Zagazig J. Agric. Res., Vol. 51 No. (4) 2024 745-752

Biotechnology Research

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ERGOSTEROL PRODUCTION IN ALGAE *Chlorella sorokinana* AND STUDY THE EFFECT OF SODIUM CHLORIDE AND GROWTH PHASES ON PHOTOSYNTHETIC PIGMENTS

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Received: 06/08/2024; Accepted: 260/08/2024

ABSTRACT: The present study aimed to detect the production of Ergosterol in *Chlorella sorokinan*a and the effect of sodium chloride (NaCl), and the difference of growth phases on the production of the compound and photosynthetic pigments. The results showed that there is a positive effect on increasing the production of the Ergosterol compound. Its maximum value was 48.591 mg. L^{-1} at the concentration of 300 ppm. Concerning the difference in growth phases, the second growth phase was the best in obtaining the maximum value of Ergosterol, which was 44. 941 mg. L^{-1}. Also, the results of the interaction between the concentrations of NaCl and the difference in the growth phases indicated that the highest value was 50.493 mg. L^{-1} at the concentration of 300 ppm at the second growth phase. In contrast, there was a negative effect of NaCl on photosynthetic pigments; the highest values of chlorophyll a and b and total chlorophyll were 0.04, 0.07, and 0.13, respectively, at the control treatment, whereas these values gradually decreased with the increase of NaCl concentration to reach the lowest values; these were 0.01, 0.04 and 0.06 mg. L^{-1} respectively at the concentration of 300 ppm. The results of the different dates of cell harvesting showed that the highest values for obtaining an increase in the concentration of chlorophyll a and b pigments and total chlorophyll were at phases 1; they were 0.06, 0.09, and 0.17 mg. L^{-1} respectively. Accordingly, we concluded that the salt stress from NaCl had a positive effect on increasing the concentration of Ergosterol, which is one of the antioxidant steroidal compounds. On the other hand, it had a negative effect on the photosynthetic pigments.

Key words: *Chlorella sorokinana*, NaCl, growth phases, Ergostrol, chlorophyll pigment.

INTRODUCTION

Microalgae are considered unicellular organisms that can carry out photosynthesis. Plus, their industrial importance is that they are sustainable sources of a wide group of primary and secondary metabolic compounds such as carbohydrates, proteins, pigments, and antioxidants. The biomass of these algae is the most economically valuable for algae-based biotechnology (**Brasil *et al.*, 2017**). *Ch. sorokinana* is regarded as one of the microalgae growing in freshwater, and it is of interest to researchers due to its rapid growth rate and the ability to withstand different environmental stresses such as light, temperature, salinity, etc. (**Spencer, 2019**). In general, this alga is a microalga suitable for laboratory growth and conducting various laboratory experiments (**Lizzul *et al.*, 2018**). The side effects in patients subjected to conventional therapies led to the search for alternative therapies from biological sources. Therefore, research is directed to many of these sources, and microalgae were the most demanding in terms of their ease of growth and their ability to synthesize compounds that have important biological activity (**Reyna *et al.*, 2018**).

Steroids are organic compounds with effective biological activities. It has been proven that microalgae can produce these compounds, considering that arachistrol is one of the steroidal compounds with effective biological

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activity (**Miller, 2014**). The biosynthesis of arachitrol is a complex pathway involving the participation of many enzymes. Also, it has been found that a low rate of biosynthesis in vivo limits cell division and the inability to adapt to different stresses (**Jordá and Puig, 2020**). Archesterol is a steroid found in cellular membranes, and it is critical for regulating cellular processes (**Choy *et al.*, 2023**). Besides, archesterol is a provitamin, which is a precursor substance that is converted to vitamin D3 under UV light plus the pharmacological effects including antioxidant, antimicrobial, antidiabetic, etc. (**Rangsinth *et al.*, 2023**).

Given that, various ecological stresses are accompanied by the accumulation of many secondary metabolic compounds like salt stress. In contrast, there has been a decrease in chlorophyll pigments (**Agathokleous *et al.*, 2020**). Chlorophylls are essential pigments in photosynthesis and play a vital role in absorbing light energy. The metabolic pathways that control chlorophyll biosynthesis are a series of interconnected reactions mediated by a group of enzymes affected by various environmental stresses (**Li *et al.*, 2024**).

The study aimed to detect the production of Ergosterol in *Chlorella sorokinan*a the effect of sodium chloride (NaCl) and the difference of growth phases on the production of the compound and photosynthetic pigments.

MATERIALS AND METHODS

This experiment was conducted in sterilized conditions (air laminar cabinet flow) in the Laboratory of Plant Cell and Tissue Culture/ Department of Biology/ Faculty of Education for Pure Sciences/Diyala University. A pure sample of *Chlorella sorokinana* was obtained from the College of Sciences at the University of Baghdad. and the samples were kept at a temperature of 2±25℃ within an alternating light-dark system 8/16 hours light-dark with a light intensity of 3000 lux.

Preparation of the Media Culture

Bg-11 culturing medium was prepared by taking 1. 6g of culturing medium by using a sensitive balance. Next, this medium was dissolved in 1 liter of distilled water in a 1000 cm flask, which was placed in a Hotplate and magnetic device to dissolve the culturing medium. After that, the flask was covered with a cellphone to sterilize the culturing medium via the autoclave. The conditions of the Autoclave are: temperature 118°C and pressure 121 bar). It was sterilized for 15 minutes, and then the culturing medium was left to cool at room temperature.

Preparation of the Isolate

Only 900 ml of culturing medium was taken and placed in each replicator, and the volume was completed with algal isolate in only one liter.

Preparation of Sodium Chloride

Different concentrations of NaCl (0, 100, 200, and 300 ppm) were weighted using a sensitive balance. The salt was added to the culturing media containing the algal isolate at different concentrations.

Cell Harvesting

Chlorella cells were harvested three times during the experiment duration along the growth phases of each group of the experimental unit. The first harvest was after 7 days (phase 1), the second harvest was after 14 days (phase 2), and the third harvest was after 21 days (phase 3) (**Jawad, 1982**).

Diagnosis and Quantification of Ergosterol

Ergosterol isolated by HPLC equipment was used using an isocratic analytical method. The absorbance spectrum was plotted at 290nm with a UV detector, and an injector sample with a 50 uL loop was used. The column used was a Supercoil C18 (Supelco; 5 µm, 250 x 4.6 mm) and, as mobile phase, HPLC-grade methanol: acetonitrile (80:20 v/v). at a flow rate of 1.0 ml/ min. (**Chiocchio and Matković, 2011**).

Estimation of the Photosynthetic Pigments

The estimation of the photosynthetic pigments, including chlorophyll a & b, the total chlorophyll was carried out by using (2.5) cm^3 of the icy acetone at the concentration of 80% with (7.5) cm^3 of the algal isolate. The mixture was put in a centrifuge at 3500 rpm for (10) minutes. Then, it was taken only (1) cm^3 of the filtrate to measure the absorbance via the spectrophotometer.

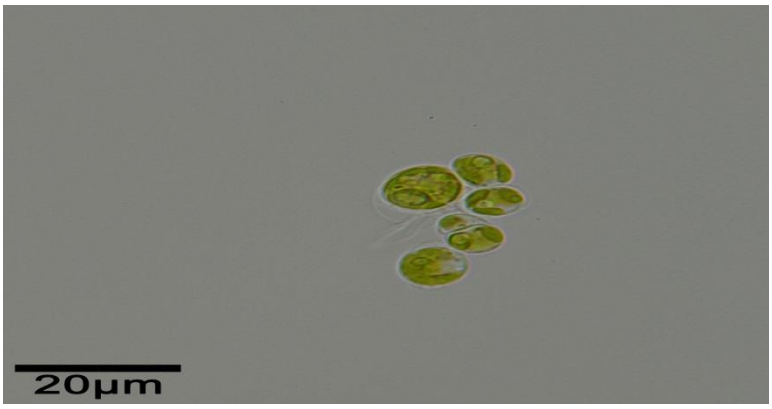

Fig. 1. The Phenotypic Shape of *Chlorella sorokinana* (light Microscope)

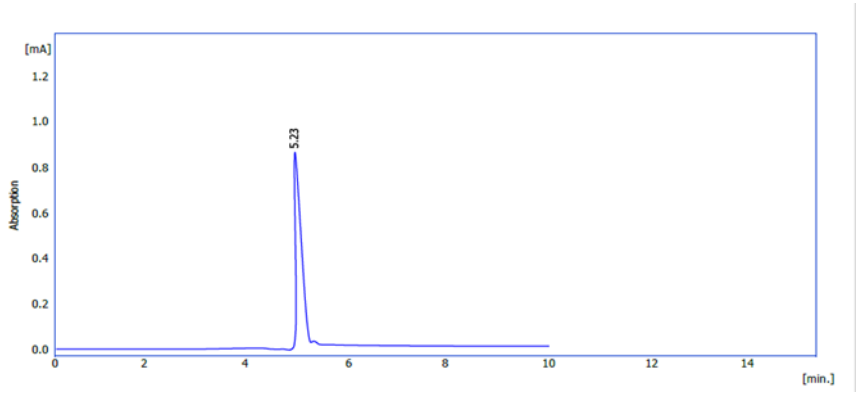

Fig. 2. Standard Curve of Ergosterol

The absorbance was measured at the following positive lengths (480_645_663) nm.

The pigments were estimated according to the following equations:

Chlorophyll a = 12.7 x absorbance at 663 nm - 2.69 x absorbance at 645 nm

Chlorophyll b = 22.9 x absorbance at 645 nm - 4.68 x absorbance at 663 nm

Total chlorophyll= 20.2 x absorbance at 645 +8.02 x absorbance at 663 nm (Eijckelhoff and Dekker, 1997).

Experiment Design

The experiment was designed using Completely Randomized Design (CRD) with three replicates for each treatment. The results were analyzed using SPSS at a significance level of 0.05.

RESULTS AND DISCUSSION

The Effect of Different Concentrations of Sodium Chloride, the Difference of Growth Phases, and the Interaction Between Them on the Ergosterol

The results that appeared in Table 1 showed that the salt stress had a positive role in increasing the concentration of Ergosterol, as the values increased to reach the maximum of 48.591m.g^{-1} at the concentration of 300 ppm of NaCl. In contrast, they decreased to reach the minimum of 35.266 m. g^{-1} at the control treatment. On the other hand, the results of the difference of growth phases also showed significant differences; the highest value was 44.941 m.g^{-1} in phase 2 of the growth. Concerning the results of the interaction, the highest value was 50.493 m.g^{-1} at the concentration

Table 1. Effect of different concentrations of NaCl, the difference of growth phases, and their interaction on the Ergosterol concentration in *Chlorella sorokinana*

Phases	Concentration NaCl (ppm) Ergosterol				Effect of phases
	Control	100	200	300	
Phases 1	33.166[l]	40.356[i]	42.400[g]	46.626[d]	40.637[c]
Phases 2	37.306[j]	44.293[f]	47.673[c]	50.493[a]	44.941[a]
Phases 3	35.326[k]	41.413[h]	45.806[e]	48.653[b]	42.800[b]
Effect of NaCl	35.266[d]	42.021[c]	45.293[b]	48.591[a]	

of 300 ppm in the phase 2 of development. This has been attributed to the fact that the microalgae - due to various environmental pressures- continuously adjust their cellular mechanisms to deal with these pressures. The accumulation of secondary metabolic compounds is closely connected with the variables occurring in their metabolic pathways. This synthesis can occur through exposure to a number of abiotic stresses (Paliwal *et al.*, 2017). Duo *et al.*, (2024) explained that microalgae have a wide range of strategies to prevent the negative effects of inappropriate conditions or when they are exposed to various environmental stresses such as NaCl.

Isah *et al.* (2018) clarified that the production of the secondary metabolites is affected by the difference in growth phases, as well as different environmental conditions. When studying the difference of growth phases of *Chlorella* sp., **Sunil and Nirpesh (2023)** showed that the production of the secondary metabolic compounds contributing in the antioxidant activity depended on the age stage of growth. It was found that there was an increase when harvesting cells at the beginning of the stationary phase. In contrast, this percentage decreased when harvesting cells at the late stage of the stationary phase. Abiotic stress stimulates the immune system to preserve cells so that they can complete their life cycle. This leads to the consumption of materials resulting from photosynthesis, which is followed by an increase in the production of secondary metabolic compounds that act as antioxidants to combat free radicals (**Al-Mohammad, 2023**).

The Effect of Different Concentrations of Sodium Chloride, the Difference of Growth Phases, and Their Interaction On the Pigment of Chlorophyll A

The results appeared in Table 2 below showed that the maximum value of the chlorophyll *a* concentration was (0.04) mg. L^{-1} at the control treatment, whereas these values gradually decreased with the increase of NaCl concentration, as the relationship was inverse between the chlorophyll *a* pigment and the salt concentration. Its lowest value reached (0.01) mg. L^{-1} at the concentration of 300 ppm. On the other hand, the different of growth phases showed significant differences when the cells were harvested; its maximum value reached (0.06) mg. L^{-1} at phase 1 of growth, while the lowest value reached (0.014) mg. L^{-1} at phase 3 of development.

Regarding the results of the interaction, the maximum value of chlorophyll a was (0.07) mg. L^{-1} in the control treatment at phase 1 of growth. According to these results, it became obvious that the addition of the salt concentration negatively affects the chlorophyll pigment. That is, adding sodium chloride led to a decrease the values, and this is consistent with the study conducted by **Fal *et al.* (2022)**. It was stated that adding salt led to a reduction in chlorophyll *a* value. This is due to the occurrence of toxic osmotic ionic stress, which caused a decrease in the rate of photosynthesis and in turn reduced chlorophyll and protein. As for the increase in the chlorophyll *a* value during the second phase of growth. This increase might be attributed to

Table 2. Effect of different concentrations of NaCl, difference of growth phases, and their interaction on the concentration of chlorophyll *a* in *Chlorella sorokinana*

Phases	Concentration NaCl (ppm) chlorophyll a				Effect of phases
	Control	100	200	300	
Phases 1	**0.07^{a}**	**0.02^{e}**	**0.004^{g}**	**0.003^{g}**	**0.06^{a}**
Phases 2	0.03^{d}	0.06^{b}	0.06 b	0.04 c	0.015^{b}
Phases 3	0.02^{e}	0.01^{ef}	0.01^{fg}	0.008^{fg}	0.014^{b}
Effect of NaCl	0.04^{a}	0.03^{b}	0.027^{c}	0.01^{d}	

the role of sodium chloride salt in the inhibitory effect on the concentration of photosynthetic pigments, which led to its decrease after a growth period. **Ahmad *et al.* (2018)** mentioned that the osmotic stress resulting from sodium chloride directly affected the decrease in the concentration rate of chlorophyll pigments. Also, this stress might damage the photosystem II. That is, each of the donor and acceptor electrons of the PSII reaction center are sensitive to sodium chloride stress (**Ji *et al.*, 2018**).

The Effect of Different Concentrations of Sodium Chloride, the Difference of Growth Phases, and Their Interaction on the Pigment Chlorophyll *B*

The results given in Table 3 explained that the highest value of chlorophyll *b* concentration was (0.070) mg. L^{-1} was used in the control treatment, while the lowest value was 0.007 mg. L^{-1} when the concentration was 300 ppm. As for the results of the difference in cells harvesting dates, they showed that the highest value reached 0.09 mg. L^{-1} in phase 1 of growth, while the lowest value was 0.02 mg. L^{-1} in the phase 3 of growth. The results of the interaction showed that the highest values reached 0.15 mg. L^{-1} at the concentration of 100 ppm in the phase 1 of growth. These results might be attributed to the role of NaCl in changing the genes of genetic reproduction of photosynthetic pigments and the negative change in the activity of the enzymes responsible for these pigments, which reduces the efficiency of the photosystem and the rate of absorption of carbon dioxide (**Kebeish *et al.*, 2014**). Concerning the results of the difference of growth phases, the explanation of the increasing values in phase 1 of growth is because of the conversion of primary metabolites into secondary metabolites and the nonoccurrence of additional growth of the cells. In contrast, their decreasing values in phase 3 are attributed to consuming the materials stored in the cells along with the consumption of nutrients in the culturing medium (**Ramawat, 2008**).

The Effect of Different Concentrations of Nacl, the Difference of Growth Phases, and the Interaction Between Them on the Total Chlorophyll Concentration

It is obvious from the results presented in Table 4 below that the addition of NaCl has an inhibitory role in the values of chlorophyll, as indicated in the results of *total* chlorophyll. The value of total chlorophyll increased in the control treatment to reach 0.130 mg. L^{-1}, whereas it decreased to reach its minimum, 0.06 mg. L^{-1} at the concentration of 300 ppm. The results of the difference in the date of cell harvesting clarified that the maximum values reached 0.17 mg. L^{-1} in the phase 1 of growth. As for the results of the interaction, in the control treatment at phase 1 of growth, the highest values were 0.229 mg. L^{-1} with significant differences of the lowest values. This decrease at the salt concentrations resulted from the effect of both chlorophyll *a* and *b*. As it is known that total chlorophyll is the result of collecting these pigments, and thus, their impact on total chlorophyll was shown. **Atteya *et al.* (2022)** stated that chlorophyll content has an inverse correlation with salinity level.

Table 3. Effect of different concentrations of NaCl and the difference of growth phases and their interaction on the concentration of chlorophyll b in *Chlorella sorokinana*

Phases	Concentration NaCl (ppm) chlorophyll b				Effect of phases
	Control	100	200	300	
Phases 1	0.03^{e}	0.15^{a}	0.03^{e}	0.01^{gh}	**0.09^{a}**
Phases 2	0.13^{b}	0.01^{fg}	0.08^{c}	0.007^{gh}	**0.04^{b}**
Phases 3	0.06^{d}	0.05^{d}	0.05^{d}	0.004^{h}	**0.02^{c}**
Effect of NaCl	0.070^{a}	0.069^{a}	0.05^{b}	0.04^{c}	

Table 4. The effect of different concentrations of NaCl, the difference of growth phases and their interaction on the total chlorophyll concentration of *Chlorella sorokinana*

Phases	Concentration NaCl (ppm) Total chlorophyll				Effect of phases
	Control	100	200	300	
Phases 1	0.229^{a}	0.020^{f}	0.076^{de}	0.018^{f}	0.17^{a}
Phases 2	0.047^{ef}	0.186^{ab}	0.140^{bc}	0.133^{c}	0.07^{b}
Phases 3	0.115^{cd}	0.067^{def}	0.053^{ef}	0.046^{ef}	0.04^{c}
Effect of NaCl	0.130^{a}	0.09^{b}	0.08^{b}	0.06^{b}	

Conclusion

The salt stress had a positive effect on the increase of the steroid Ergosterol compound, while there was an inverse effect on chlorophyll pigments. The addition of the concentration of sodium chloride salt had an inhibitory effect on the photosynthetic pigments represented by chlorophyll *a* and *b*. Concerning the difference in the date of harvesting the cells, it was found that phase 2 of growth was the best to obtain the highest values in Ergosterol and phase 1 the best to obtain the highest values in chlorophyll pigments.

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انتاج الأركوستيرول في طحلب *Chlorella sorokinana* ودراسة تأثير كلوريد الصوديوم ومراحل النمو على صبغات البناء الضوئي

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الخلاصة: هدفت الدراسة الحالية الى دراسة الكشف عن مركب الاركيسترول في طحلب Chlorella sorokinana فضلا عن تأثير كلوريد الصوديوم واختلاف مراحل النمو على انتاج المركب وصبغات البناء الضوئي، أظهرت النتائج ان هناك تأثير ايجابي في زيادة انتاج مركب الاركيسترول اذ بلغت اعلى قيمة 48.591 ملغم.لتر$^{-1}$ عند التركيز 300 ,ppm اما اختلاف المراحل العمرية كان الطور الثاني من النمو هو الافضل في الحصول على اعلى قيمة من الاركيسترول بلغت 44.941 ملغم.لتر$^{-1}$، واظهرت نتائج التداخل بين تراكيز كلوريد الصوديوم واختلاف موعد حصاد الخلايا ان اعلى قيمة بلغت 50.493 ملغم.لتر$^{-1}$ عند التركيز 300 ppm في الطور الثاني من النمو، في المقابل كان هناك تأثير سلبي لكلوريد الصوديوم على صبغات البناء الضوئي اذ بلغت اعلى قيمة لكلوروفيل a و b والكلوروفيل الكلي 0.04 و0.07 و0.13 على التوالي عند معاملة السيطرة في حين انخفضت هذه القيم تدريجيا مع زيادة تركيز كلوريد الصوديوم لتبلغ ادناها 0.01 و0.04 و0.06 كلغم.لتر$^{-1}$ على التوالي عند التركيز 300 ,ppm نتائج اختلاف موعد حصاد الخلايا اظهرت ان اعلى القيم للحصول على زيادة في تركيز صبغات الكلوروفيل a و bوالكلوروفيل الكلي كانت عند الطور الاول من النمو والتي بلغت 0.06 و0.09 و0.17 ملغم.لتر$^{-1}$ على التوالي، نستنتج من هذه الدراسة ان الاجهاد الملحي باستخدام كلوريد الصوديوم تأثير ايجابي في زيادة تركيز الاركيسترول وهو من المركبات الستيرويدية المضادة للاكسدة، في حين كان له تأثير سلبي على صبغات البناء الضوئي.

المحكمـــون:

1- أ.د. ســـــعاد خيري عبد الوهاب أستاذ علوم الحياة - كلية التربية للعلوم الصرفة – العراق.

2- أ.د. سمير احمد مرغنى محجوب أستاذ الميكروبيولوجيا الزراعية – كلية الزراعة - جامعة الزقازيق – مصر.