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Algal toxicity of *Talaromyces purpureogenus* F pigments in heterotrophic cultivation mode

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Abstract
The present study deals with the impact of *Talaromyces purpureogenus* F pigments does on growth, photosynthetic activity of *Parachlorella kessleri* GB1. The reduction in growth and pigment contents (Chl a, Chl b, and carotenoids) was observed in the positive control (herbicide). However, the negative control and pigments doses did not differ significantly in pigments contents. The growth in pigment concentration 3 mg/mL was significantly higher than negative control this might be due to the left out medium content in the fungal pigments. This research provides an understanding of fungal pigments toxicity to aquatic organisms. The heterotrophic cultivation mode for algal toxicity results is rapid to assessments of toxicants.

Keywords: Heterotrophic, *Parachlorella kessleri* GB1, Toxicity, Talaromyces purpureogenus F pigments

1. Introduction
The effluent of synthetic dyes from textile industry persistence may exhibit chronic effects such as mutagenic damage, carcinogenicity, adverse environmental and health-related impacts [1]. Therefore, microbial-based natural colorants have been predicated as environmentally friendly and biodegradable [2]. However, the application of microbial pigment as natural dyes, food colors, paints industries, and various biotechnological procedures require animal and ecotoxicity evaluation [3, 4].
Several studied reported that fungi produce mycotoxin along with pigments. During the dyeing, process pigments are discharged in the environment after use and may cause toxicity of water sources, therefore, testing of these pigments is necessary. Microalgae are highly diverse, sensitively respond to environmental changes, and are frequently used as an ideal model for monitoring microalgal growth-inhibition of various chemicals and secondary metabolites [5, 6, 7]. Microalgal communities play important roles in aquatic habitats as key primary producers and regulators of oxygen levels. Therefore, the effects fungal pigments on algae as well as subsequent impacts on higher trophic levels of the food chain.
Microalgae show sensitivity to natural products as well as municipal and industrial effluent. Toxic compounds may affect microalgal photosynthesis, growth, enzyme activity, and respiration [8]. The effective concentration of toxicants that inhibits 50% microalgal growth at 96 h (96 h EC50) is widely used as an index of toxicity [9]. *Parachlorella kessleri*, it is easy to cultivate in autotrophic and heterotrophic cultivation mode, has been reported for testing secondary metabolites, heavy metals [10]. However, the toxicity studies in heterotrophic cultivation mode have not been reported. Therefore, the objective of this study was thus to evaluate the toxicity of *Talaromyces purpureogenus* F pigment on *Parachlorella kessleri* GB1 in heterotrophic cultivation mode.

2. Materials and Methods
2.1 Microorganisms
Talaromyces purpureogenus F (NCBI GeneBank accession number MK388411.1) was used in this study. Stock cultures were maintained on Czapek's yeast autolysate agar (CYA) and subculture periodically. The pigment production was done in submerged fermentation conditions in a 3 L fermenter with CYA medium supplemented with malt extract (1.5), peptone (3), monosodium glutamate (3), and sucrose (50) g/L at pH of 5.0. The sterile cultivation medium was inoculated with 2% inoculum and the fermentation was carried out for 6 days at 30 °C with 1 vvm aeration. The fermentation was carried out at a three-stage agitation rate-controlled strategy

(0-2 days at 200 rpm, 2-4 days at 300 rpm and 4-6 days at 600 rpm). A silicone-based antifoaming agent (Antifoam A, Sigma) was added to the medium when necessary. Sampling was done after every 24 h and estimated for biomass [11], total sugar [12], and total pigment [13].

2.2 Test Organisms and Growth Conditions

The pigment toxicity test was carried out by using microalgae "*Parachlorella kessleri* GB1" according to OECD 201 method described in [14]. The microalgae *Parachlorella kessleri* GB1 (Gen Bank accession number KX151669.1) was used as an algal strain for toxicity study. The seed inoculum for *P. kessleri* GB1 was activated in Erlenmeyer flasks with 50 mL BG11 medium supplemented with 20 g/L glucose. The axenic microalgal colony was inoculated in BG-11 medium and incubated in an orbital shaker without light for 3 days at 30 ± 2 °C, and 150 rpm. The inoculum was checked for any contamination under a microscope (Olympus BX51). Different concentrations of pigments (filtered using 0.2 µ syringe filter) were mixed with sterilized BG-11 medium along with positive control [BG-11 with herbicide (CPH)], negative control [without pigment (CNH)], fungal pigment concentration P1H (1 mg/mL), P2H (2 mg/mL), and P3H (3 mg/mL). The cultivation was carried out in 500 mL Erlenmeyer flasks containing 200 mL of culture medium supplemented with 20 g/L glucose (BG11 medium; n = 3) and cultured in dark condition [15]. The variants were cultivated for 5 days at 30 ± 2 °C, and 150 rpm.

2.3 Measurements of Growth

Algal cell density was evaluated after every 24 h at 750 nm [16] using a spectrophotometer (Shimadzu UV-1800). Samples were taken (1 mL) aseptically from each flask in a glass test tubes and diluted to 10 folds. The diluted samples were centrifuged for 5 min at 8000 rpm to avoid the interference of pigment. The pellet was washed, mixed with the same amount of distilled water and the absorbance was taken at 750nm.

2.4 Measurements of Chlorophyll a, b, and total carotenoids

The pigments (chlorophyll a, chlorophyll b and carotenoids) were determined following the method outlined in Bauer *et al.* [17]. Chlorophyll a, chlorophyll b, and total carotenoids were calculated by using the following equations-

Chlorophyll a (mg/l) = 11.64 (Abs663 – Abs750) – 2.16 (Abs645 – Abs750) (1)

Chlorophyll b (mg/l) = 20.97 (Abs645 – Abs750) – 3.94 (Abs663 – Abs750) (2)

Carotenoids (mg/l) = 4 (Abs 480 – Abs750) (3)

The total concentration of each pigment was calculated by using equation (4), where CP is the concentration of pigments obtained in equations (1) – (3)

Pigment (mg/l) = CP (mg/l). Extract volume (l) /Sample volume (l) (4)

The maximum specific growth rate (µmax) and maximum biomass concentration (*X*max, g/L), maximum biomass productivity (*P*max, g/L/d) were calculated using the method of Sakarika and Kornaros [18].

2.5 Statistical Analysis

All the experiments were performed in replicates and statistical analysis was done by using the SPSS software version 20. A significant difference between the mean was determined by Tukey's test. Values are represented as ± standard error and different letters in lower case denote significant difference ($P < 0.05$)

3. Results and Discussions

3.1 Production of *Talaromyces purpureogenus* F pigments

The pigment production starts after 24 h of incubation and gradually increases until day 6 (Fig.1). The maximum extracellular pigment production of red and yellow pigment was 19.39 ± 0.42 AU_{510}/mL and 17.25 ± 0.2 AU_{410}/mL, respectively. The maximum biomass production observed was 24.0 ± 1.0 g/L. The red and yellow pigment yield was 0.483 AU_{510} and 0.4312 AU_{410} per gram of sucrose, respectively. The pigment was dried using freeze-drying. A known amount of filtered pigment was tested for its toxic effect using microalgae *P. kessleri* GB1 in heterotrophic cultivation mode.

3.2 Growth Inhibition

Algal growth bioassay is the preferred method for the assessment of the phototoxic effect in most ecotoxicological studies [19]. The effect of *T. purpureogenus* pigment (1, 2 and 3 mg/ml) on *P. kessleri* GB1 was monitored by observing the growth in terms of absorbance of the cell suspension at 750 nm.

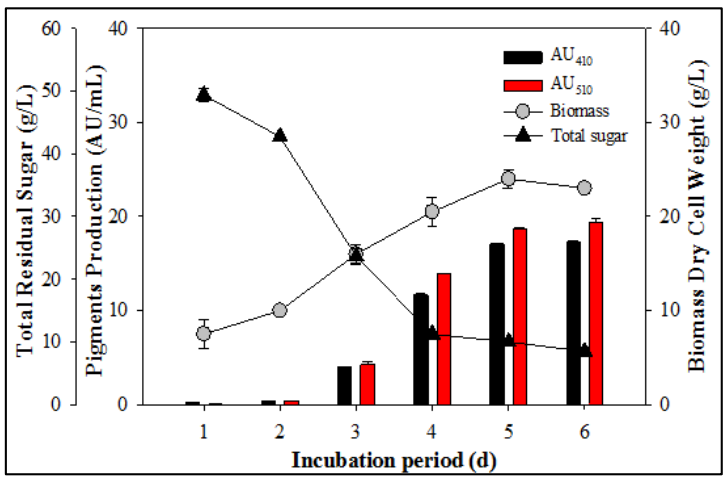


Fig 1: Time course of T. purpureogenus F biomass and pigments production (AU_{410} for yellow pigments, AU_{510} for red pigments) in CYA medium supplemented with malt extract mean ± SD; n=2.

The growth response curve of positive control (CPH) showed a significant decrease in growth compared to negative control and at all the tested doses was observed at 24 hours (Fig. 2). The bleaching out of algal cells before (0 day) and after 3 days of incubation is shown in Fig.3.

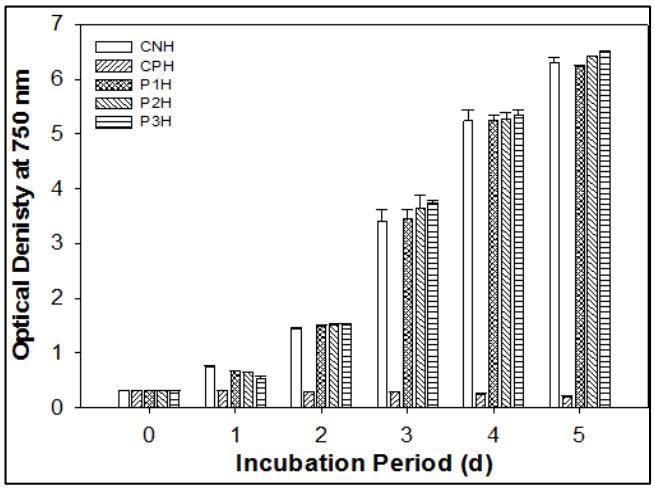


Fig 2: Growth rates of *P. kessleri* GB1 after exposer to different treatments: CNH (negative control or BG11 medium with glucose as carbon source), CPH (positive control with BG11 medium with glucose and herbicide 1.0 mg/mL), P1H (BG11 medium with glucose and fungal pigment 1 mg/mL), P2H (BG11 medium with glucose and fungal pigments 2 mg/mL), P3H (BG11 medium with glucose and fungal pigments 3 mg/mL) mean ± SD; n = 3.

Fig 3: Effect of various treatments on the 0 day and 3 days after inoculation.

Further incubation beyond 24 hours results in bleaching of algal cells (*P. Kessleri* GB1) was observed. Although in positive control (CPH) the inhibition was observed in 24 hours when compared to negative control (CNH) and pigment dose (P1H, P2H, and P3H), further incubation was extended till 5 days to check the long-term effect

It can be seen that the positive control (CPH) significantly inhibited the heterotrophic growth of the *P. Kessleri* GB1 with a specific growth rate -0.076/d. The specific growth rate of positive control was significantly lower than the growth rate of control and pigment dose (Table 1). However, the growth response in the negative control (CNH) and pigments (P1H, P2H, and P3H) showed an increase in growth at all the tested doses, confirming the health status of the organism.

In addition, the specific growth rate and doubling time of negative control (CNH), and fungal pigment doses (P1H, P2H, and P3H) shows that the addition of fungal pigment results in an increase in specific growth rate and shorter the doubling time (Table 1). This is probably due to the presence of metabolites in the pigments resulting in higher growth of *P. Kessleri* GB1. These results suggest that fungal pigment does not inhibit the *P. kessleri* GB1 at the tested dose. In contrast, a study on *marine T. purpureogenus* YL13 extracts reported algicidal activity against *Prorocentrum donghaiense* a harmful algal bloom [20].

Table 1: Effect of different treatment on the *Parachlorella kessleri* Data are represented as mean standard error and different letters in lower case denoted significant difference ($P < 0.05$).

Treatment	Specific growth rate (μ)	Doubling time	Total Chlorophyll a (mg/mL)	Total Chlorophyll b (mg/mL)	Total Carotenoid content (mg/mL)
CNH	0.621^{b}	1.12^{d}	8.29 ± 0.06^{b}	6.10 ± 0.09^{b}	2.76 ± 0.01^{b}
CPH	-0.076^{a}	-9.07^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
P2H	0.628^{b}	1.10^{c}	8.28 ± 0.00^{b}	5.89 ± 0.02^{b}	2.80 ± 0.01^{b}
P2H	0.636^{c}	1.09^{c}	8.24 ± 0.02^{b}	5.93 ± 0.02^{b}	2.83 ± 0.02^{b}
P3H	0.657^{d}	1.05^{b}	8.21 ± 0.00^{b}	5.94 ± 0.05^{b}	2.84 ± 0.05^{b}

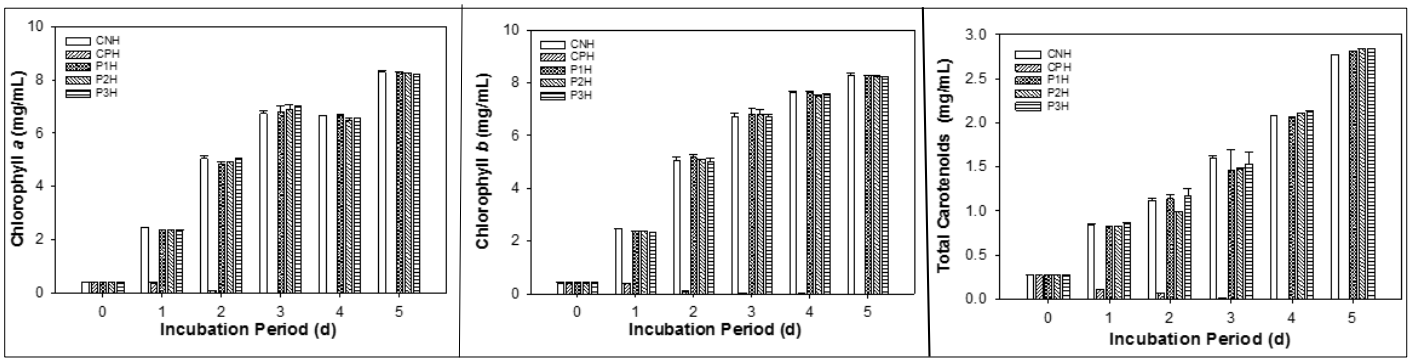
Furthermore, all the experimental treatments continued to increase in cell density until the end of the study suggesting the toxicity was not observed during a longer exposer period. The heterotrophic growth provides high degree of growth in short period. Therefore, the toxicant impact on microalgae can be detected without longer incubation period.

3.2 Photosynthesis Pigment Content and Carotenoids

The inhibitory effects of herbicides (CPH) on chlorophyll a (Fig. 4a), chlorophyll b (Fig. 4b) and carotenoids (Fig. 3c) content in *P. kessleri* GB1 increased after 24 h exposer was determined by spectrophotometer. The herbicide not only inhibits chlorophyll a, and chlorophyll b pigment but also blocked the biosynthesis of carotenoids via a process as bleaching. This effect increase was time-dependent, the longer exposer showed a further decrease in the photosynthetic pigments and carotenoids was observed in the positive control (CPH).

These results indicated that there was decrease in the ability of algal cells to phototrophic metabolism and heterotrophic metabolism. Thus, herbicide affects the entire physiological state and cell growth process. However, there was no reduction in the chlorophyll a, chlorophyll b and carotenoids of *P. Kessleri.* Whereas, the negative control (CPH) and pigment dose (P1H, P2H, and P3H) showed an increase in total carotenoids during 24 hours of incubation and gradually increase till the end of incubation.

The chlorophyll a, chlorophyll b and carotenoids (Table 1) within the pigment treated samples and negative control were not significantly different ($P< 0.05$). Therefore, it can be concluded that the fungal pigment does not inhibit the algal population growth at a concentration range from 1 to 3 mg/mL. Whereas, the positive control (herbicide treated 1 mg/mL) inhibited the algal growth and the cell concentration decline gradually.



a. b. c.

Fig 4: Photosynthetic pigment content with respect to negative control (CNH), positive control (CPH), pigment treatment P1H (1 mg/mL), P2H (2 mg/mL), P3H (3 mg/mL) exposed for 5 days in *P. kessleri* GB1: (a) chlorophyll a, (b) chlorophyll b, (c) total carotenoids

Photosynthetic activity is considered as a significant effect parameter of a variety of toxicants on algae (physiological and morphological effects). It can be carried out in autotrophic cultivation microalgae. The *Talaromyces purpureogenus* F pigment is water soluble and the culture medium is, therefore, turning to red colour. The presence of high concentration of dye affects the light transmitted in the water affecting the photosynthesis and growth of aquatic cyanobacteria [21]. However, in heterotrophic cultivation, the growth of microalgae is not influenced by photosynthesis metabolism. The cultivation under heterotrophic cultivation overcome the limit of light requirement for photosynthesis. In addition, the addition of glucose promotes higher growth in less cultivation period, therefore, the impact in algal cell growth mechanisms by toxicants results in suppression of growth/death in algal cell was detectable during 24 hours of incubation. This study could lead to future research for developing eco-friendly fungal pigments as natural dyes for natural fabrics. Also, it provides evidence for environmental risk assessment these natural fungal pigments. The heterotrophic cultivation mode can detect toxicity within 24 hours of incubation period. This method can be applied for a toxicity test for toxicants.

4. Conclusions

It has been shown in the present study that *Talaromyces purpureogenus* F at the test concentration 1 to 3 mg/ml does not show algal toxicity in heterotrophic cultivation mode. Heterotrophic cultivation mode results in high cell growth in a very short period, therefore, the toxicity can be validated within 24 hours of incubation. This finding on algal toxicity will be a benefit to the development of *T. purpureogenus* pigment as potential use as natural dye. In addition, heterotrophic cultivation mode could also be used for monitoring algal toxicity using various toxic chemicals.

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