

ORIGINAL ARTICLE

Measurement of *Anabaena* Metabolites and pigments isolated from Anzali Wetland

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ABSTRACT

Cyanobacteria or blue-green algae are one of the most diverse groups of gram-negative prokaryotic distributed around the earth. They produce a variety of secondary metabolites with diverse biological effects. Having metabolic tracts strengthens the probability of having access to new compounds in these microorganisms, with diverse applications in the pharmaceutical industry, scientific research, the production of food for humans and livestock and aquaculture. In this study, cyanobacteria *Anabaena* was isolated and purified in Sorkhankol, in the central part of Anzali Lagoon. Detecting was done using valid keys and molecular analysis of 16SrRNA. Phytochemical tests were conducted for having methanol extract. The results showed this strain with 534 nucleotides has 100% degree phylogenetic relationship with *Anabaena variabilis* ATCC29413 strain. Isolated compounds phytochemical consisting of compounds indicate alkaloid and saponin nature of these compounds. Chlorophyll content was also calculated based on Marker method, and its amount was 0.428 micrograms per milliliter. Phycobiliproteins assessment was conducted based on Siegel man method. The amount of phycocyanin was 1.373 micrograms per milliliter, that of Allophycocyanin was 0.23 micrograms per milliliter, and that of Phycoerythrin was 1.627 micrograms per milliliter.

Keywords: Sorkhankol, cyanobacteria, methanol extract, phytochemical compounds, chlorophyll, phycobiliproteins

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INTRODUCTION

Cyanobacteria are the most abundant organisms in aquatic ecosystems. These microorganisms are among the oldest living prokaryotic creatures on the Planet Earth that today play an important role in human life. The algae are a rich source of biologically active metabolites. Recent studies reveal the existence of bioactive compounds in blue-green algae in aquatic ecosystems, many of which have anti-bacterial, anti-fungal, anti-viral, anti-cancer and immunosuppressive compounds. [1] Isolation of bioactive compounds from cyanobacteria is taken place in order to discover new compounds for biological, pharmaceutical and agricultural applications. [2] By increasing the number of bacteria and fungi and viruses and the increase in their resistance to antibiotics and its derivatives, cyanobacteria are highly promising in terms of new medicine and as a rich source of new structures and metabolites with biological activities. [3]

Primary or secondary metabolites produced by these microorganisms prove the potential of bioactive compounds in industry. [4] Overall, only 10 percent of cyanobacteria isolated in laboratory environment can be cultured, and a limited number of these species have been used commercially. [5]

MATERIALS AND METHODS

Sampling

Sampling was carried out in Sorkhankol area. This area locates in the central part of Anzali Lagoon and at the geographical coordinates of east longitude E492718- E492404 and north latitude N372620 – N372304. Sampling was done using sterile screw lid containers made from surface to a depth of 50 cm.

Isolation

In order separate Marine cyanobacteria, 500 ml sample water was passed through sample from the Millipore filter with pores having diameter of 0.45 microns, then the filter paper was transferred to a flask containing 500 ml of medium BG11 (Merck Company, Germany). (BG11 Medium for blue-green algae ATCC medium 616)

Samples were placed at 25 ° C as optimum temperature for growth, the light intensity of 2500 lux with the optical frequency of 16 hours of light and 8 hours of darkness and the pH was 7.5. In order to aerate liquid medium, shakers having 150 rpm with an air pump was used. (2)

Purification

A) Serial dilution method

To serve this purpose, 9 ml of the medium BG11 was added to 10 sterile tubes. And then, 1 ml grown cyanobacteria from Erlenmeyer flask was added to tube (1). The content of the first tube was mixed well to be perfectly uniform at dilution of 10^{-1}

Then 1 ml of it was transferred in sterile conditions to the next tube. Previous was repeated for the next pipe respectively to achieved dilution of 10^{-10}

B) *Method* Pour plate: 1.5% agar was added to the liquid culture medium BG11 in order to make solid BG11, and then antibiotic Nystatin (100 micrograms per ml) was added to remove fungal infections and then antibiotic cycloheximide (100 micrograms per ml) was added to the culture medium to remove bacterial contamination. 1 ml of each corresponding dilution and 15 ml of BG11 culture medium was added to each of 10 sterile plates. And then the plates were shaken by rotational motion and were posed in a still state. And then they were posed at 30 ° C and exposed to radiation by yellow and white lights. (8)

Identification of cyanobacteria

A) Microscopic examination

For this purpose, valid identification keys such as Dyskachry and Prescott along with algae base website were used. (9, 10) we prepared slides from grown cyanobacteria and they were examined with a magnification of X100. (Figure 1)

B) Molecular analysis of 16SrRNA

DNA of cyanobacteria was extracted. (11.12) and with primer Pa (5'AGAGTTTGATCCTGGCTCAG-3') (13) and primer B23S (5'-CTTCGCCTGTGTCTAGGT-3') (14) proliferation was performed through PCR. It was sent to Iran's National Center for genetic resources in order to determine the sequencing.

Preparation of methanol extract

For this purpose, the cyanobacteria grown in BG11 medium were centrifuged at 4000 rpm, and **solvent methanol** was added to the settled Sediment, and the sediment with the solvent were transferred to sterile tube. And the samples were incubated for 24 hours at room temperature and darkness. After incubation duration, the color change was observed in solvents and colored liquid was separated from the sediment. And they dry weight was calculated using the following formula.

$$DEW = PE - P$$

DEW= Dry extract weight

PE: Weight of petri dish + extract

P: Weight of petri dish

Chemical assessment of methanol extract

- Alkaloid: **Mayer's reagent** test was used for this purpose; yellowish-white sediment indicates the presence of alkaloid. (15)
- Saponin: 1 gram of cyanobacteria powdered by lyophilizer with 10 ml of distilled water were poured into the tube, and were being severely shaken for 10 seconds, And the existence if foam to a height of 1 to 10 cm for a period of 10 minutes is indicative of saponin. (15)
- Tannin: 2 ml of extract of cyanobacteria Anabaena plus 1% dissolved gelatin along with sodium chloride were added, forming a white precipitate indicates the presence of tannins.(15)
- Flavonoid: 2 ml of extract of cyanobacteria Anabaena, and a few drops of dissolved acetate are added, the yellow represents flavonoid. (15)

Extraction of chlorophyll

Cyanobacterial biomass with 2 to 3 ml **90% acetone** were placed in the dark for 2 hours at 4 ° C, and Extracts were centrifuged for 15 min at 4000 rpm, and the supernatant liquid was poured onto the sterile plate and dry weight of the extract was calculated. (16)

Measurement of chlorophyll and phycobiliproteins

Marker method was used to measure chlorophyll, and its Light absorption was measured at a wavelength of 665 nm, and the amount of chlorophyll was calculated according to the following equation in terms of micrograms per milliliter. (17)

$$chl=13.14 \times OD\ 665$$

Phycobiliprotein assessment was based on Siegel man method. (18)

Supernatant absorption was measured at wavelengths of 562 and 652 and 615 and each of Phycobiliproteins was assessed according to the following formula in terms of micrograms per milliliter.

$$\text{Phycocyanin (PC)} = [A620 - (0.474 \times A652)] / 5.34$$

$$\text{Allophycocyanin (APC)} = [A652 - (0.208 \times A620)] / 5.09$$

$$\text{Phycocerythrin (PE)} = [(A562 - (2.41 \times PC)) - [0.849 \times (APC)]] / 9.62$$

RESULTS

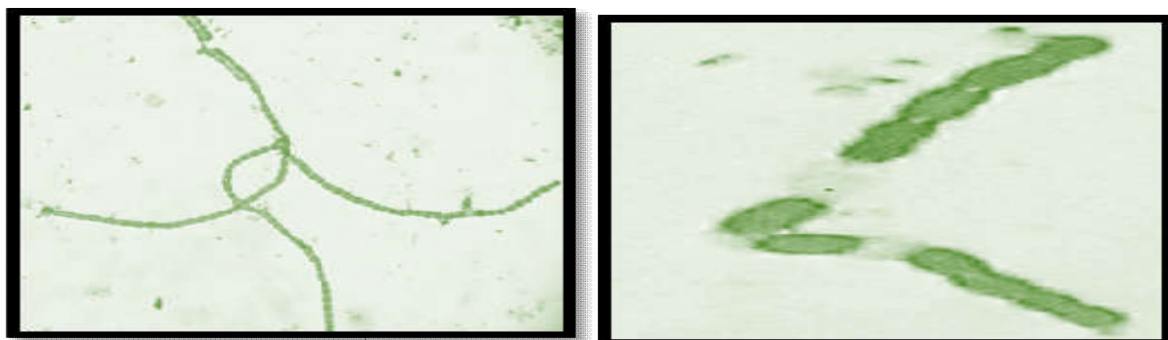


Figure 1 Microscopic features of cyanobacteria Anabaena: Vegetative cells are juxtaposed like beads, and Oval-shaped Akinetes and Heterocyst us were placed among vegetative cells, and Trichomes are in a spiral shape and free of gelatin coating.

The results of 16SrRNA gene sequences reading showed that the mentioned with 534 Nucleotides has 100% phylogenetic relationship with Anabaena strain of variabilis ATCC29413. 534 nucleotides were sequenced from 16SrRNA (sequencing was performed according to the Sanger method)

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CCCCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGACGGGGCCCGCACAAGCGGTGGAGTA
TGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAAGACTTGACATGTCGCGAATCTTCTTGAAAGGGAA
GAGTGCCTTAGGGAGCGCAACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTCGTTTTTGTAGTTGCCAGCATTAAAGTTGGGCACTCTAGAGAGACTGCCGGTGA
CAAACCGGAGGAAGGTGGGGATGACGTCAAGTCAGCATGCCCTTACGTCTTGGGCTACACACGTACTACAA
TGCTACGGACAGAGGGCAGCAAGCTAGCGATAGCAAGCAAATCCCGTAAACCGTAGCTCAGTTCAGATCGCA
GGCTGCAACTCGCTGCGTGAAGGAGG
    
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Dry weight of acetone extract: 550 mg / ml
 Dry weight of methanol extract: 300 mg / ml

The results of phytochemical measurements on cyanobacteria Anabaena isolated from Sorkhankol area

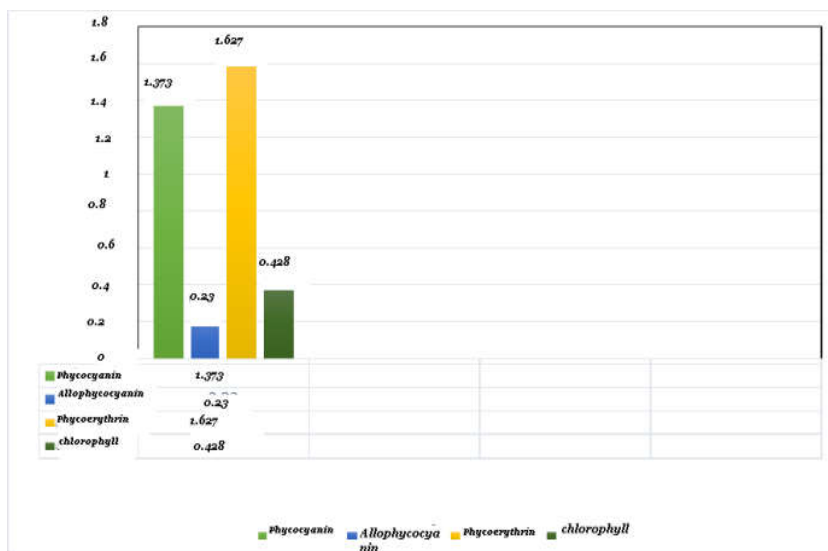
The effective ingredient in the extract	observations	Results
Alkaloid	Creation of yellowish-white sediment	+
Saponin	Creation of stable foam	+
Flavonoid	Pink colored	-
Tannin	Green or blue colored	-

The chlorophyll and phycobiliproteins proteins in cyanobacteria *Anabaena variabilis ATCC29413*

DISCUSSION

In the present study, molecular analysis 16SrRNA was performed on cyanobacteria existing in the sample using valid identification keys to the level of genus and species. (The first step was to identify the key characteristics related to genus.) The results of the reading 16SrRNA gene sequences showed that this

strain with 534 nucleotides has 100% degree phylogenetic relationship *Anabaena varibilis* ATCC29413 strain. William et al in 2006 expressed that a combination of detection methods based on morphological characteristics and molecular methods are very convenient. In this regard, they stated that in recent years, molecular information has supported and proved the validity of some cyanobacterial genera such as Pseudo anabaena, Planktothrix Microcystis, and Spirulina. [19] While in 2005 Hongmei et al reported using molecular information of 16SrRNA creates incompatibility between morphological data and molecular data [20].



Phytochemical compounds are among specific botanical compounds which act as a product of secondary metabolism. In the Phytochemical tests of this research, alkaloids and saponins were identified in the extract of *Anabaena varibilis* ATCC29413. According to research conducted by Aybana et al in 1991, alkaloids, as active ingredients in cyanobacteria, have inhibitory effects on some pathogenic bacteria and inhibit their growth [21]. The existence of a variety of alkaloid compounds in Cyanobacteria *Anabaena* has been shown in several studies, including the study in 2001, which proved the presence of compounds such as alkaloids [22].

Today the use of remote sensing and spatial evaluation determines Algal blooms, with access to accurate concentrations of phycobilins pigments to determine population density of microorganisms is of great concern. The importance of Phycobiliproteins as an anticancer compound in the form of edible color in food and pharmaceutical industries is considered by many researchers [23]. In this study, the examination of Chlorophyll content was conducted based on the Marker method and it was measured 0.428 micrograms per milliliter, and Phycobiliproteins assessment was conducted based on Siegelman method. The amount of phycocyanin was measured 1.373 micrograms per milliliter and that of Phycoerythrin was measured 1.627 micrograms per milliliter and that of Allophycocyanin was measured 0.23 micrograms per milliliter. In the studies conducted by Ojit et al in 2012, the highest amount of phycocyanin, Phycoerythrin and Allophycocyanin was seen in the strains of *Anabaena fuellebornii*, *Phormidium bohneri* and *Nostoc spongiaeforme* [24].

In Iran in the recent years, new research approach has been taken toward environmental issues and the use of microorganisms in industry. However, most of these studies have been focused on specific microorganisms and despite the variety of cyanobacteria in biological activity of these microorganisms, because our knowledge of them is little, little attention has been paid to them. Studies on the use of cyanobacteria as natural resources to control plant diseases in the country have not been many, and this study is among the first studies.

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