classified as one of the most important natural antioxidants; they protect cells and natural chemicals in the body from damage caused by free radicals[19]. [20]Mentioned the importance of Thioflavin T (ThT) in the detection of Alzheimer's disease and amyloid detection[20-23]. ThT is a benzothiazolium pigment that is used to detect the presence of amyloid found in the brains of patients who suffer from chronic neurodegenerative disease[24].These fluorescent molecules that are associated with protein amyloid are considered very desirable and they have great importance in Alzheimer research. They have two characteristics: specificity in binding as well as the light generated from this binding[25]. Proline is considered one of the amino acids that exists freely. The wide accumulation of proline contributes to change in the osmotic stress of the cell. This leads to an increase in the ability of the cells to absorb water which has an effective role in storing metabolic materials within the cell carrying out the process of osmotic regulation[26]. Because of the great role played by the amino acid as a protective response to improper conditions, it was found necessary to study ways of increasing the concentration of proline to contribute to resisting various stresses[27].

The primary objective of this study was to explore the potential production of ThT in *C. saipanensis*. Additionally, we aimed to assess the influence of NaCl concentrations and different growth phases on key parameters. Including growth rate, doubling time, proline synthesis and ThT production in algae.

Materials and Methods

Algal sample

A pure sample of *Coelastrella* alga was obtained from the College of Sciences/University of Baghdad. It was diagnosed according to the species level and was registered in NCBI under accession number LC752948.1. The experiment was carried out in the Tissue Culture Laboratory/ Department of Biology/ College of Education for Pure Sciences/University of Diyala. The sample was cultured in BG11 medium under sterile conditions and the samples were kept in the growth room at a temperature of 25±2 within a light - dark alternating system of 16/8 light hour/dark with a lighting intensity of 3000 lux.

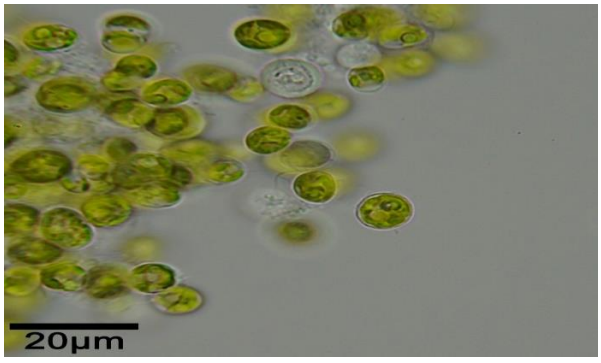

Figure 1. The Phenotypic Shape of *Coelastrella saipanensis* under the Electronic Microscope

Growth curve of C. *saipanensis*

Algal cells density was determined by optical density (OD) measurement by UV. Spectrophotometer at 540 nm everyday Growth rate (k) as well as doubling time (G) were determined based on the equation:[28]

$$K = \frac{Log\ ODt - log\ OD0}{t} \times 3.322$$

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$$G = \frac{0.301}{K} \quad 29$$

t: time (days)

OD_t: Algal growth after (t) days.

OD_0: Algal growth at zero time.

NaCl concentrations

Six NaCl concentrations were 0.00, 0.005, 0.01, 0.03, 0.06 and 0.08 M. These concentrations were added to the culturing medium that was previously prepared by adding 100 cm^3 of the algal isolate to 900 cm^3 of BG11 medium in a 1000 cm^3 beaker.

Growth Phases

The effect of cell harvesting of *C. saipanensis* was studied during the different growth phases. The cells were harvested in three stages. The first one was in the last two days of the logarithmic phase of alga growth and was called (phase 1). The second harvest was in the first two days of the Stationary phase of the alga growth and was called (phase 2). The third harvest occurred in the last two days of the Stationary phase and was called (phase3).

Preparation of algal extract

The algal extract was prepared via the hot method. This was through putting 1 gm of the dried powder of *C. Saipanensis* in a thimble of the Soxolite device plus 150cm^3 of ethanol solvent in 250cm^3 conical flask. The device was connected to a condenser. The process was conducted for 6-8 hours in 7 cycles for each sample[30].

Estimation of proline

The proline of the algal extract was estimated by adding 10cm^3 of aqueous sulfosalicylic acid at the concentration of 3% to the algae sample. Then, 2cm^3 of ninhydrin reagent solution and 2cm^3 of glacial acetic acid were added. The sample was heated with the reagent in a water bath for an hour. After cooling the sample, 4cm^3 Toluene material and was shaken for 20 seconds. Next, the sample was left at room temperature. Only 1 cm^3 was taken and the absorbance was read at the wavelength 520nm. The proline values of the sample were examined depending on the standard model (Fig. 2). The concentration of proline was calculated through the following Eq.[31]:

$$\text{The concentration of proline} = \frac{\text{Absorbance} \times 4 \times 5}{\text{Sample weight} \times 1.47}$$

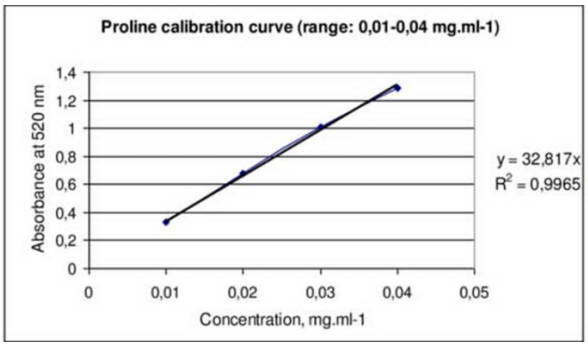

Figure 2. Standard Curve of Proline

The diagnosis and quantitative Estimation of ThT

Diagnosing of Thioflaven T was carried out from samples extracted with ethyl alcohol via using high-performance liquid chromatography (HPLC) (SYKAMN, 2010, Japan). This device is equipped with an ultraviolet detector through the use of a separating column (4.6 mm length) within the stationary phase C18. This technology is distinguished with high efficiency in terms of quantitative and qualitative estimation of the compounds required to be diagnosed by calculating curves and determining the concentration of this compound according to specified conditions as shown in Table 1. The diagnosis of Thioflaven T in the study sample was carried out depending on the measurement model, Fig. 3. The readings including the curve area and the retention time were taken. Through these readings the compound isolated from the samples was diagnosed in comparison with the retention time of the standard sample. Then, calculating the concentration was as explained in the equation below:

$$\textit{Sample concentration} = \frac{\textit{Standard material Concentration} \times \textit{Sample absorbance}}{\textit{Standard material absorbance}}$$

Table 1. HPLC Conditions of the Standard model diagnostic of ThT[32]

No.	Conditions	Thioflaven T
1	Mobile phase	Water: Ethanol
2	Sample volume	100 mm
3	Temperature	30 C°
4	The detector	UV at 280 nm

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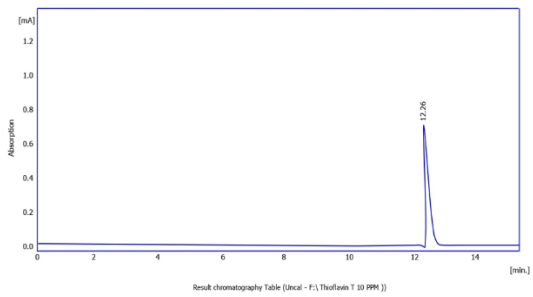

Figure 3. Standard Curve of Thioflaven T

The experiment was conducted by a completely randomized design (CRD), with three replicates per a treatment. The differences between the means were compared by using the JASP depending on the programming language R for treating data via using a Tukey test at the probability level of 0.001.

Results and Discussion

The highest growth rate of *C. saipanensis* was 0.08 at the control treatment, while it decreased to 0.03 at a concentration of 0.008 M NaCl (Fig. 4). These results indicated the opposite effect on the growth rate when the salt concentration increased. This result explained that test alga might suffer from salinity stress, particularly chloride salts which are considered one of the main salts responsible for water salinity. They exist widely in the aquatic environment whose concentration might increase due to the evaporation resulting from the continuous rise in temperature degrees[33]. Also, salinity showed the ability to change the biochemical nature of algal cells[34]. Moreover, exposing algae to levels which were different from their normal, moderate levels led to a change in the growth rate[35]. Annamalai et al. [36] Obtained the similar results of this study but they used other species, they studied the effect of sodium chloride on two species of freshwater algae; *Chlorella vulgaris* and *Chlamydomonas reinhardtii*.

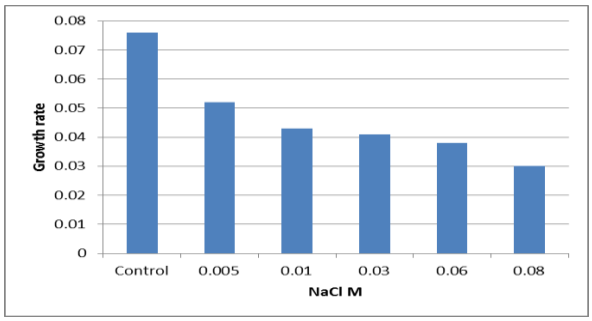

Figure 4. Growth Rate of *C. saipanensis* at Different Concentrations of Sodium Chloride

Moreover, the Nuclear Magnetic Resonance (NMR) model JEOL JNM ECA500, MHz NMR was used to detect the ThT at Central laboratory of the Faculty of Science- Alexandria University (Table 2).

Table 2. Protocol of the H- NMR

Node	Shift	Comment (ppm rel. to TMS)
3H	2.463	- CH3
6H	3.031-3.060	N-(CH3)2
3H	4.155-4.184	Benzothiazole N-(CH3)
7H	6.901-8.174	Ar

The results of doubling time showed an increase in the doubling time to be the highest (14 days) at 0.08 M NaCl (Fig. 5), while this value decreased to be the lowest (5 days) at the control treatment. This was attributed to the increase in the growth rate at the control treatment with its decrease at 0.08 M NaCl. There was an inverse correlation between the growth rate and doubling time. That is, as the growth rate increases, the doubling time decreases[37]. Doubling time was the time amount in which doubling the size of cells lasted when the relative growth rate was constant. This can be determined simply by the growth rate[38].

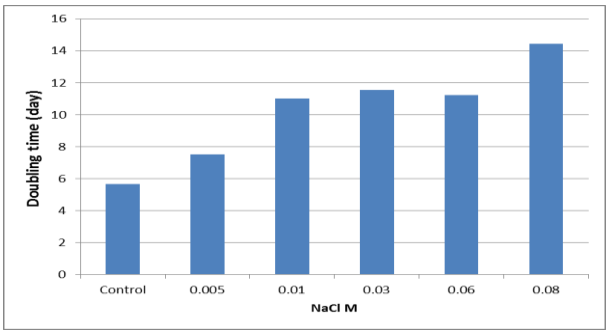

Figure 5. Doubling Time of *C. saipanensis* at Different Concentrations of Sodium Chloride

Proline

Concentrations of NaCl affected the mean rate of producing proline which increased when its concentration increased. It was found that the highest value of the proline rate was at the concentration of 0.08 M NaCl to be 0.509 mg. L^{-1} at the control treatment Fig. 6A. Given that the differences

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between NaCl concentrations were significantly high at $p < 0.001$. Proline has an important role in protecting plants that are exposed to stress. It was found that there was a relationship between the increases in the salt concentration in the culturing medium with the increase in the proline concentration. As a result, this indicated its role in the response of plants to salt stress. Moreover, differences in proline concentrations were regarded as evidence for detecting this type of stress[31]. It was believed that proline accumulation result from stresses was a resultant of basically proline biosynthesis plus the reduction of proline breakdown.

Also, proline accumulation had a role in adapting to stresses. It helps plant cells maintain cell membranes and balance both processes of oxidant and redox. It can act as a signaling molecule to modify the functions of mitochondria[39]. The amino acid (Proline) is a widespread and highly effective osmotic substance that works to protect cells from osmotic stress. In addition, exposing the cells to a high content of salt in the environment leads to the flow of water from the cytosol to the cell outside and this in turn leads to make it expose to dehydration and protein breakdown. Proline plays its role as a hydration substance. Also, the accumulation of proline in cells will oppose the loss of water under osmotic pressure[40].

The results of the difference in growth phases (Fig. 6B) showed that the highest mean value of proline was 0.3 mg. L^{-1} in the second phase of *C. saipanensis* growth, whereas this value decreased to 0.202 mg. L^{-1} in the third phase. This might be attributed to amino acids gradually decreasing with the growth of alga; and their optimal production occurred at entering the stationary phase of the alga growth[41]. Proline is considered one of the multi-functional amino acids and works to regulate many biological processes[42]. Concerning the interaction between the different concentrations of NaCl with the different stages of growth appeared in Fig. 6C, the results showed that the highest value was 0.695 mg.L^{-1} at 0.08 M NaCl at the second phase of growth. This value decreased to 0.09 mg. L^{-1} in the control treatment of the third phase of growth.

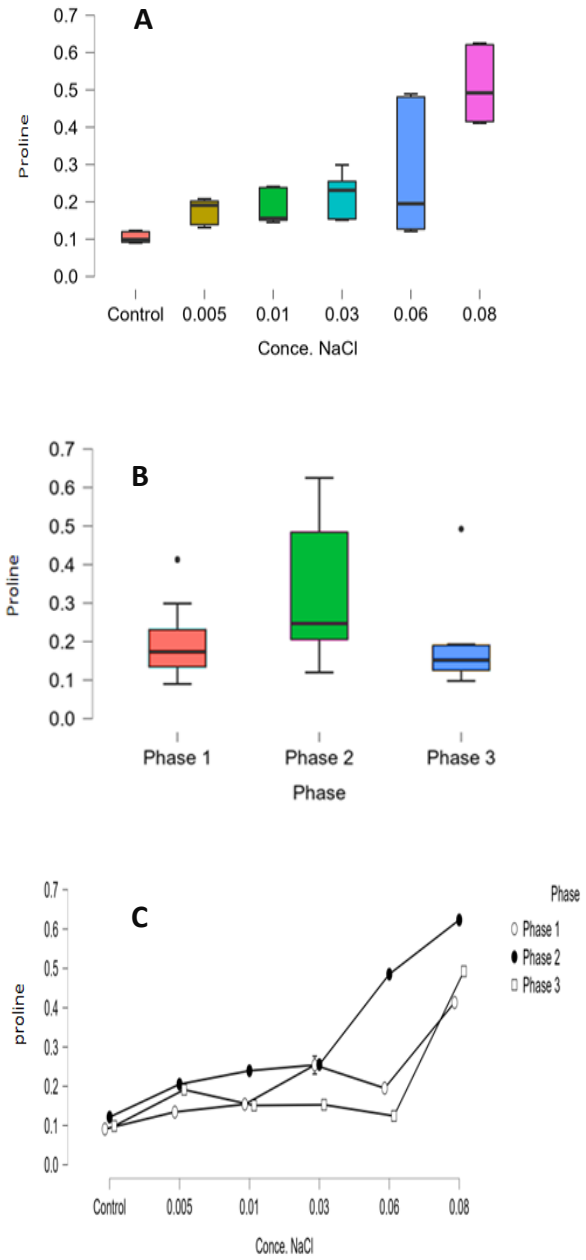

Figure 6. Shows the effect of NaCl, growth phases and their interaction on the proline concentration (mg.L^{-1}) of *C. saipanensis*

Thioflavin T

Results of HPLC and MNR revealed the existing ThT in the test alga in this study (Figures 7 and 8).

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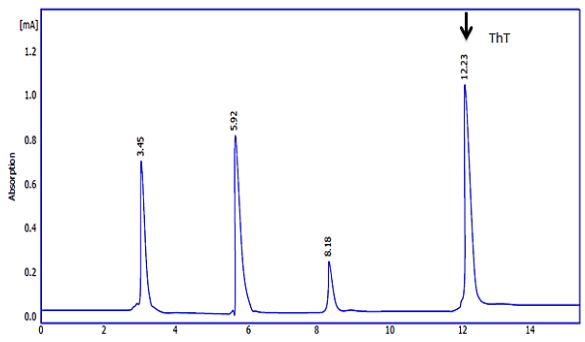

Figure 7. Sample Curve of Thioflaven T

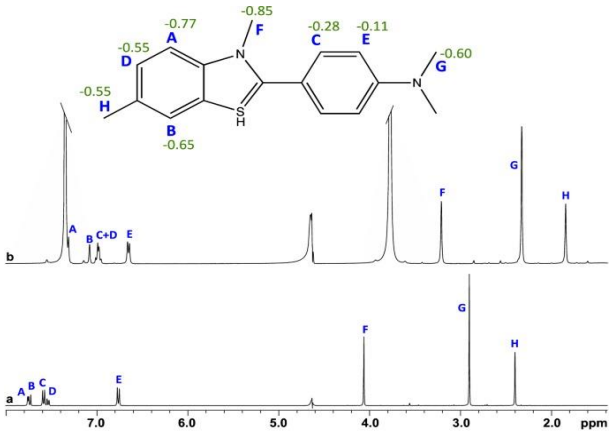

Figure 8. The results of NMR spectral for algal extract

Results of the effect of NaCl on ThT (Fig. 9A) revealed that the 0.005 M NaCl played a role in increasing this compound and recorded the highest value (25.386 mg. L^{-1}) at this concentration. Then, it started to decrease with the increase of NaCl concentration to 17.067 mg. L^{-1} at 0.008 M NaCl. Also, the differences between the concentrations of NaCl were highly significant at $p<.001$. These results show that the concentration of 0.005 M of NaCl is optimal for increasing the production of Th T.

These results might be attributed to the NaCl effect in enhancing the production of phenylalanin which is the initiator of the biological pathway of the ThT[43]. [44]Mentioned that the exposure to NaCl within certain limits of concentrations contributes to make cells tolerate stress via changing the internal cellular structure, keeping the high levels of potassium ions K^+ and calcium ions Ca^{++}, and increasing antioxidant activity. The results of the growth phases difference illustrated in Fig. 9B, indicated that the highest value of the ThT was 21.937 mg.L^{-1} at the second phase, while this value decreased to 19.963 mg.L^{-1} at the third growth phase. When studying the difference of growth phases on *Chlorella* sp alga, indicated that the production of secondary metabolic compounds that contribute to antioxidant activity depended on the age stage of growth[45]. It was found that there was an increase that reached 20% during harvesting cells at the beginning of the stationary phase. In contrast, this value decreased to 5% at the harvest of cells at the late stage of the stationary phase. The interaction between the different concentrations of NaCl with the different growth phases which was estimated in HPLC by the reference to the retention time of the compound extracted from each sample. The highest value of ThT was 27.335 mg. L^{-1} at the concentration of 0.005 M in the second phase (Fig. 9C), whereas this value decreased to the lowest was 16,000 mg. L^{-1} at the concentration of 0.08 M in the third phase of growth.

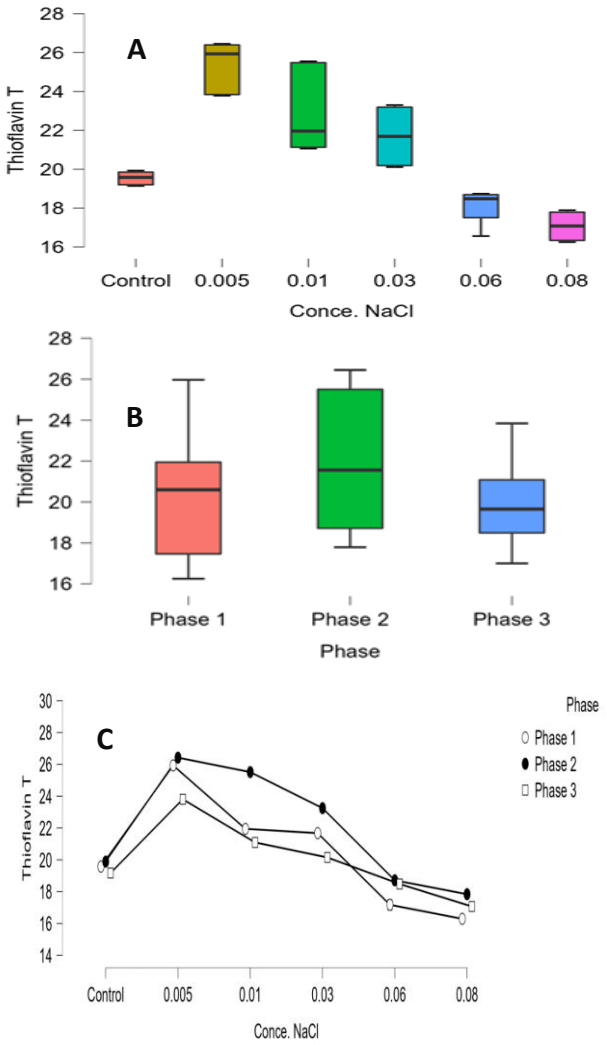

Figure 9. shows the effect of NaCl, growth phases, and their interaction on the concentration of Thioflavin T (mg. L^{-1}) of C. *saipanensis*

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Conclusion

The study confirms the presence of Thioflavin T in C. *saipanensis*, suggesting its potential use for ThT pigment production. All NaCl concentrations tested reduced algal growth rates. Lower NaCl concentrations correlated with increased ThT concentration. Additionally, higher salt concentrations led to an increase in proline. Harvesting cells at the onset of the stationary phase resulted in the highest concentrations of both proline and ThT.

Acknowledgment

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at university of Diyala, College of Education for Pure Sciences.
- No animal studies are present in the manuscript.
- No human studies are present in the manuscript.
- No potentially identified images or data are present in the manuscript.

Authors' Contribution Statement

M. M. I. and F.M.H designed the study. Z.G.F performed the experiments, analyzed the data and wrote the paper with input from all authors.

Journal Declaration:

Third author F.M.H is an editor for the journal but did not participate in the peer review process other than as an author. The authors declare no other conflict of interest.

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انتاج الثايوفلافين T في طحلب *Coelastrella saipanensis* : تأثير كلوريد الصوديوم واطوار النمو المختلفة على معايير النمو

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الخلاصة

هدفت الدراسة الحالية الى البحث عن امكانية انتاج مركب الثايوفلافين T وتأثير تراكيز كلوريد الصوديوم NaCl واختلاف اطوار النمو على معدل النمو وزمن التضاعف والبرولين لطحلب *C. saipanensis* N.Hanagata (Scenedsmacese, Shaerophleales). تم تنمية الطحلب في وسط Bg 11 ضمن ست تراكيز مختلفة من NaCl خلال مراحل النمو المختلفة, وقد كشفت النتائج عن وجود مركب الثايوفلافين T في الطحالب, واظهرت نتائج الدراسة الحالية, ان جميع تراكيز NaCl ذاتا تأثير تثبيطي على معدل النمو عدا معاملة السيطرة, ولوحظ تأثير NaCl على زمن التضاعف والتي كانت اعلى قيمة 14 يوم عند التركيز M 0.08, نتائج البرولين اظهرت ان هناك علاقة طردية بين تراكيز NaCl وقيم متوسط البرولين اذ بلغت اعلى قيمة 0.509 ملغم.لتر$^{-1}$ عند التركيز M 0.08. نتائج اختلاف اطوار النمو وجد ان اعلى قيمة لمتوسط البرولين بلغت 0.3 ملغم.لتر$^{-1}$ في الطور الثاني اما نتائج التداخل بين التراكيز المختلفة من NaCl مع الاطوار المختلفة من النمو اعلى قيمة بلغت 0.695 ملغم.لتر$^{-1}$ في التركيز M 0.08 عند الطور الثاني من النمو. ان انتاج اعلى قيمة من مركب الثايوفلافين T بلغت 25.386 ملغم.لتر$^{-1}$ عند التركيز M 0.005. اما اختلاف اطوار النمو فوجد ان اعلى قيمة لمركب الثايوفلافين T بلغت 21.937 ملغم.لتر$^{-1}$ عند الطور الثاني من النمو. في حين نتائج التداخل بين التراكيز المختلفة من NaCl مع اطوار النمو فوجد ان اعلى قيمة لصبغة الثايوفلافين T بلغت 27.335 ملغم.لتر$^{-1}$ في التركيز M 0.005 للطور الثاني من النمو. من هذه التجربة نستنتج وجود صبغة الثايوفلافين T في *C. saipanensis*, ان تركيزات كلوريد الصوديوم ومراحل النمو المختلفة لها تأثير على معدل النمو وانتاج البرولين ويسلط الضوء على امكانية استخدام الطحالب لانتاج صبغة الثايوفلافين T لاغراض مختلفة.

الكلمات المفتاحية: , *C. saipanensis* .الثايوفلافين T .كلوريد الصوديوم, معدل النمو, البرولين.