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RESEARCH ARTICLE

# The Effect of Different Light Conditions on Antimicrobial Activity of the Microalgae *Chlorella* sp. Ethanolic Extract Against *Streptococcus mutans*

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## ABSTRACT

Finding new antimicrobial agents from natural compounds with less side effects has been considered by number of researchers in the world. It is important to achieve efficient and up-to-date results in order to identify a substance with antimicrobial properties and achieve operational methods to increase these traits in a society where the prevalence of various diseases has been increased. In fact, the purpose of this study was to achieve compounds from microalgae with antimicrobial properties to be used in food and pharmaceutical industries that can have good consequences for human health. Therefore, in the current study antimicrobial activity of ethanolic extract of microalgae *Chlorella* sp., that was cultivated under different light conditions, was investigated. For this purpose, microalgae *Chlorella* sp. was separately cultivated under red, blue, green and white lights with intensity of 109 ( $\mu\text{mol-photon m}^{-2} \text{ s}^{-1}$ ) and antimicrobial potential of the microalgae extracts investigated against the activity of *Streptococcus mutans*. In addition, Minimal Inhibitory Concentration (MIC) of the extracts determined. Based on the results, the wet extracts indicated more average antimicrobial activity than dried ones. Furthermore, the wet extract of microalgae cultivated under the red light showed a stronger antimicrobial activity compared to extract obtained under the other light spectrum with the minimum inhibitory of that was 10 mg/ml. Also, extract obtained under white light had no significant antibacterial activity against the bacterial strain.

## INTRODUCTION

Owing to the alarming increase of drug resistance among microbial pathogens, many efforts have been made to find out and characterize new antimicrobial agents [1,2]. The products of marine microorganisms have shown many interesting activities, such as antimicrobial, cytotoxic, anticancer, antidiabetic, antifungal, anticoagulant and other pharmacological activities [3,4]. Algae are a wealthy source for pharmaceuticals on the basis of the presence of biologically active primary and secondary metabolic compounds [5].

Antimicrobial activities of compounds derived from microalgae have also been extensively studied by several other researchers [6-8]. The antimicrobial activity of microalgae has been attributed to compounds belonging to several chemical classes including indoles, terpene, acetogenins, phenols, fatty acids and volatile halogenated hydrocarbons [5,9].

Physical and chemical conditions of culture media affect the biochemical

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compositions in algal cultures. Manipulation of the culture condition is a possible way to enhance biological activities of microalgae. In recent years, researchers have identified the invaluable increment of microalgae as a possible source of strange antibacterial agents [10,11]. Several studies documented the antibacterial activity of *Chlorella* species [12-14]. However, few attempts were performed to manipulate the antimicrobial activity of green microalgae based on the culture conditions. Asadi, et al. [15] investigated antimicrobial effects of the *Chlorella vulgaris* extracts and the supernatant against Gram-negative bacterial foodborne pathogens. Acetone and Ethanol were used as solvent extracts, and antimicrobial activity of that was determined using well plate and disk diffusion methods. Extracts of *Chlorella vulgaris* and supernatant showed acceptable antibacterial properties against *Proteus mirabilis*, *Salmonella enterica*, *E. coli*, *Shigella dysenteriae*, and *Pseudomonas aeruginosa*. The maximum inhibition zone diameters in 2 methods were 36.8 and 35.4 mm respectively, which related to the bacterium *Proteus mirabilis*. Also, using the agar disk diffusion method Ghasemi, et al. [16] observed that the supernatant and methanolic extract of *Chlorella vulgaris* formed inhibition zones equivalent to 14, 15, 15, 14, 13 and 16 mm against the bacteria, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi*, and *Staphylococcus aureus*, respectively. The results indicated that methanolic extract of *Chlorella vulgaris* had a high antimicrobial activity against gram-positive bacteria.

Dental caries is a biofilm-induced oral disease with *S. mutans* performing an important function in the development of virulent cariogenic biofilms [17]. *Streptococcus mutans* (bacterial species) plays an important role in dental caries [18,19]. This bacterium is a gram-positive cocci, facultative anaerobic which is generally found in the human oral cavity. *S. mutans* is regarded as the main factor for tooth decay due to the production of lactic acid with binding to tooth surfaces in the presence of fermentable sugars, which contributes to corrosion of enamel [18-20]. Therefore, one of the basic biological goals in preventing dental caries is to decrease the bacterial burden of the oral cavity. Due to the increase of antibiotic resistance and side effects of some antimicrobials on one hand and the safety, availability and proportionately low costs of natural products on the other hand, for prevention of caries, various natural products have been assessed as well as incorporated into dental products. The increase of drug-resistant pathogens produces challenges to the successful treatment of microbial diseases, including tooth decay.

To combat disease caused by antimicrobial-resistant microorganisms, there is an immediate need to discover novel products which have antimicrobial properties. Thus, our goal in this study was to investigate the effect of the light quality spectrum (red, blue, green and white)

on antimicrobial potential of microalgae *Chlorella* sp. against *Streptococcus mutans*, while Minimal Inhibitory Concentration (MIC) of the extracts was obtained.

## MATERIALS AND METHODS

### Microalgae and Bacterial strain, culture, photobioreactor and light condition

The microalgae *Chlorella* sp. (PTCC 6010, Persian Type Culture Collection, Tehran, Iran) was used in this work. This microalgae species was provided by the Iranian Research Organization for Science and Technology (IROST) and pre-cultivated in Rudic medium (pH~8) [21]. This medium contained (mg/l):  $\text{NaNO}_3$ : 300,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ : 10,  $\text{K}_2\text{PHO}_4$ : 80,  $\text{NaCl}$ : 20,  $\text{KH}_2\text{PO}_4$ : 20,  $\text{CaCl}_2$ : 47,  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ : 17,  $\text{Na}_2\text{EDTA}$ : 7.5,  $\text{H}_3\text{BO}_3$ : 0.3,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ : 1.5,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ : 0.1,  $(\text{NH}_4)_6\text{MO}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ : 0.3,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ : 0.08,  $\text{Co}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$ : 0.02. Also, the *Streptococcus mutans* bacterium was obtained from Pasteur Institute of Iran.

The microalgae was cultivated in pyramid photobioreactor [22]. In this study, a continuous 24 h; red, blue, green and white LEDs with the illumination of  $109 \mu\text{mol-photon m}^{-2} \text{s}^{-1}$  were used (Table 1). Microalgae were exposed to red (R, 620-720 nm), blue (B, 450-495 nm), green (G, 495-570 nm), and white (W, 400-700 nm) light with extra white light in the first 8 days and wet biomass of these samples was used for extraction (W1, B1, R1, and G1). Then in 14 next day white lights were removed and only the colored lights stayed and both wet (W2, B2, R2, and G2) and dried (W3, B3, R3, and G3) biomass of these samples were used for extraction. The power consumption of all lamps was equal to 18 watts. Also, schematic of different light radiation to the reactor are presented in figure 1.

### Extraction method

Extraction process was done in two stages. Once, on the 8<sup>th</sup> day from the beginning, as though the extra white lights turned off. The second time was at the end of the experiment (the end of the logarithmic phase). Those algal cells from different growth modes were harvested using centrifugation at  $2500 \times g$  for 10 min.

The Extraction method was performed in two points/

Table 1: Description of sample ID.

Sign	Description
W	White light
B	Blue light
G	Green light
R	Red light
1	Colored light + white light, extraction from wet biomass
2	Only colored light, extraction from wet biomass
3	Only colored light, extraction from dried biomass

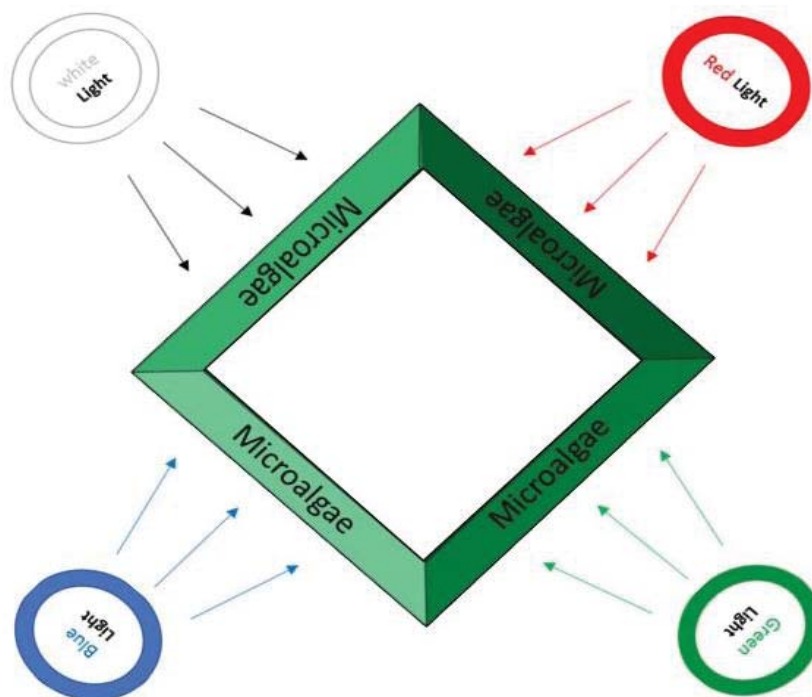


Figure 1 Schematic of the reactor.

times (from dried and wet biomass). For wet biomass- active (W1, B1, G1, and R1, W2, B2, G2, and R2), a solvent technique extraction was performed using 99.8% ethanol (Merck, Germany). After 48 h in dark and room temperature, they were suspended in solvent (10:1 v/w), then sonicated (20 KHz) (Wise clean, China) and centrifuged (SIGMA 3-30 K, Germany) for 5 min at 2500×g, respectively. Supernatants were filtered and concentrated in rotary evaporator at 45°C (Heidolph4002, Germany). The extracts were refrigerated at 4°C for the next steps of the experiment. In order to produce dried biomass-deactive (W3, G3, B3, and R3), the biomass was harvested and frozen in the freeze dryer (CHRIST, Germany) at -50°C for 72 h. Biomass powders were collected and the algal powders were suspended in the solvent in the ratio of 10:1 (v/w). Other steps were done like the previous extraction method [23].

### Preparation of bacterial inoculums

To activate the bacterial strains before inoculation, bacteria inoculums were prepared by transferring a huge number of bacterial strains from fresh culture plates to tubes containing 10 ml of BHI (Brain Heart Infusion) broth (Merck) and incubating for 72 h at 37°C (with 5% CO<sub>2</sub>). The tubes were shaken occasionally for growth promotion and better aeration. These cell suspensions were diluted with sterile BHI broth to provide initial cell count of about 10<sup>8</sup> CFU/ml.

### Antimicrobial activity of algal extract

Antimicrobial activity of different algal extracts against

microbial pathogen *S. mutans* was studied using paper disc diffusion assay [24]. Generally, the fresh culture of microbial strain was prepared and a suspension of that microorganism with the approximate cell population of 1.5×10<sup>8</sup> CFU/ml was prepared in BHI broth. The petri dish plates were prepared with 20 ml of sterile BHI agar (Merck, Germany). The microbial strain was inoculated uniformly onto the surface of BHI agar using sterile cotton swabs. The crude extracts were prepared and led on sterile discs which were placed on the surface of the solidified agar medium. The plates were incubated at 37°C for 72 h (with CO<sub>2</sub>). The assay was performed in triplicates and the diameter of the zone of inhibition formed around the disc was determined using Vernier caliper. The extraction solvent was used as negative controls and disc with anti-bacterial agent Gentamycin (10 µg- PadtanTeb) was used as positive controls [25].

### Minimal Inhibitory Concentration (MIC)

The Minimal Inhibitory Concentration (MIC) was determined for algal extracts using broth microdilution method to give a concentration between 1000 and 10 mg/ml [26,27]. Briefly, *S. mutans* from culture in sterile 96-well microtiter plates, 100 µl of algae extract were diluted with broth and placed into the well containing 100 µl of bacterial suspension in broth in order to prepare microbial inoculant, the prepared culture media for 48 h at 37°C under anaerobic condition were incubated, Then the growth of *Streptococcus Mutans* was estimated Appropriate controls of medium culture with the microorganism or each extract were included. Triplicate samples were performed for each test concentration.



## RESULTS AND DISCUSSION

### Antimicrobial activity

In our study, the antimicrobial activity of ethanolic extract of *Chlorella* sp. that cultivated in different wavelengths and methods of extraction (active/deactive) were assayed against the human pathogen by evaluating the inhibition zones, zone diameter, and MIC values. Algal extracts were tested for antibacterial activity against *S. mutans*. An example of results from the paper disc diffusion test can be seen in figure 2.

The sizes of the zone of inhibition in respect to the inhibitory effect of the microalgae extracts are presented in table 2. As presented in this table, the high inhibition zones were seen around the paper disc of the algal extract of sample R2. In fact, the extract obtained from wet biomass of microalgae that had been grown under red light (without extra white light) showed the highest antimicrobial activity against *S. mutans*. Also, the antimicrobial activity of the algae grown under green light (with extra light, G1) was remarkable. Furthermore, the samples grown under white light radiation had no activity. This phenomenon happened for both dried and wet biomass.

Generally, in all *Chlorella* sp. extracts a noticeable effect has been found which is impressive against the bacterial strain. Less inhibitory effects were recorded for all extract of *Chlorella* that grown under white lights in all modes. It is obvious that the antimicrobial spectra of the active marine bacteria are different [28].

The organic extracts of *Chlorella* sp. differ in wavelengths under which they are grown. This difference comes from the variation of the ecological conditions of the environment

of the microorganism [29]. Based on our results, it can be expressed that the antimicrobial activity of *Chlorella* sp. extracts was affected by a variety of light wavelengths positively and negatively. However, sometimes no significant effect was observed. Thus, it is clear that red light can lead to the production of antibacterial compounds in *Chlorella* sp. and the highest amount of these compounds were achieved in wet extraction method. The result gives an indication of the presence of promising antimicrobial compounds in ethanol extract of marine water algal species under our study. Further phytochemical investigations are needed to clarify the efficacious components in antimicrobial activity of these extract against bacteria.

Microalgal species and the solvents used for bioactive compounds extraction are the important factors in antimicrobial activity [30,31]. Antibiotics are natural or synthetic chemical compounds that can suppress the growth and destroy the microorganism [32,33]. The active components of various algae and cell extracts have been demonstrated to have antibacterial activity *in vitro* versus gram-negative and gram-positive bacteria [34].

The results of this research seem similar to the previous results of evaluating the antimicrobial properties of *Chlorella* sp. which may increase or decrease in different growth conditions. The present results agreed with the previous results about antimicrobial properties of green microalgae *Chlorella* sp. against bacterial strains. Several studies documented antibacterial activity of *Chlorella* species [12-14]. However, few attempts were performed to manipulate the antimicrobial activity based on the culture conditions. As an example of antimicrobial studies, Salem et al. examined the antibacterial activity of microalgae such as *Chlorella vulgaris*, against four Gram-positive bacteria

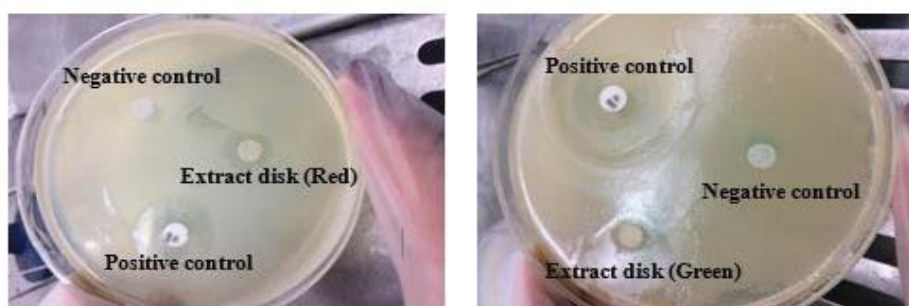


Figure 2 Disc diffusion test of microalgal extracts on *S. mutans*.

Table 2: The size of the zone of inhibition (mm) of *Chlorella* sp. extracts against *S. mutans*.

Ethanolic Extract	Red	Green	Blue	White
wet-(in 8 <sup>th</sup> day)	R1 = 12 ± 0.5	G1 = 14.13 ± 0.5	B1 = 7.1 ± 0.2	W1 = 6.3 ± 0.2
wet-(end of log phase)	R2 = 18.64 ± 0.5	G2 = 11.5 ± 0.5	B2 = 7 ± 0.2	W2 = 6.6 ± 0.2
dried-(end of log phase)	R3 = 12.6 ± 0.5	G3 = 10.5 ± 0.5	B3 = 7 ± 0.2	W3 = 6.8 ± 0.2
(ethanol = 7, Gentamycin = 20)				

(*S.aureus*, *Sarcina lutea*, *B.subtilis*, and *B.megaterium*) and one Gram-negative bacteria (*Klebsiella pneumonia*) were tested with agar well diffusion method [35]. The antimicrobial activity was tested using methanol and acetone extracts alga spices. The methanol extract was more effective against the studied bacterial strains. *Chlorella vulgaris* extract showed antimicrobial activity against *B.subtilis*, *S.aureus* and *K.pneumonia* with inhibition zone 17.5, 17 and 14.5 mm, respectively.

Syed, et al. [14] tested green microalgae *Chlorella vulgaris* against four bacterial strains (*Escherichia coli*, *Klebsiella* species, and *Bacillus* and *Pseudomonas* species) with acetone, ethanol, and chloroform extracted from microalgae, using the agar disk diffusion method. The observed highest inhibition zone was 13 mm in *Chlorella vulgaris* extracted with ethanol against *Klebsiella sp.* Alwathnani, et al. [36] studied the effect of *Chlorella vulgaris* extracts on morphological changes in human pathogens and antimicrobial activity. They evaluated the antimicrobial potential of *Chlorella vulgaris* extract against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Bacillus subtilis*. Extracts were prepared using methanol, chloroform, diethyl ether solvents. The finding revealed that the extracts could suppress the growth of tested pathogens. The maximum zone inhibition of diethyl ether extract was recorded against *E.coli* (28.6 mm) and 19.1 mm against *Streptococcus pyogenes*. Donal Mc Gee, et al. [37], extracted from 80 isolated marine and freshwater microalgae strains were tested for antimicrobial activity against 6 pathogens. These researchers reported, indicating a significantly higher bioactivity under blue light for the chlorophytes and red light for the diatom *Stauroneis sp.*

The results obtained from the present work concerning the antimicrobial properties of ethanolic extract of green microalgae that grown under different light conditions against human pathogen. It was concluded that the diameter of the inhibition zone depends mainly on the type of extraction modes (active/deactive) and type of wavelength of lights. Also, it was found that the *Chlorella sp.* grew under the red light and the extract in the active mode performed the highest antimicrobial activity against *Streptococcus mutans* (18.64 mm inhibition zone). According to the results we could test influence of red light on antimicrobial activity of algae in other different conditions. Till the results indicate an effective and beneficial effect, use it in various parts, including medicine. Based on research the red spectra were good an agent in increment activity of microalgae cell, growth rate, reproduction, and etc. This was what caused it the good antibacterial potential.

### Minimum inhibitory concentrations

The Minimum Inhibitory Concentration (MIC) of extracted from *Chlorella sp.* was grown under various light conditions is presented in table 3. As presented in this table,

**Table 3:** Minimum inhibitory concentration of microalgae *Chlorella sp.* grown under red light and green light in 2 periods.

10 mg/ml	50 mg/ml	100 mg/ml	1000 mg/ml	Sample
+	+	+	+	R2
-	+	+	+	G1

(+ means have antimicrobial properties, - means do not have)

the ethanolic extract of *Chlorella sp.* grown under red light had the minimum inhibitory concentration of 10 mg/ml. However, the extract of microalgae grown under green light with helping white light in the first 8 days has 50 mg/ml MIC. In contrast with these results, other findings showed that different wavelength can be modified to show highly potent antimicrobial properties against the bacterial strain. This study confirmed that the green microalgae *Chlorella sp.* possess biological active substances.

## CONCLUSION

This study showed the antimicrobial activity of microalgae *Chlorella sp.* when grown under both white and red lights were more than that grown under red light alone. Also, the average antimicrobial activity of wet extracts was more than dried ones. In fact, the results of our work and mention previously research showed that microalgae *Chlorella sp.* is an appropriate candidate for health and medical purposes. Furthermore, *S. mutans* showed more sensitivity to the antibiotic Gentamycin; after that, ethanol extract of microalgae *Chlorella* grown under red light showed the highest zone of inhibition of the bacteria.

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