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Biochemical characterization of five marine cyanobacteria species for their biotechnological applications

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Abstract
In the present investigation to study the growth measurements and biochemical ingredients of five species of marine cyanobacteria in terms of chlorophyll a', phycobilipigments, total carotenoid, total protein astaxanthin and polyphenolic contents were analyzed namely, *Synechococcus elongatus*, *Synechococcus aeruginosus*, *Oscillatoria subbrevis*, *Phormidium species* and *Phormidium fragile* there is repository in the germplasm collections of Jamal Mohamed College. Growth measurement of chlorophyll 'a' and protein were recorded at 2 days interval until 15th day. The amount of chlorophyll 'a' and protein were recorded maximum at 7th day and rarely 9th day. The analysis revealed that the maximum quantity of chlorophyll a' and total protein was observed in *Synechococcus aeruginosus* (58.14 ± 1.24 mg mL^{-1} and 203.51 ± 15.26 µg mL^{-1}) followed by *Oscillatoria subbrevis* (37.24 ± 2.07mg mL^{-1} and 142.81 ± 3.73 µg mL^{-1}), minimum in *Synechococcus elangatus* (10.39 ± 0.36 mg mL^{-1} and 48.92 ± 2.88 µg mL^{-1} respectively). Whereas *Phormidium fragile* showed higher quantity of phycobilipigments especially Phycoerythrin (32.28±0.34 µg mL^{-1}) followed by *Oscillatoria subbrevis* (13.03±0.60 µg mL^{-1}) respectively. Phycocyanin was recorded highest in *Phormidium species and Synechococcus aeruginosus* (14.33±0.09 µg mL^{-1} and 13.83±1.05 µg mL^{-1}) followed by *Oscillatoria subbrevis* (11.46±0.02 µg mL^{-1}), the minimum amount of Phycoerythrin in *Phormidium species* (1.57±0.67 µg mL^{-1}) and minimum phycocyanin in *Synechococcus elongatus* (2.75±0.01µg mL^{-1}). Total carotenoid was recorded highest quantity in *Synechococcus elongatus* (7.49±0.21 µg g^{-1}) followed by synechococcus aeruginosus (2.84±0.10 µg g^{-1}) very least in *Phormidium fragile* (0.93±0.25 µg g^{-1}) respectively. Astaxanthin highest quantity was present in *Oscillatoria subbrevis* (133.11±5.39 µg g^{-1}), minimum in *Phormidium fragile* (9.85±0.18 µg g^{-1}). Polyphenolic component maximum was observed in *Phormidium fragile* (583.77±66.33 µg g^{-1}), very least in *Oscillatoria subbrevis* (14.82±1.08 µg g^{-1}). The study revealed that *Oscillatoria subbrevis* and *Phormidium fragile* was a potent producer of astaxanthin, phycoerythrin and polyphenolics.

Keywords: marine cyanobacteria, antioxidative pigments, total protein

Introduction
Cyanobacteria are the large and morphologically diverse group of unique photosynthetic organisms of great importance because of their very long existence and cosmopolitan distribution in terrestrial, freshwater and marine habitats. As a basic research tool, they are largely known to provide critical insights into the origin of life, photosynthesis, nitrogen fixation and primary metabolism [1]. The innate properties of cyanobacteria make them ideal organism with potential for multifaceted biotechnological applications [2].

Cyanobacteria have gained significance as sources of wholesome food materials, fixed atmospheric nitrogen, natural colorants, bio plastics, biofuels, fine chemicals, bioactive substances, common and fine chemicals like lipids, pigments, enzymes, polysaccharides, glycerol and other novel biologically active compounds [3, 4]. Cyanobacterial biotechnology has opened up vast opportunities, whereby, these microbes could be used for eliminating human sufferings [5].

Active research in natural products of cyanobacteria has made significant advances in aquaculture. Cyanobacteria have been shown to produce a variety of compounds and some of them have been proved to possess biological activity of potential medicinal value [6]. They deserve special attention due to their antioxidant potential as they constitute a significant amount of carotenoids, phycocyanin, vitamins, flavanoids and phenolic compounds [7, 8] which are able to scavenge highly reactive free radicals that cause tissue damage [9].

Among proteins, the phycobiliproteins make the major class of water soluble proteins and they are exploited as a major source of blue pigment in food industries [10].

Cyanobacteria produce different types of pigments, amongst them chlorophyll, phycobiliproteins (c-phycocyanin, c-allophycocyanin, and c-phycoerythrin) and carotenoids are the main pigments. These pigments have commercial value as natural coloring agents, drugs, as antioxidant in cosmetic industries, to improve health and fertility of cattle and also used in the biomedical research [11, 12].

Phycobiliproteins in particular, are widely used as a natural colorant in food products such as chewing gum, fermented milk products, ice creams, soft drinks, desserts, sweet cake decoration, jellies, milkshakes, etc. [13, 14, 15, 16].

Carotenoids are the most widespread group of naturally occurring pigments produced by both prokaryotes and eukaryotes including cyanobacteria. More than 750 structurally different yellow, orange, and red-colored molecules are characterized till date [17]. Carotenoids are biotechnologically high-value compounds with an annual market estimated to exceed one billion US dollars by 2010 [18]. Astaxanthin is a substance that protects the skin against UV-in-duced photo-oxidation and it is used for anti-tumor therapies and pre-vention - treatment of neural damage interrelated with age-related macular degeneration, Alzheimer and Parkinson diseases [19, 20]. Fur-ther more, it is considered as a natural super food destined to enhance athletic performance by increasing stamina and reducing the time ofmuscle recovery [21, 22].

Astaxanthin is a most important carotenoid on the global market after β-carotene and lutein, with a predicted sales volume of 670 metric tons valued at 1.1 billion US$in 2020 [23]. Currently, astaxanthin is primarily used as a food and beverage colorant, in animal feed and in nutraceuticals [24]. Astaxanthin shows the strongest hitherto demonstrated anti-oxidant effect due toits keto and hydroxy groups at 4,4'- and 3,3'-beta-ionone ring positions, respectively. Those functional groups result in a more polar nature of astaxanthin and explain its unique antioxidative properties [25]. Among which phenols have demonstrated the highest *in vitro* antioxidant activities [26, 27]. In the case of microalgae and cyanobacteria, some studies endorse their potential as rich sources of natural antioxidants [28, 29] but the data concerning their phenolic profile are still extremely scarce. The present work has aimed to biochemical characterization of marine cyanobacteria for their biotechnological applications. The study throws light upon the cyanobacteria pigments as a source of natural colorants and proteins as sources of food and feed in selected marine cyanobacteria strains.

Materials and Methods

Source of samples

Five fast growing marine cyanobacteria species comprising (two unicellular (*Synechococcus elongatus and Synechococcus aeruginosus*) *and three filamentous isolates (Oscillatoria subbrevis, Phormidium species. and Phormidium fragile)* were selected from the Germplasm collections of Jamal Mohamed College, Tiruchirappalli, Tamil Nadu, India for the present study.

Culture conditions

Axenic cultures of the selected cyanobacteria were grown in 250 ml Erlenmeyer flasks containing 100 ml of ASN III medium [30]. Experimental cultures were incubated at 25±2°C, 14/10 light/dark cycle with illumination of 3000 lux under cool white fluorescent lamps. Cultures were mildly shaken by hand for 10 minutes every day.

Pigments analyses

Estimation of Chlorophyll

Chlorophyll *a*' was determined according to [31]. A known quantity of homogenous cyanobacterial culture was centrifuged at 5,000 ×g for 10 min. The pellet was washed twice with distilled water and then suspended in 4 ml of methanol and vortexed thoroughly. The mouth of the test tube was covered with aluminium foil to prevent the solvent evaporation. The tube was incubated in a water bath at 60°C for 1 hour with occasional shaking. The tube was cooled and centrifuged at 5,000 rpm for 5 min. The supernatant was transferred to another tube and once again 4ml of the solvent was added and extracted as above. To ensure complete extraction, 2ml of the solvent was added to the pellet and the process repeated. The supernatants were pooled and made up to 10 ml volume with methanol. Read at 663 nm in a spectrophotometer against methanol blank.

Estimation of Phycobiliprotein

A known volume of homogenized suspension of cyanobacteria was taken and centrifuged at 3000×g for 5 minutes. Phycobiliprotein was extracted completely from the pellet using 0.05M phosphate buffer by repeated freezing and thawing. The absorbency of the supernatant was read at 615, 652, and 562 nm in a spectrophotometer. The phycobiliproteins were calculated (µg mL^{-1}) using the formula [32]. Phycocyanin (PC) = (A_{615}) – (0.475 × A_{562}) /5.34, Allophycocyanin (APC) = (A_{652}) – (0.208 × A_{615}) /5.09 and Phycoerythrin (PE) = (A_{562}) – (2.41 × PC) – (0.849 × APC) /9.6.

Estimation of carotenoids

Suspended the washed culture pellet of cyanobacteria in 3ml of acetone and incubated in dark for 45min. Centrifuged the contents at 5000×g for 5 min. Stored the supernatant thus obtained in the refrigerator and repeated the extraction till acetone becomes colorless. Pooled the supernatants and final volume made up to 10 ml with acetone. Read the absorbance at 450 nm in a spectrophotometer. Calculated the amount of carotenoids in the sample using the formula A_{450} × volume of sample × 10/2500. Where 2500 is the extinction coefficient and expressed as µg mL^{-1}[33].

Estimation of total protein

A known volume of cyanobacteria culture suspension was centrifuged and the pellet was washed twice with distilled water. The pellet was treated with 10% trichloro acetic acid and left for half an hour in a boiling water bath. The precipitate was obtained by centrifugation at 5000 ×g in a cooling microfuge for 15 minutes. Then the precipitate was neutralized in a known quantity of 1N NaOH for protein estimation. All the reagents were freshly prepared prior to estimation. Protein content was estimated [34] using bovine serum albumin as the standard.

Estimation of astaxanthin

About 100 mg of fresh cyanobacterial culture was taken for astaxanthin extraction and homogenized the sample with added glass powder in a known volume of methanol/dichloromethane (3:1v/v) in the dark room. Centrifuged the content at 5000 ×g for 15 min. The supernatant was preserved in dark in the refrigerator. Re-

extracted the pellet as above till all the pigments were extracted. Pooled the supernatant together and volume noted. Absorbency was read at 480nm against methanol / dichloromethane blank in a spectrophotometer. Astaxanthin content was calculated using an absorption coefficient, A1%, of 2500. Results are expressed as mg g^{-1} dry weight [35].

Estimation of polyphenolics

Polyphenol content was determined by the method of [36] with some modification. 100 mg of fresh cyanobacteria culture was taken for extraction and homogenized the sample by with adding 50 times the fresh weight with 80% methanol (v/v). Vortexed the cyanobacteria sample for 10 min and then centrifuged at 5000 ×g for 15 min. after centrifugation add 0.5 ml of (17%) folin reagent of 12.5 µl of extract. Wait 5 minutes and the extracts added 0.25 ml of 1M Na_2CO_3. Incubated the tubes at 50ºC for 30 min, finally measured the absorbancy at 765 nm in a spectrophotometer.

Statistical analyses

All of the measurements were carried out in triplicate. Values were expressed as mean (±) standard error, using statistical software packages (Origin Pro 8.1 and SPSS 16.0.Ink program).

Results and Discussion

The present study was initiated to characterize the biochemical contents in five selected species of marine cyanobacteria comprised of two unicellular and three filamentous isolates. Marine cyanobacteria characterized for their biochemical constituents showed maximum *chlorophyll a* content in (Fig. 3) *Synechococcus aeruginosus* (58.14±1.23 mg mL^{-1}) followed by Oscillatoria subbrevis (37.25±2.07 mg mL^{-1}), phormidium fragile (28.00±0.67 mg mL^{-1}) and Phormidium sp (12.52±0.42 mg mL^{-1}) and the minimum in Synechococcus elongatus (10.39±0.36 mg mL^{-1}). At the same time, maximum total protein was observed in *synechococcus aeruginosus* (203.51±15.26 µg mL^{-1}) followed by *Oscillatoria subbrevis* (142.81±3.72 µg mL^{-1}) *Phormidium fragile* (135.04±13.17 µg mL^{-1}) and *Phormidium species* (73.82±3.57 µg mL^{-1}). And the minimum quantity of protein was observed in *Synechococcus elongatus* (48.92±2.88 µg mL^{-1} (Figure 3)). In a previous study, the algal species *Phormidium angustissimum, Lyngbya holdenii, Anabaena doliolum, Calothrix marchica* and *Fischerella muscicola* isolated from the lime sludge waste of a paper mill showed higher accumulation of chlorophyll *a*, phycocyanin, carbohydrates and protein [37].

The results of phycobiliprotein in tested cyanobacterial strains revealed maximum amount of phycoerythrin in *Phormidium fragile* (32.28±0.34µg mL^{-1}) and the minimum in *Phormidium species* (1.57±0.67 µg mL^{-1}). On the other hand *Phormidium species*, which has a substantially higher, amount of phycocyanin (14.33±0.09 µg ml^{-1}) and minimum in *Synechococcus elongatus* (2.75±0.01 µg ml^{-1} respectively). Hence, these two strains acts as rich sources of pigments and could be exploited biotechnologically. Similarly Phycocyanin (PC) content was maximum in *Lyngbya diguetii* (17.5 µg ml^{-1}) and the minimum in *Nostoc carneum* (12.1 µg ml^{-1}). Phycoerythrin (PE) content was maximum in *Oscillatoria subbrevis* (48.7 µg ml^{-1}) and minimum in *Cylindrospermum muscicola* (15.34 µg ml^{-1}). Allophycocyanin (APC) was maximum in *Lyngbya diguetii* (25 µg ml^{-1}) and minimum in *Cylindrospermum muscicola* (15.34 µg ml^{-1}). Total phycobiliproteins content was maximum in *Oscillatoria subbrevis* (81.4 µg ml^{-1}) and minimum in *Cylindrospermum muscicola* (45.98 µg ml^{-1}) [38]. When compared the pigments was the best result finded in *Phormidium fragile* (322.8 ± 0.034 µg ml^{-1} dry weight) larger than *Oscillatoria subbrevis* (48.7 µg ml^{-1}) for phycoerythrin and *Phormidium species* (143.3 ± 0.09 µg ml-1 dry weight) larger than *Lyngbya diguetii* (17.5 µg ml^{-1}) for phycocyanin.

The current total market value of phycobiliprotein products (including fluorescent agents) is estimated to be greater than US$ 60 million. [39] reported the utility of phycobiliproteins as natural dyes. Besides number of interesting investigations revealed their health- promoting properties and broad range of pharmaceutical applications. They are also used as food and feed especially aqua feed to enhance the color of the ornamental fishes. Phycobiliproteins as nonenzymatic antioxidants absorb and dissipate excitation energy as heat and efficiently transfer the absorbed energy to photosynthetic reaction centers, in order to reduce the production of singlet oxygen $^1O_2$ (ROS) [40].

The total carotenoid is *Synechococcus elongatus* (7.49±0.21 µg g^{-1}) are the maximum amount of yield followed by *Synechococcus aeruginosus* (2.84±0.10 µg g^{-1} respectively). Very least level of the carotenoid was present in *Phormidium fragile* (0.93±0.25 µg g^{-1}). Similar results were observed in *Calothrix marchica* (5.26 µg ml^{-1}) was significantly ($P<0$ 05) efficient in terms of carotenoid production and in the remaining four species carotenoid content ranged from (2.31 µg ml^{-1} 4.82 µg ml^{-1}) [37].

Astaxanthin highest quantity was present in *Oscillatoria subbrevis* (133.11±5.39 µg g^{-1}) followed by *synechococcus aeruginosus* (50.23±0.59 µg g^{-1}), minimum in *Phormidium fragile* (9.85±0.18 µg g^{-1}). Previous reports, Haematococcus pluvialis 22.7 mg g^{-1}, Chlorella zofingiensis 6.8 mg g^{-1} [41, 42], Galdieria sulphuraria 0.6±0.1 mg g^{-1}.

Polyphenolic component maximum was observed in *Phormidium fragile* (583.77±66.33 µg g^{-1}) followed by *Synechococcus elongatus* (443.26±13.69 µg g^{-1}) and *synechococcus aeruginosus* (313.30±14.03 µg g^{-1}), very least in *Oscillatoria subbrevis* (14.82±1.08 µg g^{-1}). Previously, Six microalgae from different classes, including Phaeodactylum sp. (Bacillariophyceae), Nannochloropsis sp. (Eustigmatophyceae), Chlorella sp., Dunaniella sp., and Desmodesmus sp. (Chlorophyta), were screened for their biochemical analysis. The highest concentrations of phenolics were found in Desmodesmus sp. 772±0.08 (µg/g GAE) followed by Nannochloropsis sp 645±0.25 (µg/g GAE), Chlorella sp1 576±0.12 (µg/g GAE), Chlorella sp1 586±0.06 (µg/g GAE) and Dunaniella sp 452±0.05 (µg/g GAE), the lowest concentration of phenolics was observed in Phaeodactylum sp. 316 ± 0.04 (µg/g GAE) respectively [43].

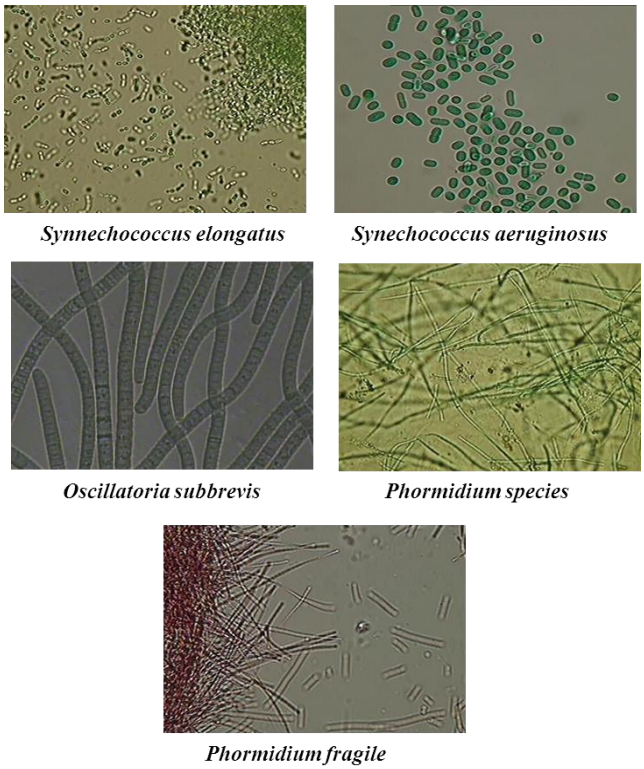

Fig 1: Photomicrographs of the cyanobacteria isolates used in the study

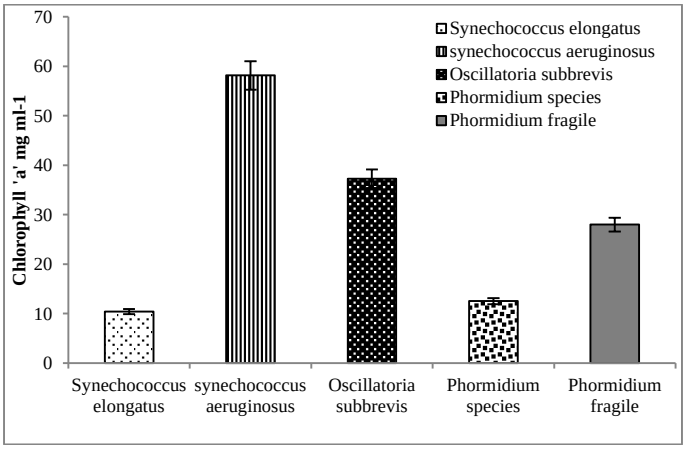

Fig 2: Chlorophyll'*a'* content in selected isolates of marine cyanobacteria

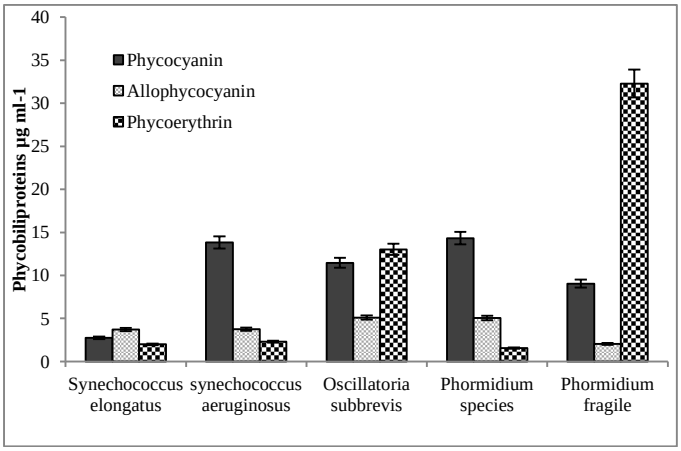

Fig 3: Contents of phycobilipigments in selected species of marine cyanobactria

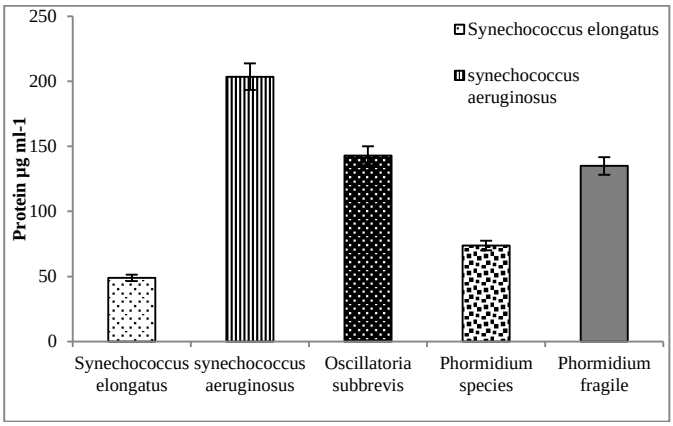

Fig 4: Protein content in selected species of marine cyanobacteria

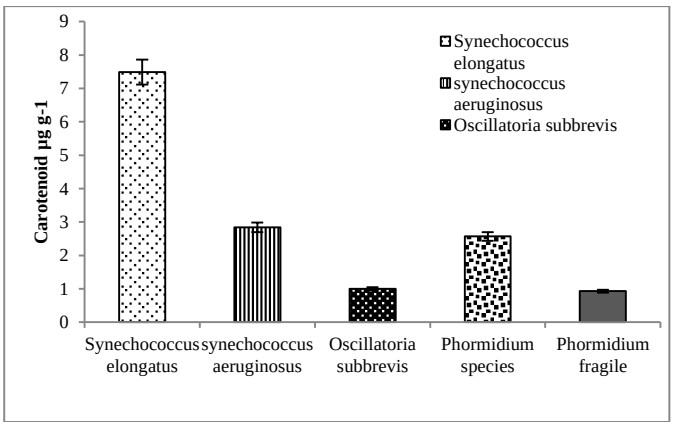

Fig 5: Carotenoid content in selected species of marine cyanobacteria

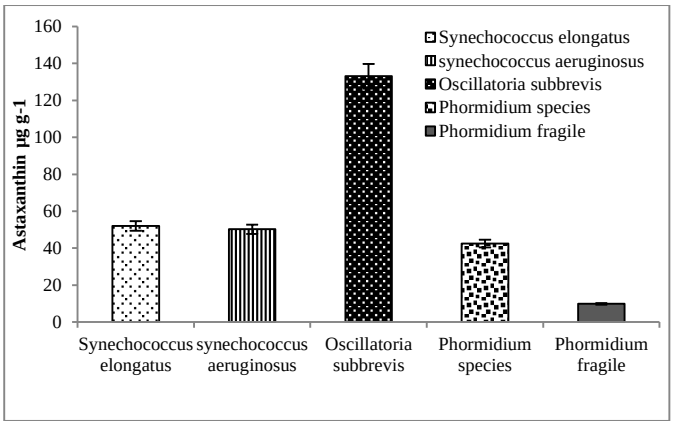

Fig 6: Astaxanthin content in selected species of marine cyanobacteria

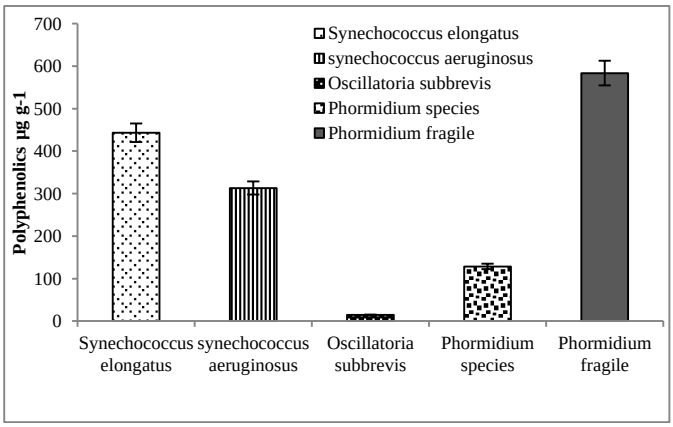

Fig 7: Polyphenolics content in selected species of marine cyanobacteria

Conclusions

In conclusion, the results of this study suggested that contains several species of marine cyanobacteria that have immense potentials. Results indicated that strain, *Phormidium fragile* showed considerable antioxidative pigments. Further studies should be made to purify natural products from these cyanobacteria. Improving knowledge of the composition, analysis, and the properties of these cyanobacteria with respect to antioxidant compounds would assist in efforts for different applications among which; pharmaceutical and casmacuetical applications.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper**.**

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