

## Some Fe chelate forms in culture media induce oxidative stress in the cyanobacterium *Spirulina platensis*

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### Abstract

This study investigated impacts of iron in different concentrations (3.5 – 100 μM) and different chelate forms including FeEDTA, FeEDDHSA and FeEDDHA on growth, chlorophyll, proline, malondialdehyde (MDA), O<sub>2</sub> production and iron concentration in dry biomass in the cyanobacterium *Spirulina platensis*. Higher growth, chlorophyll concentration and O<sub>2</sub> production observed in forms of FeEDTA and FeEDDHSA at higher concentrations (more than 50 μM). Conversely, MDA concentration and proline content were higher in FeEDDHA; in other words, the more the concentration of FeEDDHA in medium, the more the concentration of proline in the cells. There was no difference between various concentrations of the used FeEDDHA in MDA content of the cells. Chlorophyll concentration was lower in form of FeEDDHA in comparison with the other chelate forms. Iron concentration in dry weight increased as concentration of iron in medium increased in the all of used chelate forms. At lower concentrations of Fe in medium, Fe concentrations in the cells were higher in form of FeEDDHA but at higher concentrations in medium it was observed in form of FeEDDHSA. Increased amounts of MDA and proline were represented the occurrence of oxidative stress in the test microorganism under iron form FeEDDHA, while increased levels of O<sub>2</sub> production demonstrated an increase in growth via higher photosynthesis in the chelate forms FeEDTA and FeEDDHSA. The results indicated that FeEDDHSA acts as a promising source for iron in spirulina culture.

### Keywords

Chelate

Iron

Oxidative stress

*Spirulina platensis*

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### Introduction

Growth in human population and loss of different natural resources for sustainable food production, have led researchers to use other sources such as microorganisms which need different environmental conditions for growth. Obtaining these conditions is difficult, costly and complicated although it is sometimes achievable in some environments. Production of food based on cultivation of *Spirulina platensis* is feasible in different regions while optimization of the growth medium has been the focus of many studies (Richmond and Hu, 2013). A unique feature of all offered optimized mediums is an alkaline pH around 10 in which iron deposits as insoluble Fe<sup>3+</sup> oxides. High oxygen concentration in medium, which is brought about by high photosynthesis rates of the algae, limits iron availability too (Morrissey and Bowler, 2012). Indeed, in order to have a better growth of the alga, using chelate forms of iron is inevitable.

Plants overcome the limitations of iron by different strategies including energy dependent acidification of the soil, iron reduction and high affinity Fe<sup>2+</sup>

transport, phyto siderophore secretion and uptake of Fe-siderophore complexes. In cyanobacteria, uptake of iron siderophore complexes is known as the most important mechanism. Different siderophores have been identified in cyanobacteria (Simpson and Neilands, 1979; Trick and Kerry, 1992) which could be species specific (Trick and Kerry, 1992). Although the production of siderophores by different bacteria is strongly induced when having low iron availability, the uptake of iron into the cells is siderophore mediated in normal conditions (Stintzi *et al.*, 2000). The responsible transporters for iron-siderophore complexes act highly specific for the inherent siderophore of bacteria (Stintzi *et al.*, 2000). Indeed, an exchange of Fe must happen between Fe-chelate complex added to the medium and specific bacterium siderophore. This limits the application of any forms of chelate irons in the medium. On the other hand, if the iron chelate form could diffuse or penetrate into the cells in unspecific ways, release of iron in an uncontrolled manner damages cells predominately via oxidative stress. Iron easily transits between Fe<sup>2+</sup> and Fe<sup>3+</sup> inside cells. This could start the reactions known as Fenton's reactions. It produces free

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radicals which are non-specific oxidants and damage membranes and other biomolecules. In other words, free iron in the cells could act as a heavy metal and generates reactive oxygen species (ROS).

Malondialdehyde (MDA) is a cytotoxic product of lipid peroxidation and an indicator of free radical production and consequent tissue damage (Ohkawa *et al.*, 1979). Proline accumulates heavily in several plants under stress, providing protection for plants against damage by ROS. Proline plays important roles in osmoregulation (Ahmad and Hellebust, 1988; Laliberte and Hellebust, 1989), protection of enzymes (Paley *et al.*, 1984; Laliberte and Hellebust, 1989; Nikolopoulos and Manetas, 1991), stabilization of the machinery of protein synthesis (Kadpal and Rao, 1985), regulation of cytosolic acidity (Venekemp, 1987), and scavenging of free radicals (Smirnov and Cumbes, 1989). It also acts as an effective singlet oxygen quencher (Hall, 2002).

In this study, we investigated the effects of using different chelate forms of iron on growth, photosynthesis and induction of oxidative stress in cyanobacterium *Spirulina platensis*. This issue prompted us to investigate whether free radicals are generated under the stress imposed by varying concentrations (3.5, 17.5, 35, 50, 70 and 100  $\mu\text{M}$ ) of different commercially available iron chelate forms including FeEDTA (Fe Ethylenediaminetetraacetic acid), FeEDDHSa (ethylenediaminedi(2-hydroxy-5- sulfophenylacetic) acid) and FeEDDHA (Ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid) in cyanobacterium *Spirulina platensis* (PCC9108). FeEDDHA and FeEDDHSa are two commercially available forms of iron which are categorized as strong chelates (Lucena, 2006) that do not precipitate in alkaline mediums. FeEDTA is a weak chelate that is not stable at alkaline conditions but usually used in the proposed media for *Spirulina* culture. The results would be important since there are limited studies on the subject of trace elements nutrition in spirulina cultivation (Richmond and Hu, 2013).

Control of oxidative stress in the cells is important since microalgae such as *Spirulina*, are considered as sources for unsaturated fatty acids. In oxidative stress, these fatty acids are so vulnerable to oxidation which can decrease the product quality. Indeed, introducing a medium with the lowest oxidative stress is essential for mass production of microalgae.

## Materials and Methods

### *Raising and treating the test microorganism*

*S. platensis* (PCC9108) was obtained from

Institute Pasteur, France, and rose in a modified Zarrouk's medium (Venketaraman, 1983) under aseptic conditions. The stock and test cultures were maintained at 25°C in a photon fluence rate 40  $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$  following a 16:8-h light/dark regime. The volume of cultures were 150 ml in 500 ml Erlenmeyer flasks. A pH of 9.5 was maintained for the appropriate growth of the test microorganism. Iron (in different chelate forms including FeEDTA, FeEDDHSa and FeEDDHA) was added separately to the fresh growth medium in calculated amounts to obtain the total concentrations (3.5, 17.5, 35, 50, 70 and 100  $\mu\text{M}$ ). The cultures were raised in bulk and the 11-day-old cells were harvested by centrifugation of the culture media.

### *Measurements*

The progressive growth of *S. platensis* (PCC9108) was observed over a period of 11 days under control and iron treated conditions by taking absorbance at 670 nm and supported by the weight biomass data. To measure absorbance, samples were taken, centrifuged at 5000 g to precipitate cells, washed twice by water and finally cells were re-suspended in water and OD was measured against water as blank. To assess the generation of free radicals, MDA content was measured by using the procedure described by Heath and Packer (1968). The harvested cyanobacterial cells were freeze dried, homogenized in 1% trichloroacetic acid and then centrifuged at 10,000 rpm for 15 min. Supernatant was heated with 0.05 thiobarbituric acid and 20% trichloroacetic acid for 30 min at 95°C. The heated supernatant was re-centrifuged at 5000 rpm for 5 min and the absorbance was measured at 532 and 600 nm using a UV-VIS Spectrometer (RAY LEIGH; 1601 UV). Proline concentration was determined following Bates *et al.* (1973). The cells suspended in 10 ml of 3% sulfosalicylic acid were centrifuged at 4000 g for 10 min to remove cell debris. 2 ml of ninhydrin was added to 2 ml of supernatant with 2 ml glacial acetic acid and incubated at boiling temperature for 1 h. The mixture was extracted with toluene, and proline was quantified spectrophotometrically at 520 nm from the organic phase.

Oxygen production was measured using an oxygen meter (WTW with an electrode model Cellox 325, Germany) in the culture medium. The oxygen meter was calibrated before use according to the manual of the company. The media including equal biomass of grown algae in the eleventh day were blown by using pure gaseous nitrogen in an isolated chamber. The dissolved oxygen was measured repeatedly until the concentration of dissolved

oxygen was attained at the lowest level ( $1.7 \text{ mgL}^{-1}$ ). Then, we stopped the blowing of nitrogen into the media while a flow of nitrogen was retained in the chamber to protect the dissolving oxygen from air in the media. Lighting started over the flasks containing media with the intensity of  $100 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$  by using a combination of natural light supplemented by fluorescent tubes. Changes in dissolved oxygen, which indicated the photosynthetically produced oxygen, were measured periodically. Measurements were performed for 60 min and the reported data are results at the end of this time.

To measure Fe concentration in dry biomass, the harvested cells were washed by using desorption solution ( $\text{CaSO}_4$ , 5 mM;  $\text{Na}_2\text{EDTA}$ , 10 mM; pH 5.8) three times, each time for 5 min. Finally, the cells were precipitated through centrifuge at 4000 g and dried on filter nylon at  $60^\circ\text{C}$  for 24 h. The dried biomass was weighed and subjected to acid digestion as follows: Two ml nitric acid (65%) was added to the tube on dried biomass and left in lab conditions over night. The digests were heated at  $90^\circ\text{C}$  for half an hour and after cooling at room temperature, 1 ml  $\text{H}_2\text{O}_2$  (30% v/v) was added and incubated at  $90^\circ\text{C}$  until clear. Concentrations of Fe in solutions were measured by using AAS (atomic absorption Spectrophotometer; Shimadzu model 6200).

#### Statistical analyses

All of the experiments were performed in completely randomized designs. Multiple comparisons were performed by one-way ANOVA (Tukey's HSD) and MANOVA (for assessment of the simultaneous effects of both iron concentrations and iron chelate forms) by using the SPSS software (version 13, for Windows; SPSS Inc., Chicago, IL, USA). Data are representative of three independent repetitions.

## Results

#### Effects of Fe concentrations and chelate forms on malondialdehyde concentration

Observations showed statistically significant effects of Fe concentrations, chelate iron forms and simultaneous effects of both Fe concentration and chelate form on the concentration of MDA within the cells (Table 1). The greatest effect is related to chelate form of iron (65.6% of changes). Higher concentrations of MDA appeared in all of tested concentrations at the chelate form FeEDDHA. No significant difference was observed in MDA concentration between the forms FeEDDHSA and FeEDTA with the exception at the concentration 17.5

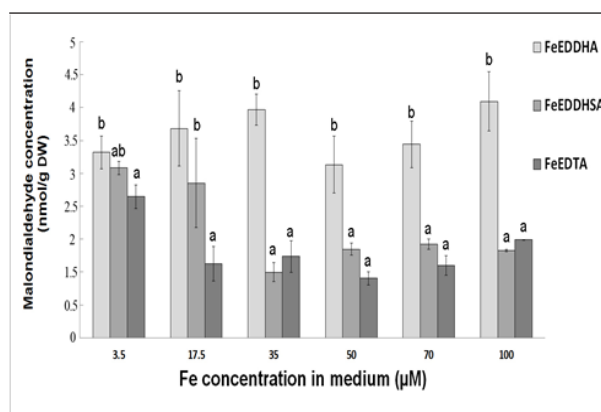


Figure 1. Effects of different Fe concentrations and chelate forms on the MDA concentration in *Spirulina platensis* (PCC9108). Shown data are average of  $n=3$  replicates  $\pm$  SD. Different letters in each series of Fe concentration indicates statistically significant difference between chelate forms based on Tukey's HSD ( $P < 0.05$ )

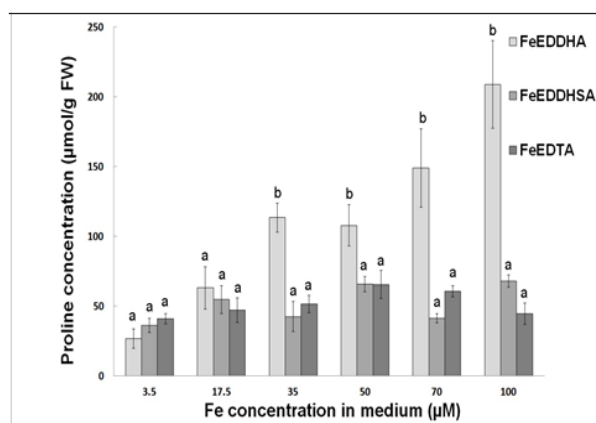


Figure 2. Effects of different Fe concentrations and chelate forms on proline concentration in *Spirulina platensis* (PCC9108). Shown data are average of  $n=3$  replicates  $\pm$  SD. Different letters in each series of Fe concentration indicates statistically significant difference between chelate forms based on Tukey's HSD ( $P < 0.05$ )

$\mu\text{M}$  which was greater in using FeEDDHA (Figure 1).

#### Effects on proline concentration

At higher concentrations of Fe in medium ( $35 \mu\text{M}$  or more) significant increases in proline concentrations were measured in form of FeEDDHA related to other forms (Figure 2). The concentrations of proline at the higher concentrations of Fe (70 and  $100 \mu\text{M}$ ) in FeEDDHA were 2 to 3 times more than the other chelate forms. No difference was observed between the forms FeEDDHSA and FeEDTA. Two-way ANOVA analysis also indicated significant effects of chelate form, concentration of Fe and interaction of the two agents (Table 1).

Table 1. Two-way ANOVA table of the effects of Fe concentration, Fe chelate form and interaction of concentration and chelate form on MDA and proline concentrations and O<sub>2</sub> production in *Spirulina platensis*. Eta<sup>2</sup> coefficient shows relative magnitude of the effects as percent

Source	df	MDA		Proline		O <sub>2</sub> production		Cell Fe concentration	
		Eta <sup>2</sup>	Sig.	Eta <sup>2</sup>	Sig.	Eta <sup>2</sup>	Sig.	Eta <sup>2</sup>	Sig.
Fe concentration	5	10.9	<0.001	24.2	<0.001	30.2	<0.001	21.7	<0.001
Chelate form	2	65.6	<0.001	37.2	<0.001	52.4	<0.001	19.4	<0.001
Con. * form	10	17.2	<0.001	32.1	<0.001	12.5	<0.001	53.7	<0.001
Error	36	6.3		6.5		4.9		5.2	
Total	54	100		100		100		100	

Table 2. MANOVA table of the effects of Fe concentration, Fe chelate form, Time and their interactions on dry weight and OD in *Spirulina platensis*. Eta<sup>2</sup> coefficient shows relative magnitude of the effects as percent

Source	df	Dry weight		OD	
		Eta <sup>2</sup>	Sig.	Eta <sup>2</sup>	Sig.
Fe concentration	5	5.9	<0.001	8.1	<0.001
Chelate form	2	11.4	<0.001	18.9	<0.001
Time	5	65.8	<0.001	53.3	<0.001
Con.*form	10	1.5	<0.001	3.4	<0.001
Con.* Time	25	2.5	<0.001	4.4	<0.001
Form* Time	10	10.6	<0.001	8.6	<0.001
Fom* Time*con.	50	1.7	<0.001	2.1	<0.001
error	216	0.9		1.3	
total	324	100		100	

#### Effects on O<sub>2</sub> production

Significant effects of Fe concentration, Fe chelate forms and their interaction on O<sub>2</sub> production were observed (Table 1) and higher magnitude of the effects was related to chelate form (52.4%). At all of the tested concentrations of Fe, the higher O<sub>2</sub> production was measured in FeEDDHSa form. At the 17.5 μM Fe, the O<sub>2</sub> production in using FeEDDHSa was almost 2 times more than the two other forms. No statistically difference was observed between the FeEDDHA and FeEDTA chelate forms except at 100 μM.

#### Effects on Fe concentration in the cells

As it is shown in Table 1, the most important factor in the Fe concentration in the cells is combination

of Fe concentration in medium and chelate form. The two other factors (i.e. Fe concentration in medium and chelate form) had less effects than their combination. Greatest concentration of Fe in the cells occurred in chelate form FeEDDHSa at higher concentrations (70 and 100 μM; Figure 3). At lower examined concentrations of Fe in medium (17.5, 35 and 50 μM), higher concentration of Fe in the cells was observed in form FeEDDHA.

#### Effects on growth

Growth and biomass production, as reported in dry weight and OD, were impressed by Fe chelate form (Table 2). The magnitude effect of Fe concentration was lower than the effect of chelate form (5.9 and 11.4 for dry weight and 8.1 and 18.9 for OD respectively).



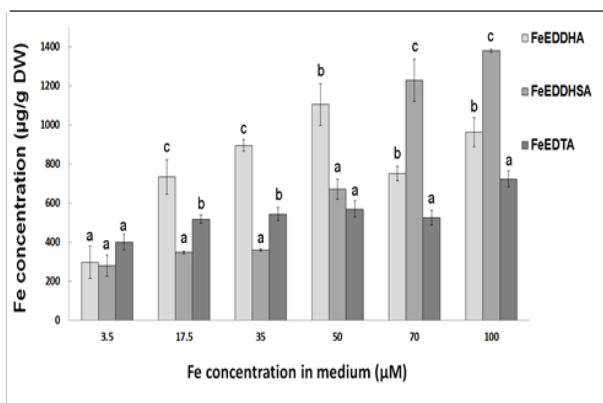


Figure 3. Effects of different Fe concentrations in medium and chelate forms on Fe concentration in the cells of *Spirulina platensis* (PCC9108). Shown data are average of n=3 replicates  $\pm$  SD. Different letters in each series of Fe concentration indicates statistically significant difference between chelate forms based on Tukey's HSD ( $P < 0.05$ )

At all of the used concentrations, the highest relative growth was observed in chelate form FeEDDHSA and the lowest in FeEDDHA. At the concentrations 70 and 100  $\mu$ M, the relative growth in the form FeEDDHSA was 2.7 times more than FeEDDHA. Increase in the concentrations of FeEDDHA had no effect on biomass production while the two other forms showed concentration-dependent responses.

## Discussion

There are some important indices in nutrition of metal elements especially in photosynthetic elements, including bioavailability, induction of oxidative stress and photosynthesis induction or inhibition. Among the metals, iron is the most limiting factor with low availability in alkaline conditions. Mandalam and Palsson (1998) suggested that in high cell density cultures of *Chlorella vulgaris*, iron is one of the cell division limiting factors. There is no report on the behavior of iron in the alkaline and oxygenated medium of *Spirulina* but it has been reported that up to 20% of iron during the initiation of the cultures may precipitates as  $\text{Fe}_3(\text{PO}_4)_2$  (Belay, 1997). Therefore, the effects of supplying iron and its chelate forms on some of the physiological properties of *Spirulina platensis* are studied here.

In this study, occurrence of oxidative stress was found in *S. platensis* (PCC9108) under some Fe nutrition conditions as indicated by the MDA production which is similar to the effect of heavy metals on higher plants. In higher plants, heavy metals induce generation of superoxide radical ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), hydroxyl radical ( $\text{HO}\cdot$ ), and singlet oxygen ( $1\text{O}_2$ ), collectively termed ROS which exert a variety of damaging effects and are

also called, oxidative stress (Devi and Prasad, 1998; Dietz *et al.*, 1999). In this study, since the highest MDA was observed in using FeEDDHA, it indicates that this form has a nonspecific Fe delivery into the cells. Uncontrolled concentrations of free or redox active Fe forms should happen in the cells when using FeEDDHA. Another finding in this study is that concentration of Fe in the cells fed by FeEDDHA was higher than the other forms at lower concentrations of Fe in medium. It suggests that in addition to cellular uptake mechanisms, some other ways (possibly diffusion) cause accumulation of Fe in the cells. This accumulation brings about oxidative stress and consequently activation of defense mechanisms such as proline biosynthesis and accumulation. In addition, it is possible that FeEDDHSA is penetrating into the cells. Since it is a strong Fe chelator, it prevents a high rate of Fe release and no oxidative stress occurs.

A high magnitude effect of Fe chelate form on proline concentration in the cells was observed (Table 1). Thus, the increased level of MDA suggests that Fe ions stimulate free radical generation in the microorganism. Also, increased MDA level has been reported in several higher plants (Luna *et al.*, 1994; Chaoui *et al.*, 1997). Proline accumulation under heavy metal stress has been reported earlier in some higher plants (Hall, 2002), although the mechanism of accumulation of proline in plants or plant parts exposed to stress is still unknown. Binding to metal ions due to the chelating ability of proline can be a defense mechanism for survival. Increase in both proline and MDA contents with increasing metal ion concentration are indicative of a correlation between free radical generation and proline accumulation. Our study also depicted an inverse relationship between biomass and proline accumulation in the test alga under stressed conditions, thus suggesting that proline might be produced at the expense of material(s) required for the development of the test strain. This might involve reduction in cell division or delay of exponential growth due to proline accumulation (Maggio *et al.*, 2002).

There are some specific mechanisms in iron acquisition by marine cyanobacteria (Morrissey and Bowler, 2012): 1- Penetration of Fe(III) into the periplasmic space, ligation to FutA (a Fe(III) binding protein) and transport into the cells via action of FutB/C (permease/ATPase) system, 2- Transport of Fe(II) by FeoA/B (small Fe binding protein/predicted Fe(II) permease) and 3- Transport by TonB system which is an iron-siderophore transporter across cell wall and plasma membrane. Based on the offered models (Stintzi *et al.*, 2000) in bacteria, Fe should be exchanged between chelators and siderophores

of bacteria and then be transferred into the cells. Weak chelators which release Fe more easily, are more capable to exchange Fe with siderophores. In this study, a more intracellular Fe concentration was observed in using stronger chelator EDDHA in comparison to weaker chelator EDTA. Indeed, it should be determined whether Fe has been exchanged more efficiently with EDDHA than EDTA or other routes have been responsible for a higher uptake of Fe by the cells. It should be also determined that how much siderophore is produced by the cells at different Fe concentrations in medium. This is important since the release of siderophores is inhibited at higher Fe concentrations in medium.

The concentrations of free essential metals such as Cu and Fe in the cells are kept to minimum by using very efficient chelating systems (Tottey *et al.*, 2002; Puig *et al.*, 2007). This prevents oxidative stress in the cells by the metals. Since in the current study both higher oxidative stress and higher Fe concentrations were observed, it suggests that at least a considerable proportion of the intracellular Fe has been free or redox active in using chelate form FeEDDHA and possibly it has entered the cells from nonspecific routes which could not be recognized and captured by the cells.

There are some indicators to assess the Fe status in the cells: Expression of the genes encoding cellular Fe assimilators, function of photosynthetic apparatus which could be measured by O<sub>2</sub> liberation (La Fontaine *et al.*, 2002; Moseley *et al.*, 2002), and the activity of some Fe status indicator enzymes such as catalase and ascorbate peroxidase (Marschner, 1995). Metal toxicity may also be observed by changes in growth of the cells. Reduction in growth of the cells by some Fe chelate forms (in this study the most important EDDHA) may be considered as a reasonable determinant of its toxic effect which has strongly affected Fe homeostasis in the cells by different mechanisms. Another finding in this study concerns the rates of photosynthesis in different Fe chelate forms.

Growth response of the cells to the Fe chelate form FeEDDHSa impressed the concentration more than the concentrations of two other used chelate forms. By increasing the concentration of FeEDDHSa, growth increased continually with a different pattern comparing with other forms. FeEDDHSa is a strong Fe chelator. It does not release Fe to the medium easily (Lucena, 2006) and so at higher concentrations has optimum Fe delivery to the cells. FeEDDHSa stimulated more oxygen production in contrast to the other Fe chelate forms. Fe is a fundamental element in different steps of photosynthesis and its

availability is a determinant factor in photosynthesis (Marschner, 1995). Indeed, it could be concluded that EDDHSa is a more capable form of Fe chelate for obtaining suitable Fe for the cells. It keeps Fe soluble at high alkaline conditions, does not induce oxidative stress and delivers enough Fe to the cells for having more growth and photosynthesis. Similar results were observed by studying different crops by Alvarez-Fernandez *et al.*, (2005) which concluded that FeEDDHSa was a more compatible iron chelate in comparison to FeEDDHMA and FeEDDHA. High concentration of Fe in the cells is important since *Spirulina* is usually considered as an efficient iron source in human nutrition (Richmond and Hu, 2013). Such an aim could be achieved by using FeEDDHSa.

## Conclusions

The results indicated that FeEDDHSa acts as a promising source for iron in spirulina culture which at higher concentrations in culture medium does not induce oxidative stress in the cells. It will be valuable to examine Fe exchange between chelators of Fe and siderophores of different strains of *Spirulina platensis* for evaluating a more effective Fe uptake by cells. Also, it is important to determine chelator uptake and metabolizing abilities of the cells to pursue the changes in their concentrations in culture media.

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