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The isolation and preliminary characterization of native cyanobacterial and microalgal strains from lagoons contaminated with petroleum oil in Khark Island

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Abstract

Introduction: Algae has many applications in terms of ecology, biodiversity, agriculture, medicine, biotechnology, industry, etc. They are potent organisms in bio-active compound production, bioremediation and primary producer. Therefore, it is important to discover local strains with biotechnological and ecological applications.

Materials and methods: Soil and water samples were collected from different sites of Khark Island (Persian Gulf). The samples were cultivated and purified using different techniques. Seven different antibiotics together with other physical methods used to purify the isolates.

Results: Throughout the project 7 strains including 2 eukaryotic algae and 5 cyanobacteria have been isolated. Imipenem and cycloheximide were the best antibiotics for purification of cultures. Three of isolates were morphologically similar to *Arthronema africanum*, *Pseudanabaena teremula*, *Anabaenopsis* sp. However, they have some different characteristics which according to the present identification keys it is not possible to identify their identity (they have nominated Kh.C.d2, Kh.T.1 and Kh.T.2).

Discussion and conclusion: According to the results, isolated strains were identified at the genus level based on morphology characters; therefore the complementary examinations such as molecular identification, ITS, 18s rRNA, 16s rRNA and sequencing can help to approve the strains identity. Upon approval of the new strains account for morphological traits are necessary for their easy identification. The Imipenem antibiotic is the best for eukaryotic algae purification and Cycloheximide is suitable for prokaryotic algae (cyanobacteria) purification.

Key words: Cyanobacteria, Culture, Khark Island, Microalgae, New species, Petroleum pollution, Purification

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Introduction

The information contained in the algal flora of Iran and Iranian coastal areas, especially in the Persian Gulf and its oil polluted reserves are limited and require more dedication to identify potential genetic (1- 7). A checklist of macroalgae in Persian Gulf and Oman Sea including Kharg Island has been published (8). The algae have many applications in different fields, secondary metabolites, fishery and veterinary food, medicine, agriculture and wastewater treatment. All of the organisms have important rules in our ecosystem and it is necessary to manage a scientific and economic program for their protection and utilization.

Oil leaching from the ships, Oil wells and offshore installations to the Persian Gulf has made many environmental damages. The precipitation of oil carbohydrates and other contaminants will destruct the precipitate ecosystem and deplete the dissolved oxygen, make acidic pH and releasing the heavy metals and toxins to the water. The photosynthesis of Dunaliella was inhibited primarily in contact with petrulium oil spilled from Prestige oil tanker in Spain, however gradually during mutations the cells become resistant to contamination (9).

Algae and plants are able to eliminate petrulium oil contaminations in soil and water through Phytoextraction or phytoaccumulation process (10). Cyanobacteria use for bioremediation to purify different environmental contaminations including oil, sewage, and other pollutants. It is important to identify and understand the algal flora of the polluted area to plan the bioremediation researches. Meanwhile, it is necessary to purify and maintain the local algal species for different purposes especially to provide for future studies.

In this systematic project, some of the algal species from the contaminated and non-contaminated lagoons to petrulium oil has been cultured and identified. The isolated axenic cultures maintained in Iranian Biological Resource Center (IBRC) for long-term preservation.

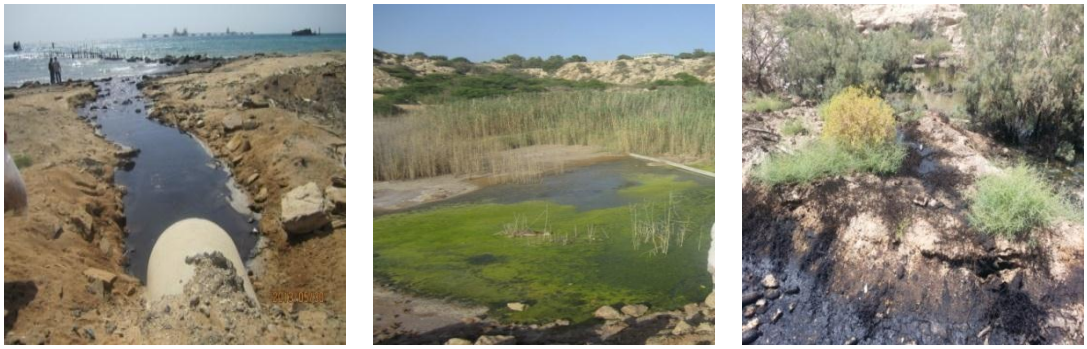
Materials and methods

Khark Island is a coral island with 32Km^2 situated in 54 km distance from North West of Bushehr port in the latitude and the longitude of 12°, 29', 30" N and 50°, 12', 45" to 50°, 20', 10" E (11). In coordination with companies and offshore oil terminals, two consecutive samplings were done from sewage lagoon and clean wetland (Baghe Pire Mard) from February 2012 to June 2013.

Sampling Stations: The geographical location and condition of stations have depicted in (fig. 1 and table 1). In the Baghe Pire Mard wetland, no oil contamination were seen and the plants were grown in a good situation.

Table 1- Geographical and pollution conditions of the sampling stations

Contamination condition	Geographical Location	Sampling station
A little contamination with plants growth	N2922/6 33/5, E5030 29/1	Oil Lagoon A
Water and soil contaminated to oil	N 29 13 16.5, E 050 19 41.3	Oil Lagoon C
Extreme contamination	N29 21/9 29/8, E50 30/3 24/2	Coastal area in south west of island
No oil contamination	E050 15 57/5, N29 15 57/5	Baghe Pire Mard wetland



South east coastal area Oil Lagoon A Oil Lagoon A

Fig. 1- Sampling stations of Khark Island contaminated with oil compounds

Fig. 2- The growth chamber for photosynthetic organisms

The water and soil samples were collected in screw cap bottles and kept in 4°C and transferred to the lab as soon as possible and cultured immediately in BBM, BG11, and Chu (12- 14) medium. Based on table 2, different antibiotics (prokaryotic and eukaryotic) were used to inhibit the growth of undesired organisms. Antibiotic solutions (50 µg/ml) were sterilized with 0.2 µ Milipore filter.

Table 2- Utilized antibiotics and their solvents

Solvent	Antibiotic
Distilled water	Ampicillin
Ethanol + DMSO	Penicillin
Distilled water	Cephalexin
Physiologic serum	Chloramphenicol
Ethanol	Imipenem
Water and alcohol	Nistatin
Distilled water	Cycloheximide

The cultured petri dishes were incubated in 16-8 hour's light-dark period at 25±2 and 2500 to 3000 lux light intensity (fig. 2).

Purification Process: According to Richmond different algological and microbiological techniques such as antibiotic, serial dilution, centrifuge, subculture with stereomicroscope and UV irradiation were used to eliminate undesired microorganisms (15). The antibiotics used in the media for purification are depicted in table 2. Cyanobacteria purification is difficult because they grow slowly and have symbiosis and hidden bacteria in their sheath. However, they grow and form a long filament with the ability to move which help to isolate and purify them by a stereomicroscope. The isolated microalgae

were cultured in liquid medium and shake manually every day. The growth of cells was determined with OD of the liquid culture with a spectrophotometer and counting the cells with Neubauer slide.

A part of sample fixed with formaldehyde 37% for bright field and phase contrast pictures with Olympus BX51 microscope. The morphologichal charcters such as chloroplast shape,cell size, flagella, unicellular, filamentous or colony, akinet, heterocyst and its position, sheath, form of apical cell, cell borders and many others were used to identify. The following morphological key books were used to identify their species; (16- 19).

Results

Based on the physic-chemical characters of the stations (table 3), oil lagoon A and south-west coastline were the most oil contaminated stations and Bghe Pire Mard had no oil pollution. The pH of all stations was alkaline and other chemical parameters are shown in Table 3.

The most effective antibiotic in eliminating bacterial contamination was imipenem (betalactame wide spectrum antibiotics that inhibit the synthesis of peptidoglycans of the cell wall) which is depicted in Table 4. Based on this table and according to Zehnder & Hughes (20) the cycloheximide is against eukaryotic cells (protein synthesis inhibition) and is suitable for cyanobacteria purification, however it weakens the eukaryotic algal growth. The efficiency of penicilin and ampiciline is low due to bacterial resistance. The chloramphenicol and cephalexin were active against cyanobacteria and useful for eukaryotic algal isolation.

The microalgae especially eukaryotic growth was inhibited by increasing antibiotic concentration from 50 to 100 µg/ml. The use of multiple antibiotics in the culture medium can be a toxic effect on microalgae, especially cyanobacteria, and they will lead to growth inhibition and death. Therefore, because of inhibition effect of high dose or multiple antibiotics, it is better to use broad spectrum antibiotic effective on more bacterial group. Resistance and sensitivity of microalgae to various antibiotics has been shown in Table 4.

Nystatin with antifungal activity was not appropriate in this study because of inhibition of the growth microalgae (both cyanobacteria and eukaryotic algae) except in one case (species KH.T.1). In this study 7 strains (5 cyanobacteria and 2 green algae) have been identified and cultured axenically.

Table 3- The physicochemical and petroleum compounds in the samples

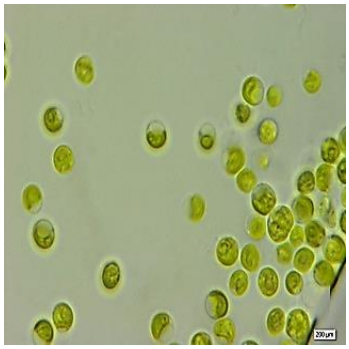
Petroleum Compounds	NaCl	$PO4^{2-}$	Mg^{2+}	Ca^{2+}	pH	TemperatureC°	Sampling area
12	18105	0.5	170	720	8.2	25	Oil Lagoon C
16	18637	0.5	194	800	8	25	Peripheral of oil Lagoon C
14	16862	0.8	211	712	8	25	Oil Lagoon A
18	17217	0.9	170	720	8	15	Peripheral of oil Lagoon A
<18	–	–	–	–	8.3	26	Coastal area south west of island
–	16316	0.9	185	811	8.3	25	Baghe Pire Mard wetland

Values is mg/l

Table 4- The sensitivity of isolated algae to antibiotics used in media

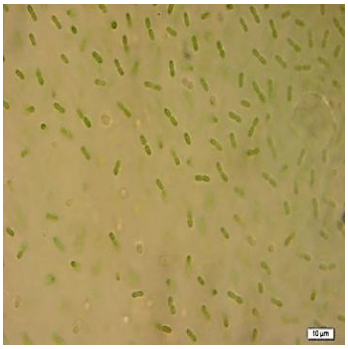
Strain	Ampicillin	Penicillin	Cephalexin	Chloromphenicol	Imipenem	Cycloheximide	Nystatin
Leptolyngbya nodulosa	+	+	−	−	+	+	−
Strain Kh.C.d2	+	+	−	−	−	+	−
Cyanobacterium aponinum	+	+	−	−	−	+	−
Strain KH.T.1	+	+	−	−	+	+	+
Strain KH.T.2	+	+	−	−	+	+	−
Chlorella sorokiniana	+	+	+	+	+	−	−
Chlorococcum ellipsoideum	+	+	+	+	+	−	−

(+)growth (–)lack of growth

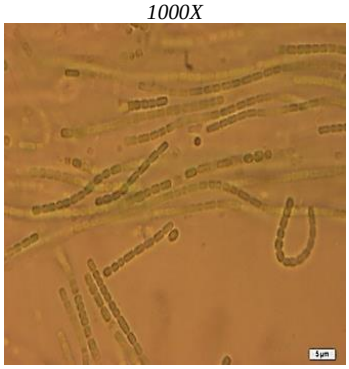


Chlorella sorokiniana
1000X

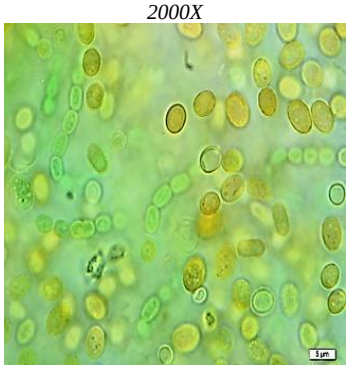
Chlorococcum sp.
2000X



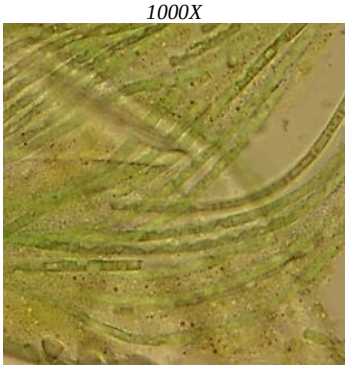
Cyanobacterium aponinum
1000X



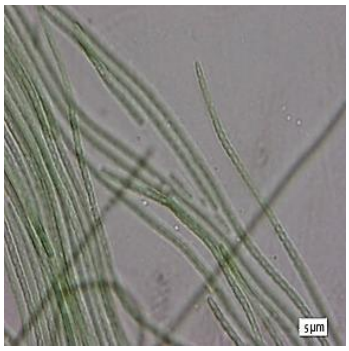
Leptolyngbya nodulosa
2000X



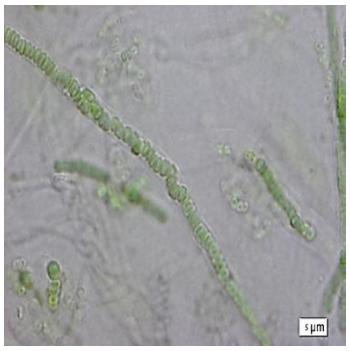
Kh.c.d2 (Anabaenopsis abijatae)
2000X



Kh.T.1 (*Arthronema africanum*)
2000X



Kh.T.2 (Pseudoanaba sp.)
1000X



Kh.T.2 (Pseudoanaba sp.)
2000X

Fig. 3- Photomicrograph of identified and cultured strains from Khark Island.

The photomicrograph at light microscopy level of isolated species is shown in fig. 3. Four species of cyanobacteria are filamentous which three of them have not been identified exactly because of their new characters which have not been mentioned in the identification keys.

Species Kh.C.D1 with 2 to 4 µm terminal heterocyst and cylindrical and barrels shape akinet (similar to *Anabaenopsis abijatae).*

The specie Kh.T.1 contain distinct gelatinous sheath around the trichome and regular cells (similar to *Arthronema africanum).*

Species Kh.T.2 had gelatinous sheath around the trichome and polymorphism shape with some short and disk cells (similar to *Pseudanabaena teremula).*

One of the cyanobacteria (*Cyanobacterium aponinum)* and two eukaryote algae were unicellular shown in fig. 3.

Discussion and conclusion

In the present study, the media BBM, BG11, and Chu No.10 were used for cultivation and purification of microalgae which their effectiveness demonstrated in previous studies (21, 22). Types of media can influence the rate of growth, biomass, cell shape, trichome, pigments and even the morphology of algae. The results showed that, compared with two other media, BG11 was suitable for the growth of cyanobacteria and BBM and Chu No.10 medium for the growth of eukaryotic algae.

The basic problems of cultivation and preparation of algal biomass are existence of a variety of organisms and contamination which deleting any one of them requires its own procedures. Based on the obtained results imipenem inhibit bacteria and cycloheximide inhibits the growth of fungi and molds which is appropriate for cyanobacterial cultures. As a conclusion, these two antibiotics recommend for algal and cyanobacterial culture purifications.

Among the oil polluted sampling areas, oil lagoons C with the lowest pollution, and Baghe Pire Mard without oil pollution had the highest number of isolates and the coastal region of South West Khark Island with the highest level of pollution, had the lowest number of isolates. Our study has confirmed the previous researches showing that high concentrations of oil pollutants can cause deactivation of critical processes such as photosynthesis and nitrate uptake and reduction in chlorophyll content in, *Nostoc moscorum, Spirolina plantensis, Anabaena* sp., *Synechococcus* sp. and affect the diversity of microalgal growth in oil polluted areas (23, 24). Microalgae in the oil-polluted environment can tolerate some degree of pollution, but petroleum compounds at high concentrations as due to problems with damage membranous will inhibit their growth (25). It is important to isolate, purify, identify and study oil decomposition microorganisms (bacteria, microalgae and etc.) in rich reserves countries (26). In the present study, a green alga *Chlorella sorokiniana* was isolated from oil contaminated areas of Khark Island. *C. sorokiniana* was present in 4 areas around and inside the oil region of

lagoon A, oil lagoon C and coastal area southwest of Khark Island. Due to the presence of live algae in water and soils contaminated with petroleum compounds in the area, these strains may have the potential to remove oil contamination and have the ability to break down oil contaminants which it requires further investigation for evaluation.

Based on morphologic investigation three suspected isolates were not identified certainly which two of them belonging to the Pseudanabaenaceae family of cyanobacteria. The first isolate (species Kh.C.d2) of the Khark Island was similar to *Anabaenopsis abijatae* with cylindrical and elongated cells, while the adjacent genera, *Anabaena*, and *Nostoc* cells are spherical. The isolated cyanobacteria similar to *A. abijatae* have terminal heterocyst however in *Anabaena* and *Nostoc* the heterocyst cells are intracellular and interstitial. The heterocyst and akinet in *A.abijatae* are far with distance but in *Anabaena*, they are close to each other. However, our isolate has two more differences; firstly the heterocyst diameter of *Anabaenopsis*is 7 to 10 µm but our isolate is 2 to 4 µm. In addition, *A. abijatae* has generally spherical akinet and less oval in shape while, on the isolated species from kharak, akint are generally cylindrical and barrels in shape and are less spherical. Also, the akinet diameter of *A. abijatae* is 12 to 14 µm but in isolate is 6 to 8 µm. Therefore, the species of *Anabaenopsis* is not clear and need more investigations.

Another isolate (species Kh.T.1) morphologically was similar to *Arthronema africanum* species with some differences. In *A. africanum* trichome is 0.8 to 5 µm thick without mucilage cover. There are irregularly swollen cells along the columnar cells, which always occur in Trichome (27), whereas in our case cyanobacteria not only has a distinct gelatinous sheath around the trichome but also no swollen cells were seen. Unlike, some areas of trichome cells have depression. Further molecular tests required to confirm the stains.

The third cyanobacterium (species Kh.T.2) was isolated from Baghe Pire Mard area with polymorph morphology which made it difficult to identify. It is morphologically similar to *Pseudanabaena teremula* from Pseudanabaenaceae family with simple cylindrical and 4 µm width trichome (seldom 6 µm), cell length is greater than the width, and the cell has a definite border (28). Differences of our isolate with *P. teremula* is the presence of obvious gelatinous sheath around the trichome, whereas it is absent in *P. teremula*. Due to polymorphism, some of the cells along the trichome are short and seeming cells are disk-shaped while in *P. teremula* all trichome cells are cylindrical shape and length is larger than the width (17).

According to the results obtained here, our isolated strains were identified at the genus level based on microscopic morphological examination and it was not possible to identify any isolate

at the species level. The isolated strains appeared morphologically distinguishable from each other ranged from microalgae with a unicellular organization to colonial

and filamentous forms with either branched or unbranched organization. Further investigations are directed at identifying any isolate at species level using precise molecular and physiological approaches as well as their possible role in natural biodegradation process of oil.

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جداسازی و شناسایی مقدماتی ریزجلبک‌ها و سیانوباکترهای بومی لاگون‌های نفتی جزیره خارک

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چکیده

مقدمه: جلبک‌ها به لحاظ اکولوژیکی، تنوع زیستی، کشاورزی، پزشکی، زیست فناوری، صنعت و سایر، بسیار مهم هستند. این موجودات توانایی زیادی در تولید زیست مواد فعال، زیست پالایی و تولیدات اولیه دارند. بنابراین، دستیابی به سویه‌های بومی ایران که دارای توانایی‌های بیوتکنولوژیکی و اکولوژیکی دارای اهمیت است.

مواد و روش‌ها: آب و خاک منطقه از ایستگاه‌های مختلف جزیره خارک (خلیج فارس) جمع آوری شدند. نمونه‌ها کشت و به کمک روش‌های مختلف خالص‌سازی شد. برای خالص‌سازی از ۷ آنتی‌بیوتیک و سایر روش‌های فیزیکی استفاده شد.

نتایج: در این پروژه ۷ سویه شامل ۵ سیانوباکتر و ۲ ریز جلبک کشت و خالص‌سازی شده است. آنتی‌بیوتیک‌های ایمیپنم و سیکلوهگزامید به عنوان بهترین پادزی‌ها برای خالص‌سازی کشت‌ها استفاده شدند. سه سویه به دست آمده به لحاظ ریخت شناسی در عین شباهت به سویه‌های *Anabaenopsis*، *Pseudanabaena teremula*، *Arthronema afaricanum*، تفاوت‌هایی هم با آنها دارند که به کمک کلیدهای ریخت شناسی امکان تعیین قطعی گونه وجود ندارد (این گونه ها Kh.T.2، Kh.T.1 و Kh.C.D2 نام گذاری شده اند).

بحث و نتیجه‌گیری: بر اساس نتایج بدست آمده و شواهد و صفات ریخت‌شناسی، سویه‌های جدا شده در سطح جنس شناسایی شده‌اند و دارای صفات حدواسط هستند. بنابراین، آزمون‌های تکمیلی مثل نشانگرهای ملکولی ITS و 18s rRNA و 16s rRNA و تعیین توالی ژن‌ها می‌تواند در تایید گونه‌ها مفید باشند. در صورت تایید سویه‌های جدید تبیین صفات ریخت‌شناختی برای آنها شناسایی آسان آنها ضروری است. بهترین آنتی‌بیوتیک برای خالص‌سازی جلبک‌های یوکاریوت، ایمیپنم و برای سیانوباکترها، سیکلوهگزامید است.

واژه‌های کلیدی: آلودگی نفت، جزیره خارک، خالص‌سازی، ریزجلبک، سیانوباکتر، کشت، گونه جدید

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