

Degradation potential, use and growth promotion and antioxidant properties of *Haematococcus Pluvialis* in algae cell culture system under the influence of phenol

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Abstract

In response to highly severe environmental conditions, the accumulation of vast levels of astaxanthin by *Haematococcus Pluvialis* is a recorded event. However, there is little investigation into the nature and possible interactions of the poisonous chemical factors on this pigment's synthesis. In this study, *H. pluvialis* was exposed to the environment phenol to determine cellular responses, synthesis of pigments, and astaxanthin. The phenol influences in different concentrations (0, 50, 100, and 150 mg l⁻¹) were checked out by two ways; a treatment (14 days) and pre-treatment (7 days + 7 days) done on green and red stages of *H. pluvialis*. The amount of carotenoid, protein, and dry weight increased, but the amount of carbohydrates and chlorophyll reduced in treated green cells with a phenol. The pre-treatment increased the amount of chlorophyll a, carbohydrates, and carotenoids in green cells, but it did not significantly affect the dry weight, protein of green cells, and the amount of protein in red cells. The phenol treatment also enhanced the carotenoid, carbohydrate, dry weight, and protein of red cells. In general, the treatment and pre-treatment of phenol in the green phase and low concentrations significantly affected the alga. However, the pre-treatment had a more considerable effect on red cells in high concentrations. While the treatment in the high concentrations had notable effects on the xanthophylls, pre-treatment had the same effects on the xanthophylls at low concentrations. These results show that *H. pluvialis* can adapt to toxic environments due to the xanthophylls.

Introduction

Phenol is one of the major organic pollutants in wastewater, discharged from many industrial processes such as textile processing, coal, resin synthesis, perfume production, chemical spills, and pharmaceutical manufacture (Sarika et al. 2020). Simple phenol (Fig. 1) has an aromatic ring with six carbons. It has a hydroxyl functional group with electron pairs of the oxygen atom that the hydroxyl functional group conjugated with the aromatic system that causes partial transfer of negative charge from oxygen to the ring and delocalization of the charge, and it additionally strengthens polarization of the O-H bond. Consequently, phenol gains an acidic character and can form toxic molecules (Anku et al. 2017).

The fundamental factor that determines phenol toxicity is their reaction with a cell's biomolecules and is related to the easiness of donation of free electrons by them from an oxidized substrate. Phenols undergo active transformation by oxidases within cytochrome P450 when they enter after the cell penetration. The effect of their practice is the organization of phenoxy radicals and intermediate metabolites, like semiquinone and quinone methides which interact with bio-molecules in the cell (Fig. 2). Also, phenols can produce ROS, like superoxide radicals or hydrogen peroxide in these reactions. Transformation of phenols leads to the enhancement of toxicity compounds by the organization of electrophilic metabolites that may damage DNA, proteins, lipids, and enzymes (Harris et al. 2020; Perillo et al. 2020). The harmful influence of phenols and their derivatives and their entry into nature leads to acute toxicity, histopathological changes, mutagenicity, carcinogenicity, and death of cells (Schweigert et al. 2001).

Introduced phenol into the surroundings or nature may be an ecological hazard because of its high toxicity, even at low concentrations. The essential factor that determines phenol toxicity is the reactivity of the compound with a cell's biomolecules and is related with easiness of donation of free electrons by phenol from oxidized substrate. A fish or algae constantly ingests organic particles suspended in water. Some pollutants, such as phenol, may be simultaneously ingested, and these substances can cause poisoning (Anku et al. 2017).

Some species can degrade phenol and toxic compounds (Dhatwalia et al. 2016; Tan et al. 2017; Mansouri et al. 2021) Many aerobic and anaerobic microorganisms in nature can use phenol as a carbon and energy source (Margesin et al. 2004; Pradeep et al. 2015). Essentially, biodegradation of phenol is its biotransformation into simpler metabolites such as H₂O, CO₂ (aerobic), or CH₄ (anaerobic) (Al-Khalid & El-Naas 2012), which this factor occurs in some organisms, but if the content of phenol increases, the oxidation also increases; thus, the growth will be gradually inhibited, and even mortality occurs (Duan et al. 2017).

The oxidation process is harmful to most living organisms, and some organisms produce antioxidants to reduce the harm of free radicals in the cells of animals and plants (Kapoor et al. 2019; Hasanuzzaman et al. 2020). It is necessary to generate, utilize and develop effective and safer antioxidants, protecting living organisms from the hazardous effects of ROS (Spinedi et al. 2021).

H. pluvialis is a microalga that contains the pigment astaxanthin. Astaxanthin (Fig. 3) has high antioxidant properties that even show antioxidant activities greater than Vitamin C (Rao et al. 2015; Zhao et al. 2019). It has functional groups with two β rings and a hydroxyl at each end (carotenoids - OH) which confers antioxidant properties (Ambati et al. 2014). The bioactive pigments can potentially improve metabolites and growth of alga in different environments (Nakano et al. 2020; Rashidian et al. 2021); Thus, algae probably have due to the pigments which have antioxidant properties an ability to counteract harmful effects of the environment and improve metabolites. For this reason, this research is to investigate the toxic effects of phenol in two time periods (7 and 14 days) to improve metabolites and astaxanthin.

Material's And Methods

Algal culture and growth conditions

The algae studied in this investigation is *H. pluvialis* from the order Volvocales. Stock cultures were kept in Bold's basal medium (BBM) (Andersen 2005), then they maintained at a light intensity of $50 \pm 3 \mu\text{mol m}^{-2} \text{s}^{-1}$ at $22 \pm 3^\circ\text{C}$ temperature under light conditions sixteen hours of light and eight darkness hours, pH 7, and aerated rate of 70 ml min^{-1} . In this study, we used the BG11 (Blue-Green) medium of negative nitrogen to induce red cells (Stanier et al. 1971).

Algae Growth Rate

The algae growth rate was measured for fourteen days in BBM with pH 7 (Rezazadeh et al. 2022). In order to perform the treatment (green stage), algae were cultured in Erlenmeyer flasks with 150 ml BBM with different concentrations of phenol (0, 50, 100, and 150 mg l^{-1}). After 14 days, the cells were harvested to check the parameters. For the pre-treatment, *H. pluvialis* was cultured in Erlenmeyer flasks with 150 ml BBM with determined concentrations of phenol for seven days. Then it was transferred to the natural BBM for the other seven days (green stage). As well as after the green stage, the cells were transferred to BG11 medium without nitrogen (Stanier et al. 1971) for another 14 days (red stage) to measure parameters in red cells. All cultures (treatment and pre-treatment) in the green and red stages were harvested by centrifugation at 3500 r/min and frozen at -30°C to measure parameters.

Analytical Methods

In this study, we measured the amount of dry weight, chlorophyll a, b, and total carotenoids (Dere et al. 1998), protein (Bradford 1976), and carbohydrate (Fales 1951). To measure the types of red stage carotenoids, the cells were dried with a freeze dryer, and the concentrations of carotenoids were checked out by reversed-phase Beckman System Gold HPLC (module 125 solvent) equipped with a diode array detector (model 168 Nouveau) according to the method of (Gilmore et al. 1991).

Statistical analysis

Each experiment was carried out with three replications, the data were analyzed by one-way analysis of variance and post-hoc mean separations were performed by Duncan's multiple range test < 0.05 .

Data are average of three replications $\pm$ SE. Mean comparison of treatment and pre-treatment data were compared with Duncan's test with 95% confidence interval. Letters Different represent a significant difference between the mean).

Results

The response of *H. pluvialis* to adding different phenol concentrations is shown in table 1. The phenol treatment significantly improved the dry weight of green cells in the low concentration. The highest dry weight was observed at 50 mg l^{-1} treatment (60% increase compared to control), and the increase of phenol concentration significantly reduced the dry weight in *H. pluvialis*. Like green cells, the phenol treatment also had significant effects on the dry weight of red cells at 50 ml^{-1} concentrations and

enhanced the dry weight by up to 45%. Table 1 shows that pre-treatment had no significant effects on green cells, but all pre-treatment concentrations significantly improved the dry weight of red cells (Table 1).

Phenol is an influencing factor on pigments. The highest amount of chlorophyll an obtained in a control sample with 0.65 mg/g fresh weight, but the pre-treatment boosted the amount of chlorophyll at low concentrations. The b-chlorophyll range also changed under the influence of phenol, and its highest amount was observed in the control sample, and chlorophyll b was restrained by enhancing the phenol. The 50 mg l⁻¹ concentration showed the highest amount of chlorophyll b in pre-treatment (12% growth compared to the control) (Fig. 4).

The amount of carotenoids significantly raised with the increase of phenol in the green cells. The highest level of carotenoids was observed in 150 mg l⁻¹ phenol (20% increase). The profile obtained from the treatment on carotenoids of red cells revealed that all concentrations of phenol improved the carotenoid content compared to controls (approximately 42%). In green cells, pre-treatment developed carotenoid levels at low concentrations, but the increase of phenol significantly limited carotenoids. With concentration increases, the carotenoid quantity of the pre-treatment improved in red cells, and the highest amount of carotenoids in pre-treatment was observed at high concentrations (Table 1).

In the therapy of green cells, the increasing concentration of phenol prevented the growth of total carbohydrates, and the lowest amount of carbohydrate was remarkable in 150 mg l⁻¹ concentration with 37% decline. The pre-treatment significantly augmented the total carbohydrates in green cells, and The highest level of carbohydrates was observed at 50 mg l⁻¹ concentration. All used pre-treatment concentrations considerably affected the total carbohydrate of red cells. The highest amount of carbohydrates was seen in 50 mg l⁻¹ concentration, with 27% improvement compared to the control. The pre-treatment of red cells also developed total carbohydrates, and the greatest extent was meaningful in 100 mg l⁻¹ concentration (28% increase) (Table 1).

Phenol treatment promoted the total protein content of green cells at the 50 mg l⁻¹ concentration. A phenol promotion caused a meaningful decrease in the total protein content in the treatment. The highest amount of protein in the treatment and pre-treatment was obtained at a 150 mg l⁻¹ concentration. However, those change is not significant at the red stage of treatment. Phenol treatment promoted the total protein content of green cells at the 50 mg l⁻¹ concentration. A phenol promotion caused a meaningful decrease in the total protein content in the treatment. The highest amount of protein in the treatment and pre-treatment was obtained at a 150 mg l⁻¹ concentration. However, those change is not significant at the red stage of treatment. Also, there was no significant effect on the total protein content of red cells in the treatment and pre-treatment (Table 1).

The astaxanthin content did not change at low concentrations, but the high concentrations of phenol dwindled the level of astaxanthin. The most lutein content was observed with an increase of 95 and 116 % in the 150 mg l⁻¹ concentration of treatment and 50 mg l⁻¹ concentration of pre-treatment, respectively. Treatment caused an expansion in Neoxanthin and Canthaxanthin in treatment with 66% and 53%. The highest amount of β -Carotene was observed in the treated algae by 100 mg l⁻¹ phenol with 81% increase compared to the control. Pre-treatment by 50 mg l⁻¹ concentration had stimulating effects on the Neoxanthin, Violaxanthin, and Lutein content, which the most development respectively observed with 84, 40, and 116%. The phenol pre-treatment increased the amount of Canthaxanthin in 150 mg l⁻¹ concentration, two-fold more than the control (Table 2).

Discussion

Phenols are a group of toxic compounds that living organisms have different reactions to this group, but in general, high concentrations of phenols have more destructive effects on living organisms (Guerin et al. 2022). In this investigation, *H. pluvialis* was grown at a low phenol concentration. This effect is due to the degradation of phenol at low concentrations and its use as a carbon source. However, an increasing concentration had an inhibition effect on algae. The phenomenon substantially resulted from the toxicity of phenol at high concentrations. Previous research has also shown that living organisms could grow at different phenol concentrations, but high concentrations negatively affect cells (Dayana Priyadharshini & Bakthavatsalam

2019; Saleem et al. 2018). The pre-treatment of phenol did not affect algal growth in the green stage. The short time of the pre-treatment and degradation of phenol are the main factors in which phenol didn't affect an organism (Bai et al. 2007). Leonard also Stated that this effect is due to the expanded rate of phenol degradation by the cells (Leonard & Lindley 1998). The improved dry weight of red cells at low concentrations of treatment and pre-treatment with rising phenol probably reflects the effect of early toxicity on green cells that remain until this stage of algal life.

The chlorophyll content declined with the gain of phenol in treatment and pre-treatment. However, the phenol had motivational effects on chlorophyll a and b in the low concentration of pre-treatment and improved chlorophylls. Phenol can sometimes be inciting agent, and the biosynthesis of pigments to elicit in low concentrations. Contrary to our results, previous researchers have reported that phenol diminishes chlorophyll contents over time (Duan et al. 2017). At high concentrations, phenoxy-radicals damage the chloroplast membrane, While Cho showed that the increase of phenol improves pigments in *Dunaliella salina* (Cho et al. 2016).

The phenol therapy declined the protein content of green cells in high concentrations. Long-term exposure to the high concentration of phenol to proteins damage, and it is consistent with reducing protein content in high concentrations. This reduction is due to increased free radicals and their destructive effects on proteins (Gill et al. 2010). The low concentrations of phenol can increase the protein content of green cells, which may be used as a carbon source for protein biosynthesis (Das et al. 2015). The treatment diminished the weight of red cells. The destructive effects of treatment may persist for a long time, and those effects will lead to a decrease in protein levels. The amount of protein in the tissue or cells depends on the kinetic balance of its catabolism. The decrease in protein content may be due to proteolysis increase to compensate lost energy of cells or the organization of lipoproteins for mending cells (Liu et al. 2018). Pre-treatment has a stimulatory effect on protein formation due to its short course. Contrary to our results, Liu exhibited that high concentrations of phenols reduced proteins in *Ceratophyllum Demersum*, but low concentrations did not have significant effects (Liu et al. 2018).

The most content of carotenoids was observed in the high concentration of treatment and the lowest concentration of pre-treatment. The researchers showed that phenol with increasing concentration positively affected the carotenoid content in *Polygonum orientale* (Wang et al. 2015). The results indicated different stimulatory mechanisms in the carotenoid biosynthesis pathway between treatment and pre-treatment. The increase in carotenoid content can be caused by tension at the highest concentration of phenol treatment which can protect an organism against damage (Murray et al. 2007). However, the low concentrations of pre-treatment caused the growth stimulation. In addition, wang's examination showed that the phenol's time and concentration positively affected the carotenoid content (Y. Wang et al. 2019). Thus, the induction of carotenoids may be a response against phenoxy radicals produced by phenol (Cho et al. 2016).

The most important part of the evaluation in this paper showed that the carotenoid content had a direct relationship with concentrations of phenol treatment and pre-treatment on red cells. The phenol pre-treatment had better effects on carotenoids in the red stage than the treatment. The enhancement of carotenoids is probably due to phenol's stimulating effects, which remain for a long time. To evaluate better on the carotenoids of *H. pluvialis*, another series of experiments were carried out on *H. pluvialis*. It was found that treatment of phenol and pre-treatment considerably affect primary and secondary carotenoids in red cells.

This study showed that the carbohydrate of green cells increased at low concentrations of pre-treatment phenol. An increase in carbohydrates may be due to a phenol breakdown that creates a precursor to carbohydrates (Megharaj et al. 1992; Ramos et al. 2016), but Abdel-Hamei (2007) expressed that the content of carbohydrates in the liver, gills, and muscle of blue tilapia juveniles generally reduced due to the increase of phenol (Abdel-Hameid H 2007). The results showed that the phenol treatment and pre-treatment stimulated the quantity of carbohydrates in red cells. The increase may be a confronting mechanism for the stress stimulated by phenol.

Abbreviations

Haematococcus Pluvialis, *H. pluvialis*; Reactive oxygen species, ROS; Bold's basal medium, BBM; High Performance Liquid Chromatography, HPLC

Statements And Declarations

We declare that the authors have no conflict of interest. The authors were only directly interested in working on algae and plants and had no other direct or indirect interest in the other's jobs. We declare availability of data and material (data transparency). The study conception and design, material preparation, data collection and analysis were performed by [Hamzeh Rezazadeh] and [Hakimeh Mansouri]. The first draft of the manuscript was written by [Hamzeh Rezazadeh]. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Effects of phenol pre-treatment and treatment on dry weight, and the content of carbohydrate, protein and carotenoid of green and red cells of *H. pluvialis* algae. Values are means of three replications $\pm$ standard deviation. Different words show the significant difference with $p \leq 0.05$ in one-way ANOVA and Duncan's analyses. Means followed by the same letter in each column are not significantly different

Sort	Stage	Dens	Parameter			
			Dry weight	Carbohydrate	Protein	Carotenoid
Treatment	Green	0	0.24 ± 0.05 c	3.5 ± 0.03 a	4.20 ± 0.13 b	0.34 ± 0.01 d
		50 mg/l	0.4 ± 0.11 a	2.85 ± 0.06 b	4.92 ± 0.002 a	0.37 ± 0.04 c
		100 mg/l	0.3 ± 0.1 b	2.51 ± 0.14 c	4.37 ± 0.24 b	0.38 ± 0.02 b
		150 mg/l	0.18 ± 0.11 d	2.18 0.42 d	3.46 ± 0.014 c	0.418 ± 0.01 a
	Red	0	0.4 ± 0.0 c	1.3 ± 0.03 d	1.52 ± 0.01 a	13.85 ± 0.1 b
		50 mg/l	0.59 ± 0.15 a	1.9 ± 0.05 a	1.68 ± 0.01 a	19.58 ± 0.46 a
		100 mg/l	0.52 ± 0.01 b	1.65 ± 0.01 b	1.24 0.002 b	19.40 ± 0.12 a
		150 mg/l	0.38 ± 0.03 c	1.41 ± 0.004 c	0.97 ± 0.01 c	19.22 ± 0.24 a
Pre-Treatment	Green	0	3.33 ± 0.05 A	3.11 ± 0.06 B	4.32 ± 0.01 A	0.36 ± 0.01 B
		50 mg/l	3.35 ± 0.02 A	3.63 ± 0.59 A	4.32 ± 0.09 A	0.4 ± 0.15 A
		100 mg/l	0.34 ± 0.02 A	2.47 ± 0.01 C	4.48 ± 0.19 A	0.32 ± 0.02 C
		150 mg/l	0.36 ± 0.05 A	1.81 ± 0.01 D	4.65 ± 0.04 A	0.28 ± 0.13 D
	Red	0	0.41 ± 0.06 D	2.2 ± 0.2 B	1.65 ± 0.12A	18.20 ± 0.33 D
		50 mg/l	0.46 ± 0.01 C	2.6 ± 0.08 B	1.67 ± 0.01 A	19.14 ± 0.31 C
		100 mg/l	0.49 ± 0.01 B	3.23 ± 0.09 A	1.72 ± 0.17 A	20.48 ± 0.27 B
		150 mg/l	0.51 ± 0.01 A	2.4 ± 0.29 B	1.82 ± 0.02 A	21.55 ± 0.2 A

Table 2. The results of HPLC analysis of carotenoid extracts in red cells. Values are means of three replications ± standard deviation. Different letters show the significant difference with $p \leq 0.05$ in one-way ANOVA and Duncan's analyses. Means followed by the same letter in each column are not significantly different

Sort	Con (mM)	Neoxanthin	Violaxanthin	Antheraxanthin	Lutein	Canthaxanthin	β -Carotene	Astaxanthin
Treatment	0	0.42 $\pm$ 0.02 b	1.3 $\pm$ 0.18 a	0.62 $\pm$ 0.02 a	2.1 $\pm$ 0.16 c	2.35 $\pm$ 0.31 c	0.32 $\pm$ 0.1 b	58.42 $\pm$ 1.94 a
	50 mg/l	0.48 $\pm$ 0.01 b	1.4 $\pm$ 0.2 a	0.54 $\pm$ 0.06 b	3.03 $\pm$ 0.8 b	2.12 $\pm$ 0.18 c	0.37 $\pm$ 0.06 b	57.57 $\pm$ 1.83 a
	100 mg/l	0.54 $\pm$ 0.01 b	1.4 $\pm$ 0.2 a	0.32 $\pm$ 0.04 c	3.1 $\pm$ 0.33 b	2.85 $\pm$ 0.16 b	0.58 $\pm$ 0.03 a	56.91 $\pm$ 1.75 a
	150 mg/l	0.70 $\pm$ 0.02 a	1.6 $\pm$ 0.2 a	0.31 $\pm$ 0.01 c	4.1 $\pm$ 0.07 a	3.61 $\pm$ 0.3 a	0.3 $\pm$ 0.08 b	53.04 $\pm$ 2.1 b
Pre-Treatment	0	0.32 $\pm$ 0.04 b	1.5 $\pm$ 0.27 b	0.93 $\pm$ 0.1 a	1.8 $\pm$ 0.3 b	2.52 $\pm$ 0.13 b	0.28 $\pm$ 0.15 a	58.07 $\pm$ 1.25 a
	50 mg/l	0.59 $\pm$ 0.08 a	2.1 $\pm$ 0.23 a	0.65 $\pm$ 0.15 b	3.9 $\pm$ 1.2 a	1.9 $\pm$ 0.16 b	0.26 $\pm$ 0.04 a	56.48 $\pm$ 1.84 a
	100 mg/l	0.28 $\pm$ 0.01 b	1.4 $\pm$ 0.03 b	0.5 $\pm$ 0.02 b	1.5 $\pm$ 0.3 b	1.63 $\pm$ 0.15 b	0.21 $\pm$ 0.03 a	57.59 $\pm$ 1.5 a
	150 mg/l	0.27 $\pm$ 0.02 b	1.6 $\pm$ 0.01 b	0.69 $\pm$ 0.03 b	3.44 $\pm$ 0.9 a	5.2 $\pm$ 1.73 a	0.2 $\pm$ 0.01 a	53.57 $\pm$ 1.03 b

Figures

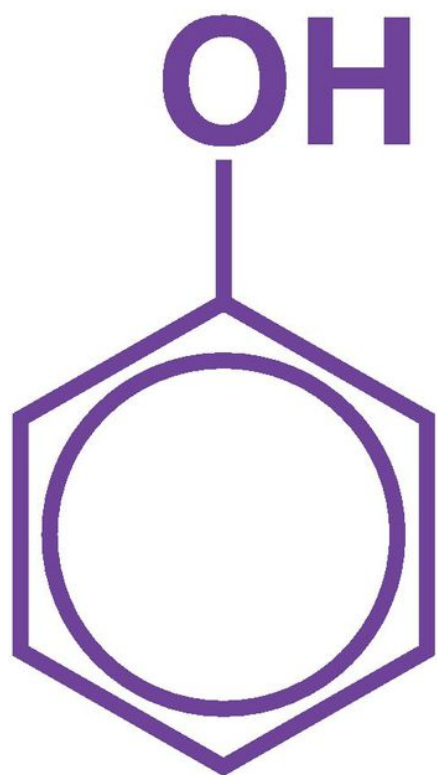


Figure 1

Structure of phenol

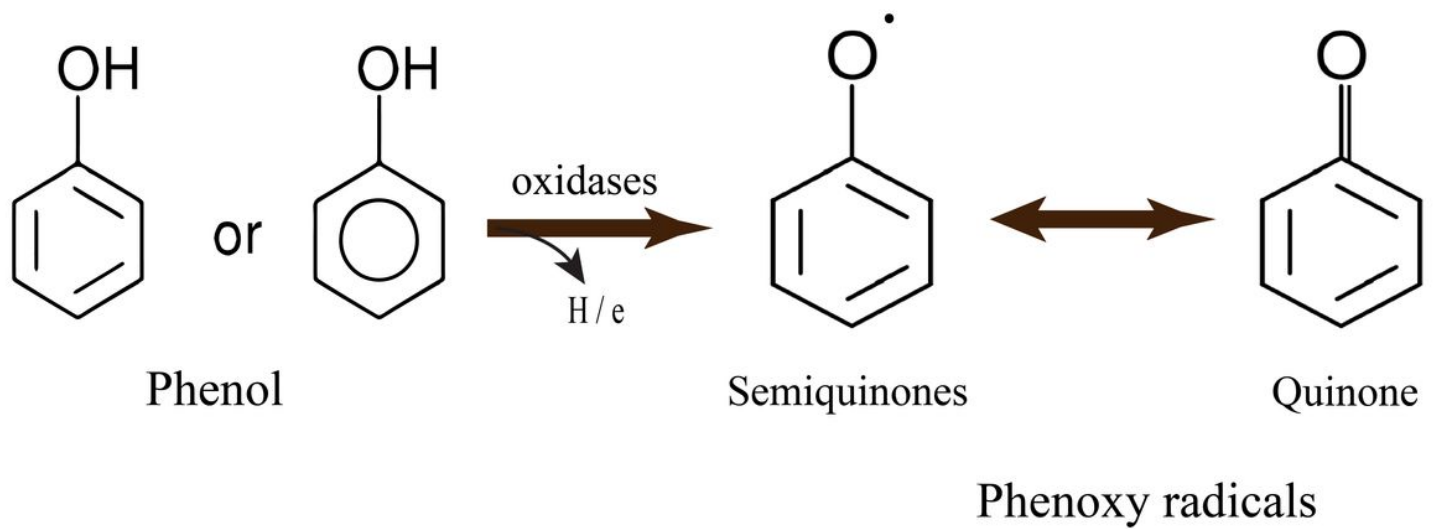


Figure 2

Phenol transformation to phenoxy radical by oxidase enzymes

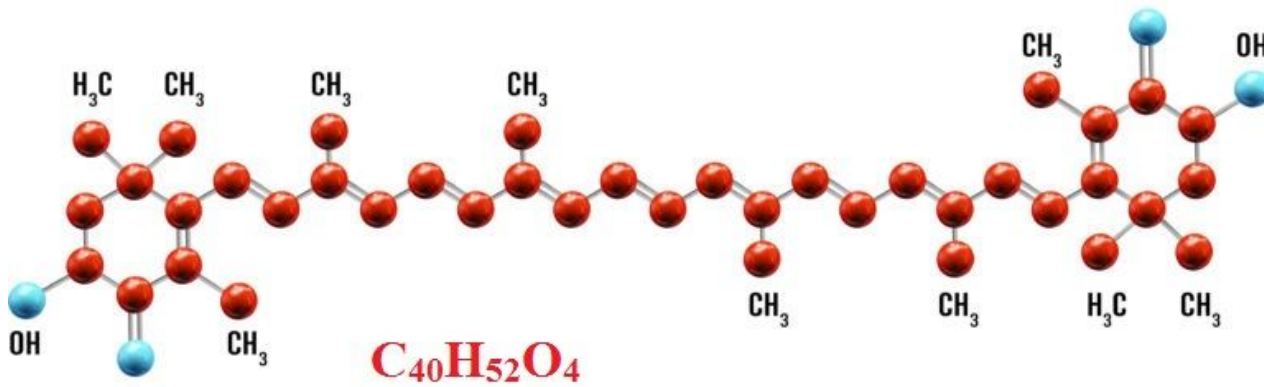


Figure 3

The chemical structure of astaxanthin

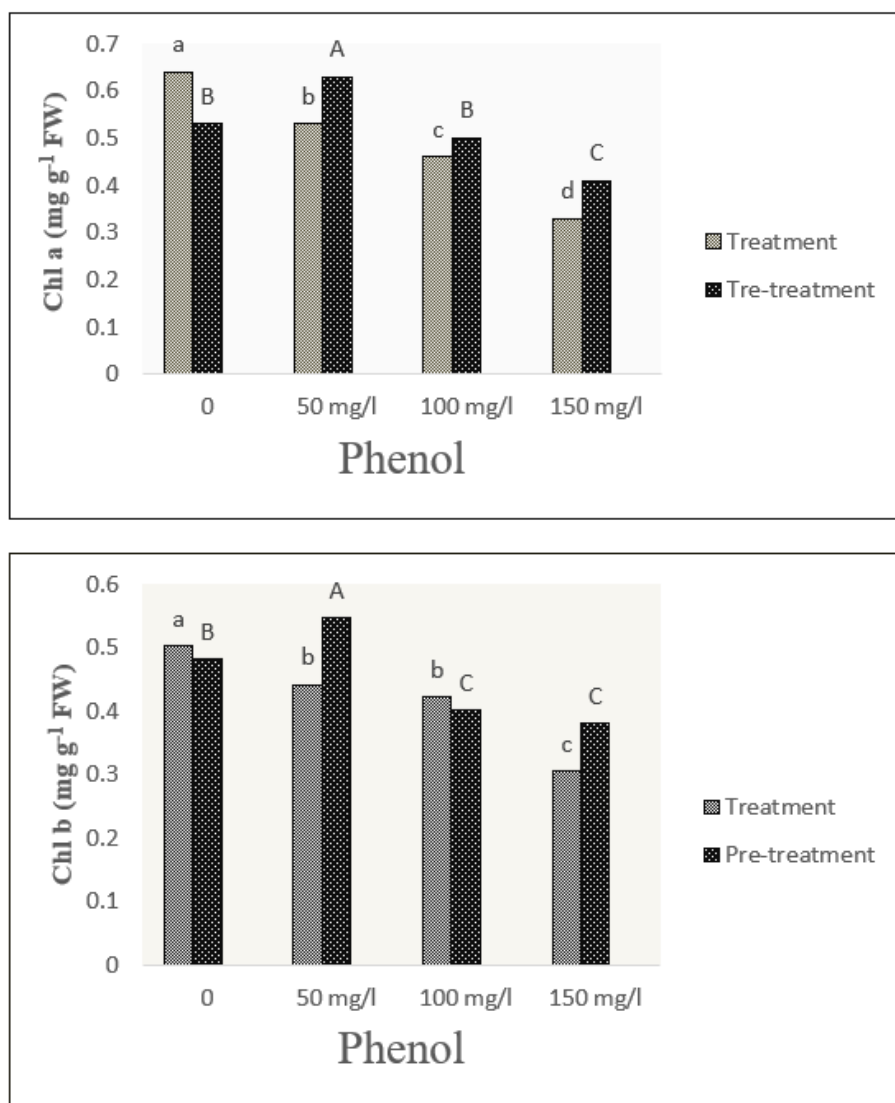


Figure 4

Effects of phenol on chlorophyll a (a) and b (b) content in *H. pluvialis* algae. Values are means of three replications $\pm$ standard deviation. Different words show the significant difference with $p \leq 0.05$ in one-way ANOVA and Duncan's analyses. Means followed by the same letter in each figure are not significantly different

Supplementary Files

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- [Graphicalabstract.jpg](#)