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INFLUENCE OF IRON ON GROWTH AND SIDEROPHORE PRODUCTION BY PHORMIDIUM SP.

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ABSTRACT
Iron is an essential limiting nutrient for the growth of cyanobacteria. A cyanobacterium *Phormidium* sp. NMU 203 isolated from Lonar Lake, MH, India was used in the study. The growth pattern and siderophore production by cyanobacterial isolate *Phormidium* sp. NMU 203 was studied in response to different iron concentrations. 80 µM iron concentration supported the maximum growth in terms of chlorophyll *a* and carotenoids content. The cyanobacterial isolate was found to synthesize siderophore to acquire iron from the environment. We observed that the siderophore biosynthesis was dependent on iron concentration in the growth medium. Iron starved condition exhibited the maximum siderophore production. Increase in iron concentration reduced the siderophore production. Iron deprived algal culture medium at pH 8 was found to be efficient for siderophore production. Cyanobacterial isolate produced Csaky's and Neiland's positive extracellular material, which indicated that the excreted siderophores belonged to hydroxymate type.

KEYWORDS: Iron, Siderophore, *Phormidium.*

INTRODUCTION

Cyanobacteria are oxygenic photosynthetic prokaryotes and present in almost every habitat on the earth around 3.5 billion years. The most common habitat of cyanobacteria is aquatic and terrestrial environment (Gademann *et al*., 2009). Cyanobacteria also withstand in many adverse conditions such as alkaline lake, hot spring, etc. Iron is one of the essential elements for growth and metabolism of cyanobacteria. It is important for various cyanobacterial metabolic processes such as photosynthesis, synthesis of photosynthetic pigments, nitrate reduction, nitrogen fixation and other oxidation reactions (Rueter and Peterson, 1987; Leão *et al.*, 2007). Cyanobacteria typically have a higher iron quota than other phytoplankton (Achilles *et al*., 2003). The bioavailability of iron is of more concern for the growth of cyanobacteria. Alkaline pH and redox potential reduce iron concentration due to oxidation of bioavailable iron; ferrous to ferric state, and formation of insoluble ferric hydroxides (Xu *et al*., 2013). These create iron starved condition and affect the growth of cyanobacteria. Many researchers assessed the effect of iron on the cyanobacterial physiology. Saxena *et al.* (2006) observed iron induced changes in growth, CO_2 fixation, nitrogen fixation and photosynthetic activity in cyanobacterium *Anabaena* PCC 7120. Decrease in iron, chlorophyll and total nitrogen content in *Ulva pertusa* due to iron stress has been reported by Jing-wen *et al.* (2002). The low solubility (10^{-18} M) of ferric iron (Fe^{3+}) has led to the evolution of molecular systems in cyanobacteria specifically designed to acquire iron from the environment (Webb *et al.,* 2001). Characterization of iron uptake systems in phytoplankton is still ongoing, but reduction of ferric to ferrous state of iron with the help of siderophore has been studied in all uptake pathways (Hopkinson and Morel, 2009). Siderophore are the iron chelating compounds secreted by cyanobacteria to acquire iron under iron starved condition. Siderophore utilizes different chemical moieties to bind iron which includes hydroxymate and catecholate types. Siderophore iron complex is taken up by the cell and release the iron in cell (Wilhelm *et al.*, 1996; Xing *et al.*, 2007). Synthesis of iron chelating siderophores in cyanobacteria has been reported in numerous studies. Goldman *et al.* (1983) revealed the structure of schizokinen, a citrate derivative siderophore from *Anabaena* sp. PCC 7120. Raghuvanshi *et al.* (2007) studied iron mediated regulation and dihydroxymate type siderophore production in *Anabaena cylindrica*. A marine cyanobacterium *Synechococcus* has been reported for the production of hydroxamate and cathecol like siderophore, as well as atypical siderophore in iron limiting culture conditions (Wilhelm *et al.*, 1996; Leão *et al.*, 2007). Ito and butler (2005) reported the structure of three new siderophore synechobactin A, B and C from *Synechococcus* sp. PCC 7002. Beiderbeck *et al.* (2000) isolated two complex siderophores 'anachelin H' and 'anachelin 1' from cyanobacterium, *Anabaena cylindrica*. Trick and Kerry (1992) isolated dihydroxymate siderophore from growth medium of cyanobacteria *Synechococcus* spp. PCC 7942 and *Anabaena variabilis* ATCC 29413. Understanding the cyanobacterial interactions with iron and their metabolic strategies to cope up with the iron stress has been the centre of the recent past research activities. The present study is an attempt to assess the effect of growth medium and their properties on siderophore production in *Phormidium* sp.

MATERIALS & METHODS

Organism, culture media and growth condition

Cyanobacterial culture *Phormidium* sp. NMU 203 was isolated from alkaline soda lake of Lonar (District

Buldhana, Maharashtra, India). Culture was enriched in BG-11 medium at 25°C with 40 µmole/m^2/s light intensity and alternating photoperiod 16 h light and 8 h dark. The BG-11 medium (pH 7.5) contained (in g/l) 1.5, $NaNO_3$; 0.04, K_2HPO_4; 0.075, $MgSO_4.7H_2O$; 0.036, $CaCl_2$; 0.006, citric acid; 0.006, ferric ammonium citrate; 0.001, disodium salt of EDTA; 0.02, of Na_2CO_3. Trace metal solution containing (g/l) 2.86, H_3BO_3; 1.81, $MnCl_2$; 0.39, Na_2MoO_4; 0.222, $ZnSO_4.4H_2O$; 0.079, $CuSO_4.5H_2O$; 0.0494, $Co(NO_3)_2.6H_2O$) was added to BG-11 medium at a concentration of 1 ml/l (Rippka *et al.*, 1979) . The culture was purified and maintained in BG-11 medium by repeated sub culturing.

Morphological characterization of culture

The cyanobacterial isolate NMU 203 was microscopically examined for morphological characters using trinocular brightfield research microscope (Nikon, Eclipse-200) and florescent microscope (Motic advanced florescent microscope). For scanning electron microscopic analysis, cyanobacterial cells was kept overnight in 2% glutaraldehyde at 4°C and dehydrated by successive passages through increasing concentrations of ethanol (10 to 90% v/v) and finally air dried. The specimen were examined under a scanning electron microscope (FESEM, Model: S-4800 type-II Hitachi) at 15 kV (Cappitelli *et al.*, 2009). Cyanobacterial culture NMU 203 was identified morphologically according to Desikachary (1959).

Inoculum preparation and siderophore production

In order to provide favourable condition for siderophore synthesis the *Phormidium* sp. NMU 203 was grown in iron free medium for 7 days. The composition of iron free medium at pH 7.1 was (in g/l) 0.25, K_2HPO_4; 1, $NaNO_3$; 0.51, $MgSO_4.7H_2O$; 0.05, NH_4Cl; and 0.05, $CaCl_2$. All glass wares used in THE experiments were soaked overnight in 6N HCl and rinsed with glass distilled water to remove surface iron contamination. For all the experiments, Milli Q water was used. To remove the amount of iron adsorbed extracellularly on the cell surface the culture inoculum was washed three to four times with fresh iron deprived algal culture medium. The culture flasks were incubated at 25°C with 40 µmole/m^2/s light intensity and alternating photoperiod of 16h light and 8h dark .

Measurement of growth

Culture of *Phormidium* sp. NMU 203 (5 ml) was centrifuged at 10,000 rpm for 10 min. The supernatant was decanted and pellet was resuspended in 80% acetone and kept overnight at 4°C in the dark. After 24 h the sample was centrifuged at 10,000 rpm for 10 min. Absorbance of supernatant was measured at 665, 630 and 645 nm. For carotenoids content, the cell pellet was resuspended in 95% ethanol and kept overnight at 4°C in the dark. After 24 hrs, sample was centrifuged at 10,000 rpm for 10 min and the absorbance was measured at 470 nm using UV-visible spectrophotometer. The content of chlorophyll *a* and carotenoids was calculated as per following equations Eq. 1 (Kaushik, 1987) and Eq. 2 (Xing *et al*., 2007), respectively.

Chlorophyll *a* (mg/l) = $(11.6 \times A_{665}) - (1.31 \times A_{645}) - (0.14 \times A_{630})$ ….. Eq. 1

Carotenoids (mg/l) = $(1000\ A_{470} - 2.05$ Chl *a*$)/245$ ….. Eq. 2

Siderophore assay

Culture supernatant was used for the detection of siderophore using Chrome Azurol Sulphonate (CAS) shuttle assay. CAS solution was prepared according to Schwyn and Neilands (1987). One ml of CAS reagent was added in 1 ml of supernatant and incubated for 1 h. Change in color from blue to orange indicated presence of siderophores in sample. Quatitation of siderophore produced was made by measuring the absorbance at 630 nm and percentage of siderophore (% SU) production was calculated using Eq. 3 (Xing *et al*., 2007). Wherein, 'Ar' was the absorbance of reference uninoculated medium and 'As' the absorbance of the test sample.

% Siderophore units = $(Ar - As)/Ar \times 100$ ….. Eq. 3

Effect of growth medium on siderophore production

Four different media viz. Algal culture medium (ACM), BG-11, Zarrouk's medium and Chu-10 medium (pH 7.5) were used to check the effect of growth medium on siderophore production by *Phormidium* sp. NMU 203. The algal culture medium contained (g/l) 1, $NaNO_3$; 0.25, K_2HPO_4; 0.513, $MgSO_4.7H_2O$; 0.05 $CaCl_2.2H_2O$; 0.05, NH_4Cl and 0.003 g $FeCl_3$. The composition of Zarrouk's medium was (in g/l) as 18, $NaHCO_3$; 0.5, K_2HPO_4; 2.5, $NaNO_3$; 1, K_2SO_4; 1, NaCl; 0.2, $MgSO_4.7H_2O$; 0.04, $CaCl_2$; 0.01, $FeSO_4$; 0.08, Na_2-EDTA and 1 ml trace metal solution (Zarrouk, 1966). The Chu-10 medium (Safferman and Morris, 1964) consisted of (in g/l) 0.232, $Ca(NO_3)_2.4H_2O$; 0.044, $Na_2SiO_3.5H_2O$; 0.01, K_2HPO_4; 0.025, $MgSO_4.7H_2O$; 0.0035, citric acid; 0.0035, ferric citrate; 0.02, Na_2CO_3 and 1 ml trace metal solution. All media used were made iron free by removing iron source in each medium. The concentration of inoculums, growth conditions and siderophore quantification were similar as stated earlier. Effect of pH on the synthesis of siderophores by *Phormidium* sp. NMU 203 was assessed by growing the culture in iron free medium adjusted to different pHs (4-10).

RESULTS

Influence of iron on growth and siderophore production in *Phormidium* sp. NMU 203

The cyanobacterial isolate NMU 203 was purified by subculturing in BG-11 medium. Purified culture was subjected to morphological characterization following bright field, fluorescence and scanning electron microscopy (Fig. 1). Microscopic analyses revealed that cyanobacterial species is filamentous, unbranched, tube like sheaths, firm and composed of cylindrical cells. The culture was identified on the basis of microscopic characteristics as *Phormidium* sp. NMU 203. The experimental data on growth performance of *Phormidium* sp. NMU 203 in terms of chlorophyll *a* and carotenoids content are depicted in Fig. 2. It also shows the siderophore production as the function of increasing concentrations of iron in growth medium. It can be seen that the content of chlorophyll *a* and carotenoids increased with increase in iron concentration up to 80 µM. The culture grown at high concentrations of iron and uninoculated media showed no change in color in CAS

assay, which was indicative of no synthesis of siderophores. The culture produced siderophore under iron starved condition and also up to 20 µM iron beyond which synthesis was inhibited (Fig. 2). The quantity of siderophore produced in absence of iron in the medium was higher (69%) when compared with the culture grown in presence of 20 µM iron (8.9%). This indicated that the iron starved condition was favourable for maximum siderophore production. The cell free supernatant of *Phormidium* sp. NMU 203 showed Csaky's and Neiland's test positive. The positive response to these tests indicated that the siderophores of *Phormidium* sp. NMU 203 belonged to hydroxymate type.

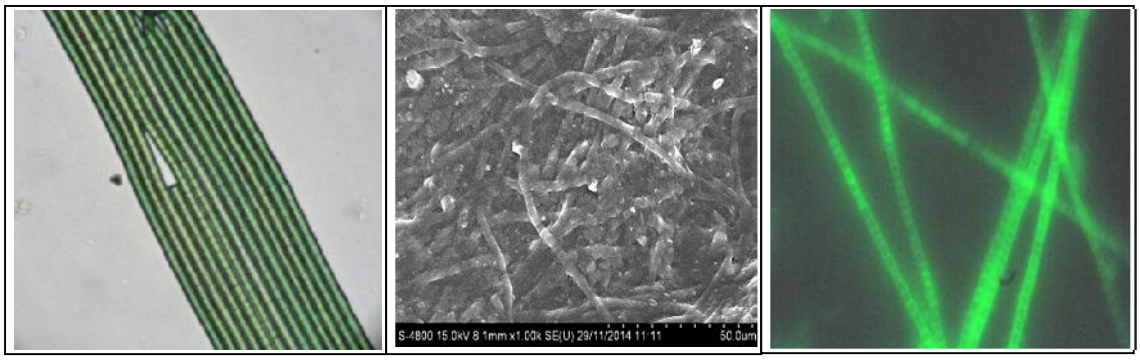

FIGURE 1. *Phormidium* sp. NMU 203 (a) Light micrograph (b) Scanning electron micrograph (c) Fluorescent micrograph

Effect of growth medium on siderophore production

Growth medium with various constituents and their concentrations as well as associated growth conditions have influence on growth profile of microorganisms. In the present study various growth media were tested for growth and siderophore production by *Phormidium* sp. NMU 203. The experimental data are presented in Fig. 3. It can be seen that the content of chlorophyll *a* and carotenoids increased in the medium containing iron source. Cyanobacterial culture *Phormidium* sp. NMU 203 grew efficiently in algal culture medium, BG-11 medium and Zarrouk's medium. However, the growth of culture in Chu-10 with and without iron was negligible. It could be noted that absence of iron in all growth media decreased chlorophyll *a* as well as carotenoids in culture. Algal culture medium without iron source (ACM*) was found to be efficient for the synthesis of siderophore (68.30%) (Fig.3). Siderophore synthesis was not detected with the culture grown in Chu-10 medium with and without iron.

Effect of pH of growth medium on siderophore production

The growth response of cyanobacteria and siderophore production was mainly affected by pH of the medium. The data on effect of pH of the algal culture medium (ACM) on the chlorophyll *a* and carotenoids content of *Phormidium* sp. NMU 203 are shown in Fig. 4. Chlorophyll *a* was estimated on the last day. It can be seen that the growth performance of *Phormidium* sp. NMU 203 was improved with increase in pH of growth medium. Chlorosis of cyanobacterial cells was observed at acidic pH and under alkaline condition growth of culture was luxuriant. Maximum siderophore activity (70%) was obtained with the culture grown at pH 8. Higher alkalinity (pH >8) resulted in decreased siderophore activity.

DISCUSSION

Iron is the one of the most essential micronutrient required for vital cell metabolic activities. Availability of iron has been considered to be the critical factor deciding cellular activity. Most of the microorganisms including cyanobacteria do produce iron chelating metabolites termed as siderophores to avail the iron present trace quantities or unavailable form (Wilhelm *et al*., 1998; Raghuvanshi *et al.,* 2007). Number of cyanobacteria flourish in alkaline conditions. The net availability of iron gets decreased under alkaline conditions due to its insolubility (Rueter and Petersen, 1987). In such situations acquiring iron through siderophores appeared to be the main physiological adaptation in cyanobacteria. The present study deals with the growth and siderophore production in *Phormidium* sp. NMU 203 in presence and absence of iron in growth medium. Reports have been revealed the potential of *Phormidium* in the production of iron chelating siderophores (Zimmerman *et al.,* 1995; Harland *et al.*, 2013).

In the present study the synthesis chlorophyll *a* and carotenoids was found dependent on concentration of iron in the medium. Increase in chlorophyll *a* upto 50 µM iron in *Anabaena* PCC 7120 and further increase in iron decreased the chlorophyll *a* content has been reported by Saxena *et al.* (2006). Similar observation where in increased iron, nitrogen and phosphorus resulted in elevated chlorophyll *a* and carotenoids reported for *Anabaena fols-aqua*e and *Calothrix* sp. (El-Sayed *et al.*, 2010). The addition of iron in the medium can stimulate the growth of cyanobacteria. Chlorophyll does not contain iron itself but iron is required for iron dependant enzymes used for biosynthesis of chlorophyll (Rueter and Petersen, 1987). Presence of iron is necessary for functionality of iron-dependent enzymes necessary for biosynthesis of chlorophyll *a*. However, iron deficiency results in chlorosis (Boyer *et al*., 1987). Hence iron plays important role for existence of cyanobacteria.

Several studies have revealed the effects of iron deprivation on freshwater and marine cyanobacteria (Zimmerman *et al.,* 1995; Saxena *et al.,* 2006; Raghuvanshi *et al.,* 2007; El-Sayed *et al.,* 2010; Harland *et al.*, 2013). These reports confirmed on iron stress induced phenotypic changes, decreased growth performance and synthesis photosynthetic as well as accessory pigments

and associated synthesis of iron chelating siderophores (Webb *et al.*, 2001). The experimental results showed that in *Phormidium* sp. NMU 203 growth and synthesis of chlorophyll *a* and carotenoids was dependent on availability of iron upto 80 µM concentration. Decreased growth at higher iron concentrations could be attributed to the iron acting as cyanotoxin inhibiting various essential cell metabolic activities such as nitrogen assimilation, carbon dioxide fixation and photosynthetic activities (Saxena *et al.*, 2006). The cyanobacterium *Phormidium* sp. NMU 203 did not show the production of siderophores was not seen in the presence of iron in the medium. The siderophore synthesis was found to be associated with iron limiting / depleting condition. Presence of 20 µM iron also resulted in the decreased siderophore synthesis. In contrast to the present observation the report on *Anabaena cylindrica* shown that the siderophore synthesis was increased with increased iron concentration to 60 µM (Raghuvanshi *et al.*, 2007). It has also been seen that *Anabaena cylindrical* synthesized siderophores in iron starved condition which was seen on 5th day of incubation. The growth and siderophore production by *Phormidium* sp. NMU 203 was found different when in various growth media (Fig. 3). The diverse growth response could be due to change in overall composition and pH of medium. No growth in Chu-10 medium with and without iron source might be due to the presence of silicates. Synthesis of siderophores predominantly in iron depleting conditions was found in *Phormidium* sp. NMU 203. This confirms the role and importance of siderophores as an effective mean to deal with iron stress condition.

CONCLUSION

Cyanobacteria have ability to produce siderophore under iron starved condition. A cyanobacterium *Phormidium* sp. NMU 203 isolated from Lonar Lake, MH, India was studied for growth and siderophore production at various nutritional conditions. Synthesis of chlorophyll *a* and carotenoids increased with an increase in iron content of growth medium. The absence of iron in all growth media decreased chlorophyll *a* as well as carotenoids in culture. Algal culture medium without iron source was found to be efficient for the synthesis of siderophore (68.30%) (Fig.3). Siderophore synthesis was not detected with the culture grown in Chu-10 medium with and without iron. The synthesis of siderophore was also found dependent on pH of growth medium. It was maximum at pH 8. Higher acidity as well as alkalinity hampered production of siderophores. This study indicated that synthesis and release of siderophores appeared to be an effective cultural adaptation to deal with iron stress especially associated with alkalinity and salinity of Lonar lake.

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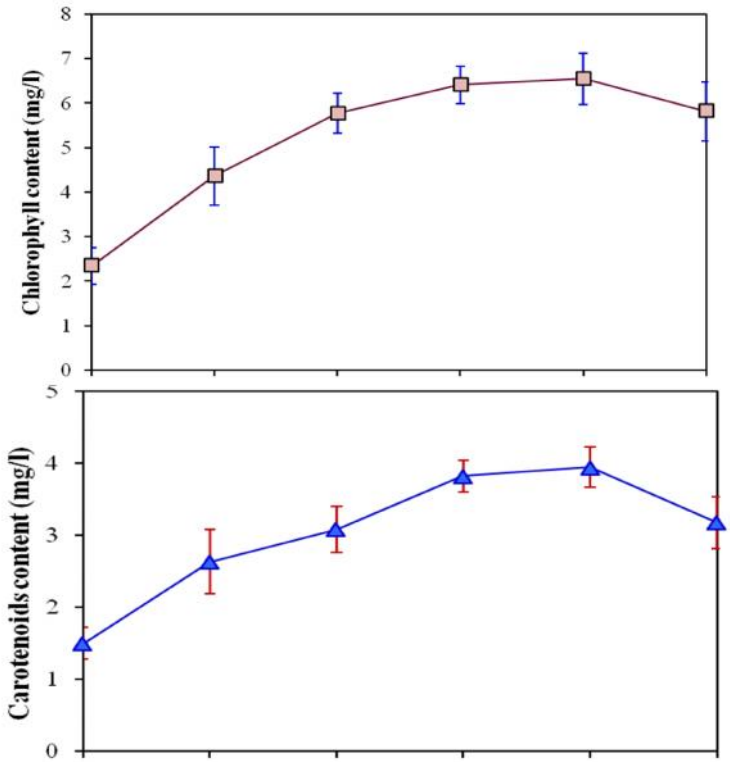

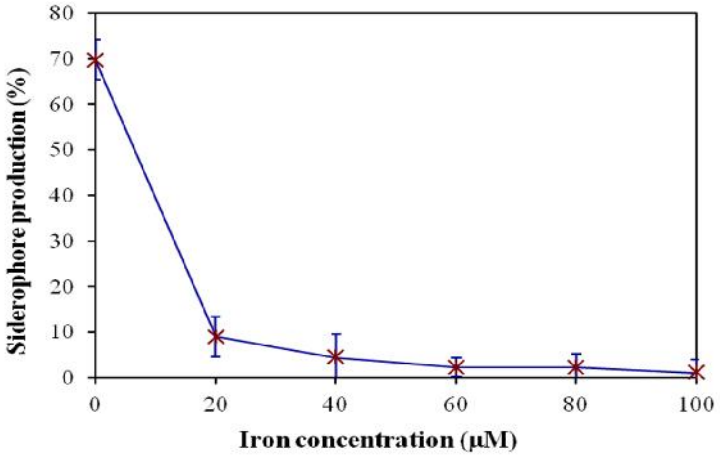

FIGURE 2. Influence of iron on chlorophyll *a*, carotenoids and siderophore production by *Phormidium* sp. NMU 203. Values plotted are of mean of three replicates and error bars represent standard deviation.

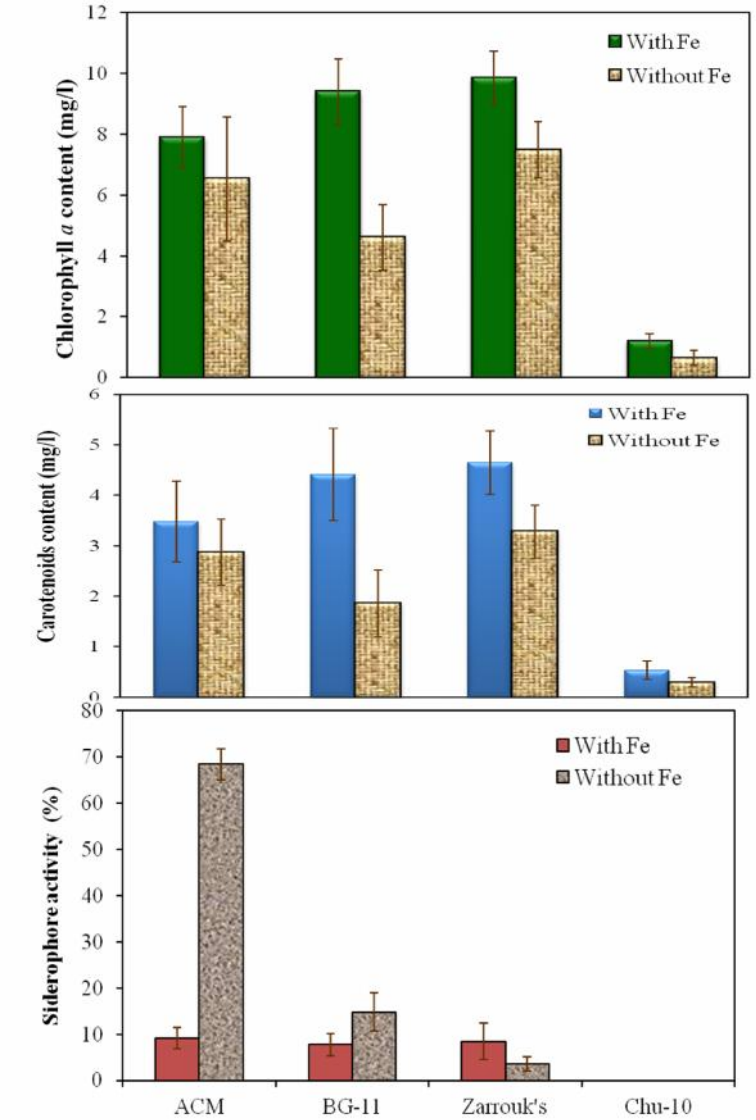

FIGURE 3. Influence of growth medium on chlorophyll *a*, carotenoids and siderophore production by *Phormidium* sp. NMU 203. Values plotted are of mean of three replicates and error bars represent standard deviation. ACM, Algal culture medium.

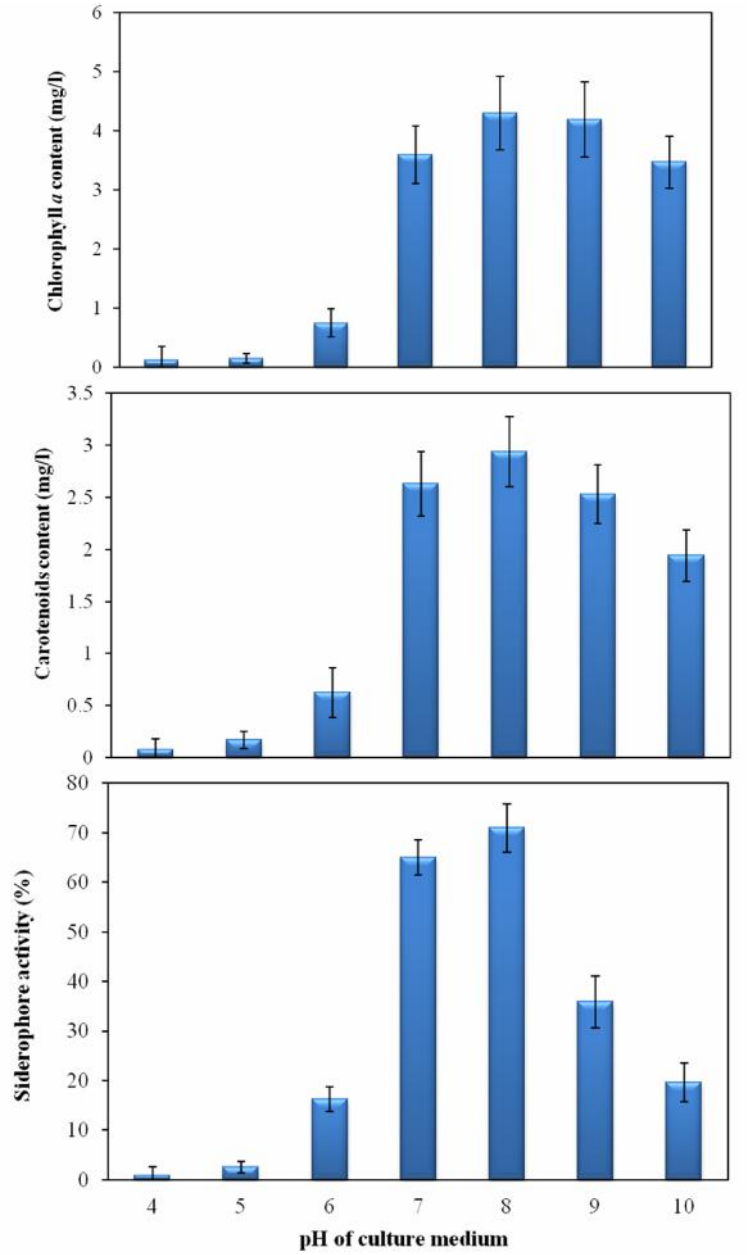

FIGURE 4. Influence of pH of growth medium on chlorophyll *a*, carotenoids and siderophore production by *Phormidium* sp. NMU 203. Values plotted are of mean of three replicates and error bars represent standard deviation.

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