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Antagonistic activity of bioactive compounds extracted from cyanobacterium *Oscillatoria* isolated from oil refinery waste

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ABSTRACT

The aim of the present study was to isolate cyanobacteria from oil refinery waste water and detection of antagonistic activity of their extracts on four species of pathogenic bacteria. The cyanobacterium was isolated and purified on Blue green algae medium number 11 (BG-11) medium. Methanol and water extracts prepared from the cultured cyanobacterium after 45 days growth on BG-11 medium. The bioactive antagonistic effect of extracts was investigated on *Escherichia coli* (PTCC 1399), *Pseudomonas aeruginosa* (PTCC 1707), *Bacillus cereus* (PTCC 1015) and *Staphylococcus aureus* (PTCC 1112) via well diffusion method. The chemical composition of the effective extracts was detected by gas chromatography Mass spectrometry (GC-MS). The isolated bacterium detected as *Oscillatoria* based on microscopic morphological characteristics. The methanol extract of the cyanobacterium showed considerable antagonistic effect on Gram-negative bacterial species (growth inhibition zone of 22.33±0.4 mm for *Escherichia coli* (PTCC 1399), and 18.6±1.52 mm for *Pseudomonas aeruginosa*, (PTCC 1707); while little effect on Gram-positive bacterial species (growth inhibition zone of 9.3±0.57 mm for *Bacillu scereus* (PTCC 1015) and 7.9±0.3 mm *Staphylococcus aureus* (PTCC 1112). The water extract of the cyanobacterium had no antagonistic effect on all experimented bacterial species. The chemical composition of the methanol extract detected as: 28.11% Dodecamethyl-cyclohexasiloxane, 25.76% Hexasiloxane, 3.75% Tetracosamethyl-cyclododecasiloxane (three related compounds) and 3.91% Bisabolol oxide A (unrelated compound). Minimal nutritional and environmental requirement, are advantages which set cyanobacteria as suitable candidates for production of antiviral, anti-tumor and antibacterial bioactive materials.

1. Introduction

Cyanobacteria are prokaryotic microorganisms with the structure similar to Gram-negative bacteria in their cell wall (Hoiczyk and Hansel, 2000). They show diversities in shape (unicellular to multicellular, coccoid to branched filaments, nearly colorless to intensely pigmented), nutrition (autotrophic to heterotrophic) and life condition (psychrophilic to thermophilic, acidophilic to alkylophilic, planktonic to barophilic, freshwater to marine including hypersaline). These properties are advantages for using cyanobacteria in different biotechnological processes (Thajuddin and Subramanian, 2005).

Cyanobacteria are distributed globally in soil and water including see and fresh water. Adaptation to different conditions is partially

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due to their ability to produce different secondary metabolites (Chlipata et al., 2012).
The aim of the present study was to isolate cyanobacteria from oil refinery waste water and detection of antagonistic activity of their extracts on four species of pathogenic bacteria.

2. Materials and Methods

2.1. Sampling and isolation of bacteria

Waste water samples with pH value of 7.3, BOD=7 mg L^{-1} and COD=38 mg L^{-1}, obtained in January to Jun 2014 from aeration pound in Isfahan oil refinery, Iran. The samples were cultured in BG-11 medium in the presence of light (3klux for 16 hours) and darkness (8 hours) periodically. Medium contained the following nutrients (values in parenthesis showed the concentration): $MgSO_4.7H_2O$ (0.075 g L^{-1}), $NaNO_3$ (1.5 g L^{-1}), $CaCl_2.2H_2O$ (0.036 g L^{-1}), $K_2HPO_4.3H_2O$ (0.04 g L^{-1}), Na_2EDTA (0.001 g L^{-1}), Na_2CO_3 (0.02 g L^{-1}), Ferric ammonium citrate (0.006 g L^{-1}), citric acid (0.006 g L^{-1}) as macronutrients along with 1 ml of micronutrients solution containing: H_3BO_3 (2.86 g L^{-1}), $ZnSO_4.7H_2O$ (0.22 g L^{-1}), $MnCl_2.4H_2O$ (1.81 g L^{-1}), $CuSO_4.5H_2O$ (0.08 g L^{-1}), $Na_2MoO_4.2H_2O$ (0.39 g L^{-1}) and $CO(NO_3)_2.6H_2O$ (0.049 g L^{-1}) (Bhardwaj et al., 2010; Dezfooli et al., 2012). All chemicals were obtained from Merck company (Germany).

Purification stage was done by addition of 0.1 g L^{-1} Imipenem and Cyclohexamide antibiotics to BG-11 agar plate media for inhibition of the growth of bacteria and fungi respectively. UV irradiation (254nm UVC lamp, for 10 min) used for further purification (Ferris et al., 1991; Naghavi et al., 2012).

2.2. Extract preparation

The purified cyanobacterium was inoculated in 1000 ml flasks containing 500 ml BG-11 medium and cultured for 45 days in 25˚C in the presence of light (3klux for 16 hours) and dark (for 8 hours) periodically. The bacterial cell biomass was separated by centrifugation (5000 rpm, 15 min). For preparation of water extract, the supernatant was dried at 40˚C and dissolved in 1 ml distilled water. The methanol extract was prepared by addition of 30 ml methanol (99.8%, Merck, Germany) to the bacterial cell biomass and shaked for 20 min at 150 rpm. The extract was filtered using Whatman paper (No 589.2), dried at 40˚C, dissolved in 1 ml methanol and kept at 4˚C before use (González et al., 2001).

2.3. Antibacterial effect testing

Agar well diffusion method was used for detection of the effect of water and methanol extracts on two strains of Gram-negative (*Escherichia coli* PTCC 1399 and *Pseudomonas aeruginosa* PTCC 1707) and two strain of Gram-positive (*Bacillus cereus* PTCC 1015 and *Staphylococcus aureus* PTCC 1112) bacteria. The number of 1.5×10^8 (McFarland Standard No. 0.5) bacterial cells was cultured on Mueller Hinton Agar (MHA) medium (Scharlau, Spain) in three directions. Wells with 6×6 mm in size and 250 mm distance from each other were punched aseptically in the medium. The amount of 100 µl of each extract was treated on the mentioned Gram-negative and Gram-positive bacterial strains. Gentamicin (15 mg ml^{-1}) which is a protein synthesis inhibitor for Gram-negative and Gram-positive bacteria was used as positive control. Distilled water for water extract and methanol for methanol extract were used as negative controls (Kognou et al., 2011; Salimi et al., 2013). All data were extracted by mean average of triplex experiments.

2.4. Chemical analysis

The chemical composition of the effective extract was detected by gas chromatography Mass spectrometer (Mass spectrometer Aglient 5975C coupled with gas chromatograph Aglient7890). HP-5MS column (30 m length with 0.25 mm inner diameter and 0.25 µm film thickness) was used.

3. Results

3.1. The isolated cyanobacterium

Microscopic characteristics including filamentous growth without branch formation, separated arranged trichoms, lack of heterocyst or akinete, and gliding movement resulted in the detection of *Oscillatoria* sp. The microscopic view of the bacterium is shown in figure 1.

3.2. Antibacterial effect of the extract

The averages inhibition zone diameters of the extracts are shown in table 1. Maximum results were achieved by methanol extract on Gram-negative bacterial isolates by the cyanobacterium which was isolated in warmer mounts of the year (May- Jun 2014).

3.2. Chemical composition of the extract

Gas chromatography analysis of methanol extract detected 4 major peaks with retention times of 14.269, 17.058, 19.532 and 20.763 (figure 2). Mass spectrophotometer analysis of the peaks detected 4 compounds including Dodecamethyl-cyclohexasiloxane,Hexasiloxane, Tetracosamethyl-cyclododecasiloxane and Bisabolol oxide A. Chemical characteristics and the structure of the compounds are illustrated in table 2 and figure 3.

4. Discussion

In the present study, waste water samples from aeration pounds in oil refinery plant, Isfahan, Iran were used for isolation of cyanobacteria; and then the antagonistic activity of their extracts was studied on some species of pathogenic bacteria. The waste which was used for isolation of the cyanobacterium had been exposed to sunlight. It has been shown that photo-oxidation increased the bioavailability of microorganisms to petroleum hydrocarbons, which enhance oil biodegradation in aquatic environments (Maki et al., 2005). Green color of the waste which used for isolation of cyanobacteria in our study also indicated the presence of photosynthetic microorganisms in such environments.

As cyanobacteria are resistant to beta-lactam antibiotics, because of specific characteristics of their cell walls and in some degrees are resistant to UV irradiation, we used imipenem and UV light (254nm UVC lamp, for 10 min) for controlling bacterial growth in culture media as well as Cyclohexamide for fungal growth inhibition (Ferris et al., 1991).

It has been shown that nutrients including nitrogen, phosphorus and in some cases iron are limiting factors affecting oil biodegradation processes (Atlas, 1984). However cyanobacteria are able to grow heterotrophically and biotransfer aliphatic compounds to aromatic compounds in crude oil (El-Sheekh and Hamouda, 2014). This ability makes cyanobacteria as suitable candidates for oil biodegradation in aquatic environment including oil waste removal plants.

The methanol extract of cyanobacterium *Oscillatoria* which isolated and detected in the present study, was able to inhibit the growth of Gram-negative (*Escherichia coli* PTCC 1399 and *Pseudomonas aeruginosa* PTCC 1707) and Gram-positive (*Bacillus cereus* PTCC 1015 and *Staphylococcus aureus* PTCC 1112) bacterial strains. However, water extract showed any antibacterial activity. It has been shown that water extract of *Anabaena variabilis* and *Oscillatoria angustissima* had no effect on the tested bacterial strains (Khairy and El-Kassas, 2010). The role of cyanobacteria in the production of antiviral, anti-tumor, antibacterial, anti-HIV and food additive have been well established (Singh et al., 2005). However there are different results obtained by antibacterial testing of cyanobacterial alcoholic extracts. Methanol extract of thermophilic cyanobacteria inhibited the growth of Gram-negative bacteria, and had no effect on Gram-positive strains (Bhardwaj et al., 2010). Martins et al. (2008) were found that nine cyanobacteria isolates have antibiotic activity against two Gram-positive bacteria, *Clavibacter michiganensis* subsp. *insidiosum* and *Cellulomonas uda*. It has been found that the effect of standard antibiotics was more than that of cyanobacterial extracts on *Bacillus subtilis* and *Escherichia coli*. While, this effect were higher than those of standard antibiotics on *Staphylococcus aureus*, *Streptococcus mutans* and *Micrococcus mutans* (Madhumathi et al., 2011; Abed-El-Aty et al., 2014).

The variable results in different studies may be due to cyanobacterium species, solvent composition and the bacterial species used for antibacterial activity (Madhumathi et al., 2011; Reehana et al., 2012).

In the present study, we achieved maximum inhibitory activity by the cyanobacterium which isolated in warm months of the year (May-Jun 2014). The compounds which detected by GC-Mass analysis, are related in their structure (figure 3). Dodecamethyl-cyclohexasioxan and tetracosamethyl-cyclododecasiloxane have been previously reported as bioactive compounds (Moustafa et al., 2013; Patil and Jadhov, 2014; Esmaeili et al., 2012). It seems that dodecamethyl - cyclohexasiloxan and

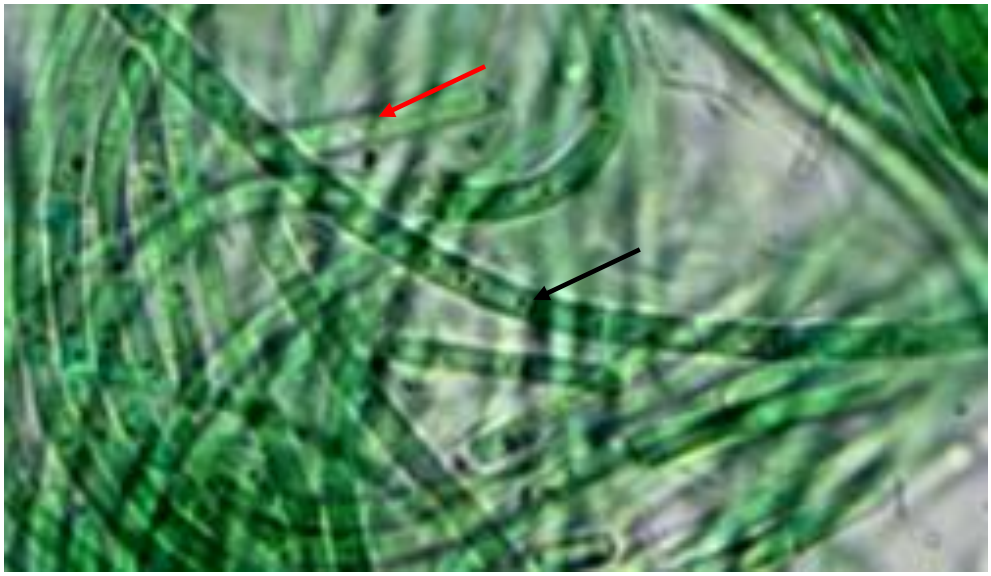

Figure 1. Filamentous growth of the isolated cyanobacterium (*Oscillatoria*). Trichom (red arrow) is seen which is separated (black arrow) and arranged.

Table 1. Mean average of inhibition zone (mm) acquired by the effect of water and methanol extract on bacterial isolates. The results are maximum effect which obtained in warmer mounts of the year.

Bacterial strain	Methanol extract	Water extract	Gentamicin (15 mg ml^{-1})
Escherichiacoli ATCC1399	22.33±0.4	6±0	26.5±0.3
Pseudomonas aeruginosa ATCC107	18.6±1.52	6±0	24.6±0.5
Bacilluscereus ATCC1015	9.3±0.57	6.5±0	29.3±0.5
*Staphylococcusaureus*ATCC1112	7.9±0.3	6.3±0.3	25±0.4

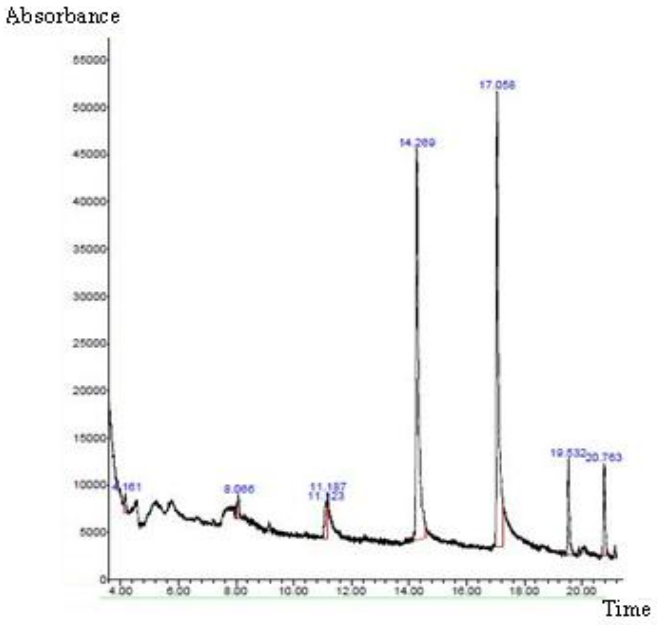

Figure 2. Four major peaks which detected by Gas chromatography analysis with retention times of 14.269, 17.058, 19.532 and 20.763.

Table 2. Characteristics of several kinds of compounds detected by GC-MS.

Peak number	The compound	Chemical composition	Retention time	Area (%)
1	Dodecamethylcyclohexasiloxane	$C_{12}H_{36}O_6Si_6$	14.269	28.11
2	Hexasiloxane	$C_{12}H_{38}O_5Si_6$	17.05	25.76
3	Tetracosamethylcyclododecasiloxan	$C_{24}H_{72}O_{12}Si_{12}$	19.532	3.75
4	Bisabolol oxide A	$C_{15}H_{26}O_2$	20.763	3.91

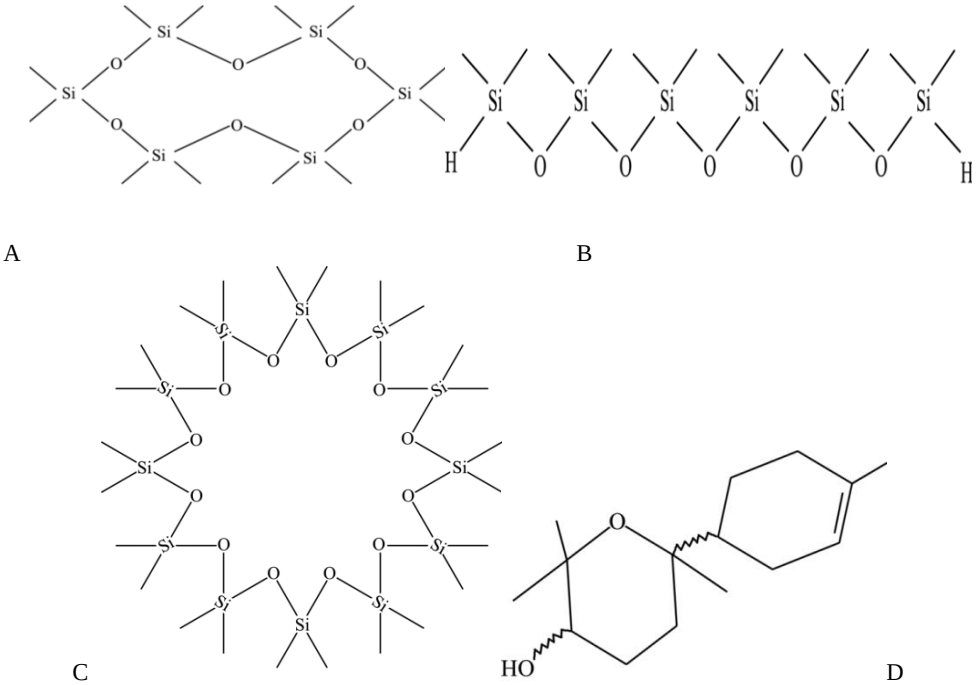

Figure 3. Molecular structure of chemical compounds detected by GC-Mass. A: Dodecamethylcyclohexasiloxane, B: Hexasiloxane, C: Tetracosamethylcyclododecasiloxane and D: Bisabolol oxide A. The three first compounds (A, B and C) seem related in composition and Bisabolol oxide A (D) is an unrelated compound.

tetracosamethyl-cyclododecasiloxane are cyclic forms of hexasiloxane. Bisabolol oxide A which was detected as an unrelated compound in the structure of isolated cyanobacterium also known as a bioactive compound (Dezfooli et al., 2012).

In this study we extracted bioactive compounds from cyanobacterium existing in oil refinery waste. Two of three related compounds (dodecamethyl-cyclohexasiloxan and tetracosamethyl-cyclododecasiloxane) had been previously extracted from different biological origins and defined as bioactive agents. Hexasiloxane has been reported for the first time in this study which was isolated from cyanobacterium. Bisabolol oxide A was an unrelated compound which had been previously known as bioactive agent from different resources and also detected in current study. As cyanobacteria are able to grow in minimal nutritional and environmental conditions, they are suitable candidates for production of bioactive materials.

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Refereces

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