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Determination of Superoxide Dismutase Activities in Different Cyanobacteria for Scavenging of Reactive Oxygen Species

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Abstract: There is considerable interest in cyanobacteria as sources of antioxidant pigments, antiinflammatory substances, enzymes such as superoxide dismutase (SOD) and vitamins. Antioxidant enzymes have significant role in the removal of reactive oxygen species produced during visible and ultraviolet irradiance stress in cyanobacteria. One of the most ubiquitous protective enzyme is superoxide dismutase (SOD). Most of cyanobacteria are known to have both FeSOD and MnSOD which are associated with photosystem I and II. These enzymes are responsible for scavenging superoxide radicals.*Spirulina platensis* and *Synechococcus nidulans* are widely used cyanobacteria as food supplement and cosmetic due to their bioactive compounds. Although the chemical composition of *S.platensis* has been investigated by many researchers, research on the relationship between protein content and comparison of SOD activity with other cyanobacteria is limited.Among several SOD producing cyanobacteria, the most promising organisms were investigate in terms of SOD activities in this study. The aim of the present work was to investigate and compare the SOD activities of different cyanobacteria including *S. platensis, Pseudanabeana* sp., *S. nidulans* and associate with protein contents. SOD activities of these cyanobacteria were determined by xanthine/xanthineoxidase method.Modified Lowry method was used for protein determination. The analysis results showed that the maximum specific SOD activity obtained was 50.4 U/mg from *S.nidulans* and the minimum specific SOD activity was 18.4 U/mg from *Pseudanabeana* sp. Total protein amount of *S. nidulans*, *S. platensis*, *Pseudanabeana* sp. was determined as 0.25 mg/ml, 0.27 mg/ml and 0.43 mg/ml respectively. As a result, *Synechococcus nidulans* was more efficient at scavenging peroxide radicals than *Pseudanabeana* sp.

Key words: SOD activity, *Spirulina platensis*, *Synechococcus nidulans*, *Pseudanabeana* sp.

Introduction

Cyanobacteria are oxygen evolving photosynthetic microorganisms and produce reactive oxygen species (ROS) that can damage cellular components such as phospholipids, DNA leading to cell death. Normal cellular metabolism involves the production of reactive oxygen species. Superoxide (O_2^-) is produced from electron reduction of oxygen and leads to hydrogen peroxide (H_2O_2) and the toxic product peroxynitrite ($ONOO^-$) formation. Also the combination of H_2O_2 with metal ions (iron) or the breakdown of $ONOO^-$ can produce the highly toxic hydroxyl radical (•OH) which are classified as reactive oxygen species (ROS). They have excellent antioxidative system and scavange ROS produced during photosynthesis.

Although there are several antioxidant mechanisms that consist of both enzymatic and non-enzymatic such as superoxide dismutase (SOD),

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catalase, ascorbic acid, tocopherol and etc. Cyanobacteria possess an effective stress combat system to cope with this pressure by the help of a cascade of antioxidants, where the SODs act initially followed by the catalase and peroxidases [1,2].

Mann and Leilin first discovered it as a blue/green protein, in 1938. However, its catalytic activity and its name, SOD, was identified by Mc Cord and Fridovich in 1969. SOD is the primary defence system against oxygen damage [1,3,4]. Superoxide dismutases (EC 1.15.1.1) which are one of the most important metalloenzymes in antioxidant defense system, found ubiquitously in aerobic organisms and catalyze the dismutation of O_2 to H_2O_2 and O_2. SOD is in the range of molecular mass 17–85 kDa and plays a major role in defense against oxygen radical-mediated toxicity [5,6]. SOD is not only the first step for the protection but also the only enzyme capable of catalyzing this reaction. Therefore SOD plays a key role in the antioxidant network of cyanobacteria [7].

SODs are classified as four types according to the metal ion cofactor required for their activity: copper–zinc (Cu/ZnSOD), manganese (MnSOD), iron (FeSOD), and nickel (NiSOD). Most of cyanobacteria are known to have both FeSOD and MnSOD to scavenge superoxide radical. FeSOD is associated with photosystem I and MnSOD is primarily associated with photosystem II. The cellular SOD profile varies between organisms and with environmental conditions [6,8]. In human body, under physiological conditions, a balance exists between the level of ROS produced during normal cellular metabolism and the level of endogenous antioxidants, which serve to protect tissues from oxidative damage. Disruption of this balance, either through increased production of ROS or decreased levels of antioxidants, produces a condition referred to as oxidative stress and leads to variety of pathological conditions including cardiovascular diseases, neurological disorders, lung pathologies, and accelerated aging. Besides, in many cases, ROS production is genetically programmed and is induced during development [9]. ROS can react with a variety of cellular macromolecules such as lipids, proteins, DNA, and, leading to the disruption of cell membranes, inappropriate activation or inactivation of enzymes, and genetic mutations. Therefore, these diverse ROS can cause several pathophysiological damages in tissues [2]. In a similar study, it has also been determined that the formation of active oxygen species at respiratory sites and electron transport system in photosynthesis was stimulated by UV radiation [10].

Once UVR reaches the inside the cell, it interacts with oxygen and other organic compounds to produce toxic ROS such as superoxide (O_2^-), hydroxyl radical (OH^-) or hydrogen peroxide (H_2O_2) finally resulting in oxidative stress (as discussed above). To overcome the oxidative stress cyanobacteria have developed an antioxidant system as a second line of defense mechanisms against UVR. This system includes non-enzymatic and enzymatic antioxidants. The enzymatic antioxidants include superoxide dismutase (SOD).

Recent comparative genomic analysis by Priya *et al*. revealed that NiSOD is the only SOD found in primitive cyanobacteria, Fe and Mn occupy the higher orders of cyanobacteria and Cu/ZnSOD is rare in cyanobacteria [11].

It was found that the presence of SOD activities shows the dismutation of superoxides to hydrogen peroxide (H_2O_2). It could be possible to change the activities of SODs by eliminating the micronutrients as source for metal prosthetic groups from the growth medium in cyanobacteria. This case provides a survival from such an oxidative stress that the microorganism may use alternative defence mechanisms such as accumulation of carotenoids [12].

Recently, new treatments are being developed focused on SOD in scavenging ROS. There are several approaches being improved such as enhancing the SOD abilities of cells with often using natural extracts, using SOD mimetics and supplementary enzyme therapies [13]. This enzyme already has been shown to influence aging, cancer, and some very important diseases, such as artheriosclerosis, cataract, retinal damage, essential hypertension, amyloidosis, ischemia, and age-dependent immune deficiency disease [14,15].

L'Oreal recognized the importance of this enzyme and obtained a patent related to a cosmetic compositions which contain SOD. It is thought that such formulations combat skin ageing and/or

to protect the skin against the effects of free radicals for a marine source of SOD in 1997 for its general use in cosmetics (EU patent no. 2 287 899) [4].

Most of cyanobacteria are considered as rich sources of natural antioxidants. *Spirulina platensis*, a commercial source of single cell protein, is an organism well adapted to various physiological stresses. This cyanobacteria expresses various enzyme antioxidants such as SOD, catalase, ascorbate peroxidase and a non-specific peroxidase [8]. *Pseudanabaena* is a unique divergent branch occurring at the very beginning of the evolution of photosynthetic organisms [16]. A nitrogen fixing cyanobacterium, *Anabaena* sp. PCC 7120 has differentially expressed MnSOD and FeSODs. Both FeSOD and Cu/ZnSOD are discovered in marine cyanobacterium *Synechococcus* sp. WH 7803 [17,18,19]. Detailed studies on the mechanisms of UV induced impairment of photosynthesis and antioxidant response have been predominantly conducted on crop plants, while comparative studies on these aspects in micro-organisms particularly cyanobacteria are still scarce. In addition, *Synechococcus* shows high SOD activity under different light and UV stress conditions. Therefore *Spirulina platensis* and *Synechococcus* could be model organisms to study antioxidant capacity and compare to other cyanobacteria.

The aim of this study was to investigate and compare the specific growth rates, protein contents, determine the SOD activities with crude extracts of cyanobacteria *Spirulina platensis*, *Synechococcus nidulans* and *Pseudanabeana* sp. Although there are recent studies related with the SOD activity of different cyanobacteria concerning *Spirulina platensis* and *Synechococcus nidulans*, no experimental studies has been encountered about the SOD activity of *Pseudanabeana* sp. in the literature. Consequently, this study will also make a significant contribution to the literature for *Pseudanabaena* sp.

Materials and methods

Strain and culture conditions

Spirulina platensis EGE-MACC 38, *Synechococcus nidulans* EGE-MACC 7 and *Pseudanabeana* sp. EGE-MACC 40 were obtained from the Ege University Microalgae Culture Collection, Izmir, Turkey.

Spirulina platensis and *Synechococcus nidulans* were cultivated in Zarrouk medium [20], BG-11 medium [21] and BBM medium [22], respectively. The cultures were grown at 25°C under the light intensity of 28 µmol photons m^{-2} s^{-1} in 1-L glass bottles for 10 days. Air was supplied to the cultures at a flow rate of 1 L min^{-1} (1.67 vvm). *Pseudanabeana* sp. was also grown at 30°C in ASN III medium [23] at an air flow rate of 1 L/min (1.25 vvm) under continuous illumination (28 µmol photons m^{-2} s^{-1}) in 2L flask for 10 days of batch production.

Cell disruption and crude extract preparation

Cultured cells were harvested with centrifugation for 30 min at 5000 rpm and washed thrice with 1xPBS solution. Then 1g biomass was suspended with 5 ml sonication buffer (10 mM potassium phosphate with 0.1 mM EDTA at pH 7.8). For cell disruption, 1 g centrifugated biomass was placed in a 20 ml tube containing 5 ml sonication buffer. Ultrasonic homogenizer (Bandelin Electronic HD-2070) was operated with 80 % power output for 10 min at 8 pulsation cycle. The horn was placed in the center of the tube. Cooling was achieved by placing the tube in an ice water bath during ultrasonication in order to avoid the increase of temperature that could affect the cell viability. Sonicated biomass was diluted with potassium phosphate buffer (Dilution Factor; DF:10) for SOD activity determination.

Growth measurements and protein determination

The cell concentration was determined spectrophotometrically at 600 nm. Samples were taken at indicated times, and following growth parameters were measured immediately.

The specific growth rate (µ) of the cells was calculated from the initial logarithmic phase of growth, as

$$\mu = \frac{\ln x_2 - \ln x_1}{dt}$$

where X_2 is the final cell concentration, X_1 is the initial cell concentration and dt is the time

required for the increase in concentration from X_1 to X_2. Doubling time (Dt) was also calculated as Dt = ln 2/μ.

Protein amount of cultures was determined spectrophotometrically by modified Lowry method. This colorimetric assay is based on the biuret reaction by subsequent reaction with the Folin phenol reagent (Folin–Ciocalteu reagent). Bovine serum albumin (BSA) was used as standard in the assay [24].

SOD activity determination

SOD activities were determined by xanthine/ xanthine oxidase (X–XOD) method. RANSOD® enzyme kit (RANDOX Laboratories Ltd.) was used. This method employs reaction of xanthine with xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye (Figure 1). The inhibition of the rate of reduction of INT under the conditions of the assay refers to inbition % values (Table 1).

The superoxide dismutase activity was measured by the degree of inhibition of the above reaction. Assay was carried out at 37°C using double beam spectrophotometer (Shimadzu Digital, UV-160A) at 505 nm which includes heat jacket. Sample measurements were carried out in triplicates and standard deviation of data was determined for each sample group.

Results and discussion

Spirulina platensis, Synechococcus nidulans and *Pseudanabeana sp*. were cultured under the optimum growth conditions for 10 days of cultivation period. During cultivation the specific growth rates were calculated. After incubation period, protein contents and SOD activities were determined.

The growth rate determination of cyanobacteria

The growth rates were determined by optical density measurement. Maximum and minimum optical densities at the end of the cultivation period were obtained from *Pseudanabeana* sp. and *Synechococcus nidulans* with the values of 1.90 and 0.33, respectively (Figure 2). During the 10 days of cultivation period, it was found that *Synechococcus nidulans* grow faster than *Spirulina platensis* and *Pseudanabeana* sp.

The specific SOD activities of cyanobacteria

Results of the study indicate that *Spirulina platensis, Pseudanabeana* sp. and *Synechococcus* sp. extracts have antioxidant properties and high protein contents. It is well known from

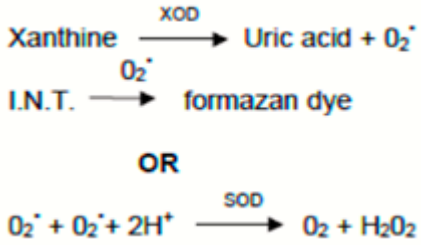

Figure 1. Mechanism of superoxide dismutase determination

Table 1. Results of obtaining percentage inhibition, SOD activity, and protein amount of different cyanobacteria

Microalgae	Inhibition %	SOD activity (U/mL)	Total protein amount (mg/ml)	Specific activity (U/mg protein)
Spirulina platensis	57.33±1.88	8.0±0.14	0.27	30.0±5.2
Pseudanabeana sp.	56.92±1.33	7.9±0.073	0.43	18.4±1.7
Synechococcus nidulans	68.60±0.93	12.6±0.125	0.25	50.4±5.0

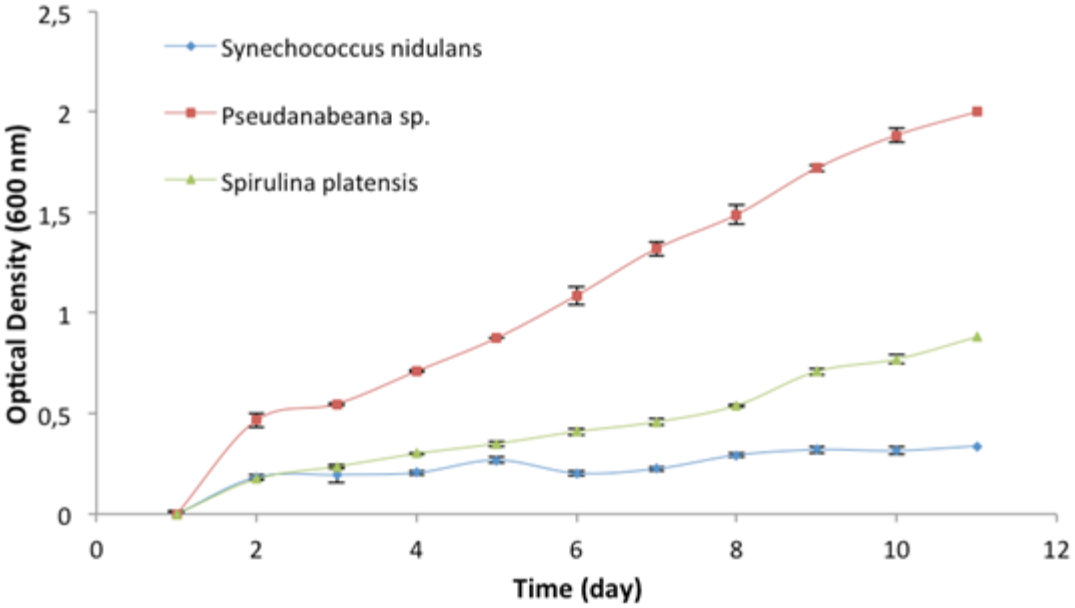

Figure 2. Optical densities (OD) of different cyanobacteria at 600 nm

the literature that *Spirulina platensis* and *Synechococcus* sp. show strong antioxidant properties due to their SOD activities. However, poorly known filamentous cyanobacteria *Pseudana-beana* sp. is new to literature and there is lack of knowledge about its antioxidant pro-perties. Therefore, based on previous studies, SOD activities *of Spirulina platensis, Synecho-coccus* sp and *Pseudanabeana* sp. were investi-gated. Besides, the SOD activity of *Pseudana-beana* sp. was compared with comprehensively studied cyanobacteria *Spirulina platensis*, and *Synechococcus* sp. The specific SOD activities of cultured cyanobacteria were determined by xanthine/xanthine oxidase (X–XOD) method as potential for antioxidant mechanism. Protein concentrations were used for calculation of specific SOD activity.

As shown in figure 3, the highest SOD activity was obtained with the value of 50.4 U/mg from *Synechococcus nidulans* and the lowest SOD activity (18.4 U/mg) was obtained from *Pseudanabeana* sp. On the other hand, the highest protein content (0.43 mg/ml) was obtained from *Pseudanabeana* sp. The SOD activities of 50.4 U/mg and 30 U/mg, which correspond to the

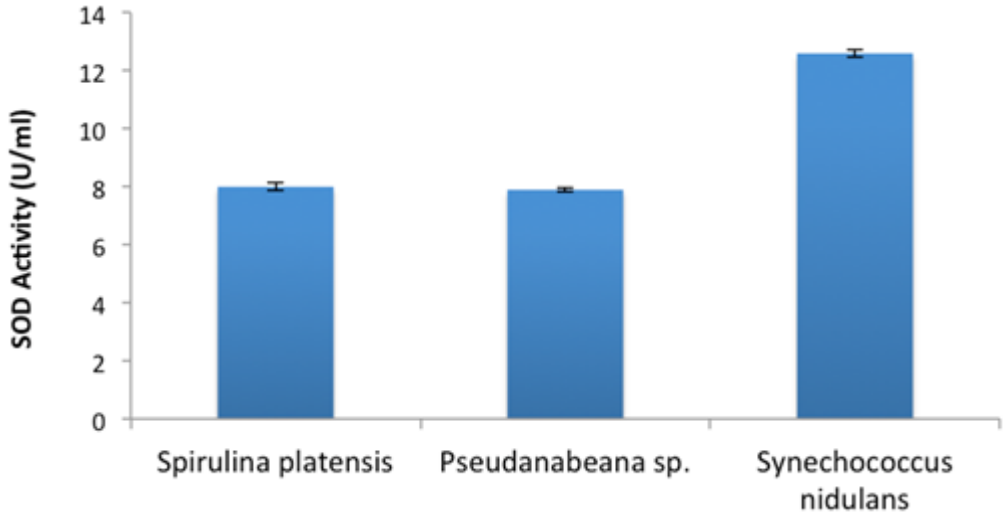

Figure 3. SOD activities of *Spirulina platensis, Pseudanabaena* sp., *Synechococcus nidulans*

percentages inhibition of 68.60 and 57.33, were obtained from *Synechococcus nidulans* and *Spirulina platensis*, respectively (Table 1). Though inhibition percentages and volumetric SOD activities of *Spirulina platensis* and *Pseudanabeana* sp. are close to each other, their specific SOD activities are different. The reason is that, these microorganisms have different protein amounts. On the other hand, the specific SOD activity of *Pseudanabeana* sp. is lower in accordance with the spesific SOD activity of *Spirulina platensis*. From the results obtained, there has been an inverse proportion between protein amount and spesific SOD activity when volumetric SOD activities and percentage inhibitions are similar. As a result of, these microorganisms can be ideal model systems for studying the regulation of oxidative stress in many organisms.

Several experiments have been carried out by different authors for different species in order to determine the SOD activity. For instance, the SOD activitiy was 42.5 IU/mg for *Phanerochaete chrysosporium* [14]. As reported by Al-Hamdani *et al*. [25], the percentage inhibition of *Spirulina maxima* extract was found 2.30 after the 7 days of cultivation period. For temperate phytoplankton cultures the inhibition was more variable: *Nannochloropsis* SOD inhibited 32 % at 1°C and 5 % at 30°C, whereas *Synechococcus* SOD was clearly more activated and inhibited 99 % of NBT-formazan at 1°C and 30 % at 30°C [26]. The photosynthetic cyanobacterium, *S. platensis*, is an ideal model organism to study environmental regulation of oxidative stress in both bacteria and plants [8].

It has been reported in several studies that SOD activity is higher in cold treated than warm treated plants [27-29]. Also, higher SOD activity has been reported in frost resistant species of certain genera than in frost-sensitive species of the same [30] and even in differently cold-adapted strains or cultivars within the same species [31,26].

The increased concentration of H_2O_2 was associated with decreased growth, chlorophyll concentration, and SOD activity. Hydrogen peroxide might cause membrane damage and thus death of the cells [32]. Earlier studies showed that high cell concentrations were needed for a reproducible SOD assessment, regardless of the assay used. Therefore, low cell densities may cause occuracy problems in SOD activity measurements. Thus, it is essential to optimize cell collecting and disruption procedures.

Conclusion

The results indicated the maximum specific SOD activity obtained was 50.4 U/mg from *Synechococcus nidulans* and the minimum specific SOD activity was 18.4 U/mg from *Pseudanabeana* sp. Total protein amounts of *Synechococcus nidulans, Spirulina platensis, Pseudanabeana* sp. were determined as 0.25 mg/ml, 0.27 mg/ml and 0.43 mg/ml respectively.

From the obtained results, there has been an inverse proportion between protein amount and specific SOD activity when volumetric SOD activities and percentage inhibitions are similar. It is clear that cell disruption and protein extraction procedures affect the SOD activity of cyanobacteria. Therefore it is crucial to optimize cell collecting and disrupting procedures. Optimization of protein extraction procedures from cyanobacteria should be carried out in further studies in order to observe the correlation between SOD activity and protein content.

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