Growth promotion effect of steelmaking slag on *Spirulina platensis*

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**Abstract.** A growth promotion effect of steelmaking slag on *Spirulina platensis* M135 was investigated. The growth promotion effect was obtained that was 1.27 times greater than that obtained by the control by adding 500 mg L$^{-1}$ of steelmaking slag and culturing for 60 days. The lipid content decreased in a concentration-dependent manner with steelmaking slag, whereas the carbohydrate content remained constant. The protein content of *S. platensis* M135 increased in a concentration-dependent manner with steelmaking slag when cultured at day 45. The superoxide dismutase activity of *S. platensis* M135 exhibited a decreasing trend in a time-dependent manner and an increasing trend in the control. The superoxide dismutase activity was lower than that of the control at day 1 but was higher at day 30. No genetic damage was observed up to 500 mg L$^{-1}$ of steelmaking slag at 30 days of culture. Recovery from genetic damage was observed at 1,000 mg L$^{-1}$ of steelmaking slag but not at higher concentrations.

1. **Introduction**

Global warming because of the increasing levels of greenhouse gases, including carbon dioxide, is a serious concern. The potential for carbon capture by biomass production has been attracting much attention; in particular, the higher productivity of microalgae than that of plant biomass has been of interest. *Spirulina* is one of the most popular edible microalgae. It is rich in protein and can grow in severe conditions such as high temperatures, salinity, and highly alkaline pH. Because of these specific culture conditions, *Spirulina* can avoid contamination of other microbes; thus, it can be easily commercialized. Screening for strains with higher growth rates and higher concentrations of targeted products is required for commercialization. Genetic modification is expected to be applicable to *Spirulina*, since the genetic structure of *Spirulina* (*Arthrospira*) *platensis* NIES-39 has been elucidated [1]. But application of genetic modified microorganism should be severely restricted. Much more simple approach is supplementation of growth enhancer to culture medium. *Spirulina* growth promotion is easily achieved by adding a carbon source such as glucose. Chen *et al.* reported a growth promoting effect of 2.38 times than that of the control by adding 2 g L$^{-1}$ glucose and of 1.53 times that of the control by adding 4 g L$^{-1}$ acetic acid [2]. Nevertheless, even though higher productivity is achieved, these approaches are costly. Therefore, more cost effective methods to produce biomass have been...
investigated such as the utilization of seawater or organic wastewater as a culture medium. Ak reported that cultivation costs could be reduced by 20% using wastewater [3].

Iron and steel slag is a by-product of iron and steel manufacturing processes and can be categorized into blast furnace slag and steelmaking slag (SMS). SMS consists of both converter slag that is generated by a converter and electric arc furnace slag that is generated during the electric arc furnace steelmaking process that uses steel-scrap as its raw material. In Japan, 15.627 million tons of SMS slag was annually produced in 2014, which was used in a wide range of applications such as cement, civil engineering works at ports, concrete aggregate, and ground improvement [4]. But SMS can be applicable as a fertilizer because it contains the plant nutrients such like CaO, SiO$_2$, MgO, and FeO. Fertilization effect of SMS for seaweed bed restoration has been actually proved in various Japan seacoasts [5-8].

The ecological impact of SMS and its component on marine organisms and on some seawater microalgae has been concerned. For example, ferric ethylenediaminetetraacetic acid (Fe-EDTA) effectively increased the growth of the macro algae Sargassum horneri [9]. Liu et al. reported a growth promoting effect of FeCl$_3$-EDTA on Chlorella vulgaris [10]. The effect of 10 mg L$^{-1}$ iron nanoparticles (Total Fe concentration <1.4 × 10$^{-5}$ M) on the growth and metabolic status of various marine microalgae such as Pavlova lutheri, Isochrysis galbana, and Tetraselmis suecica has been investigated [11]. Iron binding at the cell surface has been reported to be a critical step in the uptake mechanism of marine microalgae [12]. To maximize the iron fertilizer effect, the size of the SMS should be small to increase the surface area for elution. Normally the size of the SMS should be carefully determined or controlled to avoid sudden increase in alkalinity because of CaO reacting with water. But this disadvantage of SMS inducing high alkalinity in water is expected to be advantage for growth of *Spirulina*, specifically. Therefore, the effect of SMS on growth, and the metabolite and genetic status of *S. platensis* M135 was investigated in this study.

2. Materials and methods

2.1. Materials

The cyanobacteria *S. platensis* strain M135 was supplied by the Algal Biotechnology Department, Institute of Biotechnology, Vietnam Academy of Science and Technology, Hanoi, Vietnam. *S. platensis* M135 were cultured in 250 mL flasks with 100 mL of SOT medium with the following composition (mg L$^{-1}$): NaHCO$_3$, 16,800; K$_2$HPO$_4$, 500; NaNO$_3$, 500; K$_2$SO$_4$, 1,000; NaCl, 1,000; MgSO$_4$·7H$_2$O, 200; CaCl$_2$·2H$_2$O, 40; FeSO$_4$·7H$_2$O, 10; Na$_2$EDTA·2H$_2$O, 80; H$_3$BO$_3$, 2.86; MnSO$_4$·5H$_2$O, 2.5; ZnSO$_4$·7H$_2$O, 0.22; CuSO$_4$·5H$_2$O, 0.08; Na$_2$MoO$_4$·2H$_2$O, 0.02. *S. platensis* M135 was cultivated under the following conditions: light intensity of 1,500 Lux from a white fluorescent lamp with 12 h/12 h light/dark cycles at 28°C. All flasks were cultured under static conditions and were shaken by hand twice a day.

SMS was provided from Nippon Steel Company Oita Factory. The SMS composition was determined by x-ray fluorescence spectrometry (XRF) (ZSX101e, RIGAKU, Japan). Table 1 shows the chemical composition of the SMS, which exhibits the typical characteristics of arc furnace oxidizing slag.

2.2. Methods

2.2.1. Cell growth. Cell growth was determined by measuring the optical density at 556 nm using a spectrophotometer (U-1100 Spectrophotometer, HITACHI Ltd, Japan).

2.2.2. Metabolite analysis. The experiment examined the effect of 0, 50, 100, 500, 1,000, and 10,000 mg L$^{-1}$ SMS during 60 days of cultivation.

The total lipid content was analysed using a Soxhlet apparatus following the method of Bligh and Dyer [13]. To quantify the intracellular carbohydrates, aliquots of 100 mg of lyophilized cells were
added into a 2.5 M HCl solution (20 mL) and hydrolyzed in boiling water for 30 min. Then, the pH of the sample was adjusted to 7 using a NaOH solution (5 M) and water was added up to 50 mL. Finally, the mixtures were centrifuged for 5 min at 5000 × g, and the supernatant was used for carbohydrate determination using the DNS method [14].

2.2.3. SOD activity assay. Samples were harvested by centrifugation at 12,000 rpm for 5 min at 4°C and were then washed twice with sterile distilled water. Aqueous extracts of S. platensis M135 were prepared by homogenizing the microalgae (0.1–0.5 g fresh weight) and mixing with an equal volume of glass beads in 1.2 mL sterile distilled water at 4°C. The time for homogenization was optimized using a light microscope to verify complete cell breakage. The homogenates were then centrifuged at 12,000 rpm for 10 min at 4°C and then stored at −20°C until the superoxide dismutase (SOD) assay [15].

2.2.4. DNA profile of S. platensis M135. Variations in the DNA profile of S. platensis M135 were compared between the control and the different concentrations of SMS. Samples with different concentrations of SMS cultured for 30, 45, and 60 days were analysed, respectively. DNA of each sample was extracted using the ZR Plant/Seed DNA MiniPrep Kit for Random amplified polymorphic DNA based on polymerase chain reaction (RAPD-PCR). After the PCR reaction, the PCR product was analysed using agarose gel electrophoresis [16, 17].

3. Results and discussion

3.1. Growth promotion effect of SMS on S. platensis M135

Figure 1 shows the growth curves of S. platensis M135 grown with various concentrations of SMS. Growth promotion effects of SMS were obtained at all concentrations compared to the growth levels of the control without SMS. Figure 2 shows the growth promotion ratio of S. platensis with SMS based on the growth of control regarded as 100% at 60 days of culture. A growth promotion effect was observed

![Figure 1](image-url). Growth curve of S. platensis M135 with steelmaking slag(SMS) (■: 50 mg L⁻¹, △: 100 mg L⁻¹, ○: 500 mg L⁻¹, ◇: 1,000 mg L⁻¹, and ×: 10,000 mg L⁻¹) and without SMS (●). The data are means ± standard deviations.
after 5 days and the highest growth-promoting ratio of 1.62 was achieved with SMS of 10,000 mg L\(^{-1}\).
A growth promotion effect of 1.27 times greater than that with the control was obtained with 500 mg L\(^{-1}\) SMS at 60 days. The growth profile of this experiment agreed with the growth promotion effect of different iron forms with EDTA on \(S. \text{platensis}\) [18]. Thus, the iron dissolution from SMS is considered to be the growth promoter. Erosion of wustite and brownmillerite interfaces lead to Mg, Al, Si, and Ca dissolution from SMS slag to water [19]. Considering the composition of wustite [(Fe, Mg, Mn)O/MgO–FeO] and brownmillerite [Ca\(_2\)(Al, Fe)\(_2\)O\(_5\)·(4CaO·Al\(_2\)O\(_3\)·Fe\(_2\)O\(_3\))] which are contained in this SMS, Fe should also be eluted and available for \(S. \text{platensis} \) M135. Based on the solubility diagram for iron, the estimated form of iron eluted from SMS could be Fe(OH)\(_4\)\(^-\). A time course of this dissolution might explain the growth promotion effect during culture. The growth promotion ratio was not proportional to the SMS concentration. Zhang et al. reported that the dissolved amount of Si and Fe decreases with increasing the ratio of slag/seawater, this is because iron dissolution is not linked to the slag concentration [20].

**3.2. Metabolite analysis**

Figure 3 shows the metabolite (carbohydrate and lipid) content of \(S. \text{platensis} \) M135 at 60 days of culture. SMS decreased the lipid content of \(S. \text{platensis} \) M135, but there was no change in the carbohydrate content from that of the control. The lipid and carbohydrate contents of the M135 strain are considered higher than those of other strains. The lipid content of \(S. \text{platensis} \) M135 of this study was comparable to a previously reported value [21], even though treated with SMS. While, another study with different microalgae \(Chlorella\), the lipid content increased up to 7.27 times with 0.67 mg L\(^{-1}\) Fe\(^{3+}\) [10].

| Table 1. Elemental composition of SMS. |
|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Element         | Fe  | Ca  | Mn  | Si  | Mg  | Al  | S   | P   |
| mass %          | 45.44 | 39.22 | 7.15 | 4.18 | 1.96 | 1.40 | 0.36 | 0.29 |
| ±3.53 ±2.77 ±0.33 ±0.98 ±0.31 ±0.23 ±0.09 ±0.08 |
Figure 4 shows the protein content of *S. platensis* M135 at 1, 30, 45, and 60 days of culture. The protein content increased with concentration-dependent manner of SMS until 45 days, but then decreased from that with the control at 60 days. A slow change in the protein content occurred because of the iron form of SMS and its time consuming dissolution. Similarly, pigment-protein complex of

![Graph showing protein content in the dry biomass of S. platensis M135 at 1 (■), 30 (■), 45 (■), and 60 days (□) of culture.](image)

**Figure 3.** Carbohydrate (a) and lipid (b) content in the dry biomass of *S. platensis* M135 at 60 days of culture, respectively.

![Graph showing carbohydrate and lipid content.](image)

**Figure 4.** Protein content in the dry biomass of *S. platensis* M135 at 1 (■), 30 (■), 45 (■), and 60 days (□) of culture.

Figure 4 shows the protein content of *S. platensis* M135 at 1, 30, 45, and 60 days of culture. The protein content increased with concentration-dependent manner of SMS until 45 days, but then decreased from that with the control at 60 days. A slow change in the protein content occurred because of the iron form of SMS and its time consuming dissolution. Similarly, pigment-protein complex of
phycocyanin content increased at the early stages of culture but decreased at the latter stages with different forms of iron and EDTA [18]. A chelating agent like EDTA is not provided when applying SMS. The strong chelator EDTA has been found to diminish the growth promotion effect of iron from SMS with compost [22]. Protein content increases should also be associated with iron dissolution. This idea is also supported by the increase in marine phytoplankton fluorescence intensity by iron from SMS (SMS diameter: 5–20 μm) [23]. Another explanation for the change in protein content is the pH increase. The highest amino acid content, corresponding to the protein content was obtained at pH 9 with S. platensis, but the amino acid content decreased with pH change [24]. The pH of the medium was affected by Ca and Mg from the SMS. Despite the continued buffering action of Mg, the Fe from SMS increased the protein content, while Ca release lead to a higher pH than that in the control, which also affected the protein content.

3.3. SOD activity

Figure 5 shows the SOD activity of S. platensis M135 at 1, 30, 45, and 60 days. SOD activity decreased with a time-dependent manner but showed an increasing trend in the control. The SOD activity was lower than that in the control at day 1, but it was higher at 30 days. Similarly, iron promoted the growth of Heterosigma akashiwo [25] and Chattonella marina [26] while reducing hydrogen peroxide production, which indirectly in accordance with our results of SOD activity.

What is the relationship between the SOD activity and microalgae growth with SMS? How can microalgae utilize inorganic iron? Possibly, the release of organic Fe chelators from various types of microalgae including S. platensis M135 help the uptake of iron from the medium. Based on a study of organic Fe chelator release from Scenedesmus, Benderliev postulated that the contact of inorganic Fe (II) and humic Fe (II) with the microalgae cell surface might result in the generation of strong oxidants such as superoxide radicals and hydroxyl radicals that tend to inhibit the release of chelators from cells [27]. The contact of Fe (III) with the cell’s surface triggers another set of reactions that stimulate the release of chelators from cells. Thus, contact with iron from SMS might trigger organic Fe chelator release from S. platensis M135 and lead to growth promotion.

![Figure 5. Superoxide dismutase (SOD) activity of S. platensis M135 dry biomass at 1 (■), 30 (■), 45 (■), and 60 days (□) of culture.](image-url)
3.4. Effect of SMS on DNA profile of S. platensis M135
Figure 6 shows phylogenetic trees of six samples of S. platensis M135 cultured with 0, 50, 100, 500, 1,000, and 10,000 mg L\(^{-1}\) SMS for 30, 45, and 60 days. The degree of polymorphism in the samples treated with different concentrations of SMS increased with a time-dependent manner of 7\%, 28\%, and

![Phylogenetic tree A](image)

![Phylogenetic tree B](image)

![Phylogenetic tree C](image)

**Figure 6.** Phylogenetic tree of S. platensis M135 cultured with SMS concentration from 0, 50, 100, 500, 1,000 and 10,000 mg L\(^{-1}\) after 30 days of cultivation (A), 45 days of cultivation (B) and 60 days of cultivation (C).
33\% at 30, 45, and 60 days, respectively. Genetic similarity coefficient of samples treated with 10,000 mg L\(^{-1}\) SMS decreased from 0.923 to 0.788 (or 0.824) and then to 0.444, which differed from that of the samples treated with 0–1,000 mg L\(^{-1}\) SMS. For 0–1,000 mg L\(^{-1}\) SMS, after 60 days, the DNA levels are not different from those of the control. This may be an adaptive process of \textit{S. platensis} M135 to lower concentrations of SMS.

4. Conclusions

SMS continuously promoted the growth of \textit{S. platensis} M135 without largely affecting its metabolite and DNA profile. A growth promotion effect of 1.27 times greater than that with the control treatment was obtained with up to 500 mg L\(^{-1}\) SMS at 60 days of culture. The lipid content decreased in an SMS-concentration-dependent manner. The protein content increased in an SMS-concentration-dependent manner at 45 days of culture. No genetic damage was observed with up to 500 mg L\(^{-1}\) SMS, and recovery from genetic damage was observed with 1,000 mg L\(^{-1}\) SMS at 30 days of culture.

References

[1] Fujisawa T, Narikawa R, Okamoto S, Ehira S, Yoshimura H, Suzuki I, Masuda T, Mochimaru M, Takaichi S, Awai K, Sekine M, Horikawa H, Yashiro I, Omata S, Takarada H, Kato Y, Kosugi H, Tanikawa S, Ohmori K, Sato N, Ikeuchi M, Fujita N and Ohmori M 2010 DNA Research \textbf{17} 85

[2] Chen T, Zheng W, Wong Y S, Yang F and Bai Y 2006 Bioresource Technology \textbf{97} 2260

[3] Ak I 2012 Aquaculture International \textbf{20} 413

[4] Press release from Nippon Slag Association, 2003, http://www.slg.jp/e/images/Steel\%20Slag\%202014FY.pdf (Accessed 18 August 2015)

[5] Takahashi T and Yabuta K 2002 \textit{NKK Technical Report-Japanese Edition} \textbf{87} 43

[6] Miyata Y, Sato Y, Shimizu S and Oyamada K 2009 \textit{JFE Technical Report} \textbf{13} 41

[7] Nakamura Y, Taniguchi A, Okada S and Tokuda M 1998 \textit{ISIJ international} \textbf{38} 390

[8] Sugie K and Taniguchi A 2011 \textit{ISIJ international} \textbf{51} 513

[9] Nagai T, Miki O and Okumura C 2014 \textit{Journal of Water and Environment Technology} \textbf{12} 285

[10] Liu Z Y, Wang G C and Zhou B C 2008 Bioresource Technology \textbf{99} 4717

[11] Kadar E, Rooks P, Lakey C and White D A 2012 \textit{Science of the Total Environment} \textbf{439} 8

[12] Sutak R, Botetbol H, Blaiseau PL, Léger T, Bouget F Y, Camadro J M and Lesuisse E 2012 \textit{Plant Physiology} \textbf{160} 2271

[13] Bligh E G and Dyer W J 1959 \textit{Can. J. Biochem. Physiol.} \textbf{37} 911

[14] Miller G L 1959 \textit{Analytical chemistry} \textbf{31} 426

[15] McCord J M and Fridovich I 1969 \textit{Journal of Biological Chemistry} \textbf{244} 6049

[16] Hien H T M, Lan V T and Hong D D 2000 \textit{Journal of Biology} \textbf{22} 269

[17] Sayin S and Doganay S 2011 \textit{Fresenius Environmental Bulletin} \textbf{20} 1888

[18] Mombelli D, Mapelli C, Barella S, Gruttadaura A, Le S G and Garcia D E 2014 \textit{Journal of Hazardous Materials} \textbf{279} 586

[19] Ogbonda K H, Aminigo R E and Abu G O 2007 \textit{Bioresource Technology} \textbf{98} 2207

[20] Twiner M J and Trick C G 2000 \textit{Journal of Plankton Research} \textbf{22} 1961

[21] Liu W, Au D W T, Anderson D M, Lam P K S and Wu R S S 2007 \textit{Journal of experimental marine biology and ecology} \textbf{346} 76

[22] Benderliev K 1999 \textit{Bulg J Plant Physiol} \textbf{25} 65