Molecular characterization and expression analysis of iron superoxide dismutase gene from *Pseudochlorella pringsheimii* (Trebouxiophyceae, Chlorophyta)

Mostafa M. S. Ismaiel^{1,2} · Michele D. Piercey-Normore^{1,3}

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Abstract The FeSOD isoforms of *Pseudochlorella pringsheimii* were identified, a preliminary characterization of the enzyme was conducted, and the relationship among the FeSOD gene from *P. pringsheimii* and that of other organisms was examined. The FeSOD has an open reading frame of 612 bp that encodes 203 deduced amino acids with a molecular mass of 23 kDa. Expression of the recombinant FeSOD gene was done successfully in *Escherichia coli*. The purified FeSOD has a specific enzyme activity that reached 688 U mg⁻¹ protein (in vitro assay). Alkaline conditions showed the highest activity for the recombinant FeSOD. Moreover, it showed a relative thermostability up to 50 °C, while at 50 and 70 °C, the activity was reduced by 32 and 68%, respectively, after 1 h as compared to the maximum. Phylogenetic analysis revealed three main clusters i.e., the prokaryotic Cyanophyta, bacteria, and the eukaryotic Chlorophyta intermingled with plant species and a dinoflagellate. *P. pringsheimii* was closely grouped with *Chlorella pyrenoidosa*, however, other species showed a relative disparity. Alignment of FeSOD gene sequences of the different species showed

many conserved regions which could be used for FeSOD sequences among unexplored species and may be useful for the taxonomy of the revised coccoid *Chlorella* species.

Keywords Phylogeny · Reactive oxygen species (ROS) · SOD isoforms · Taxonomy · Thermostability

Introduction

Superoxide dismutases (SODs) are one of the antioxidant enzymes that essentially occur in aerobic organisms and are involved in in vivo strategies to remove the damage resulting from the reactive oxygen species (ROS). Hydroxyl (·OH) and superoxide radicals (·O₂⁻) are examples of the ROS which are generated normally as a result of aerobic-pathway metabolism (Fridovich, 1995). SODs are considered as the first cellular guard which dismutate the harmful superoxide radicals (·O₂⁻) to O₂ and H₂O₂. The toxicity of H₂O₂ is then diminished by another set of enzymes including catalases and peroxidases (Bannister et al. 1987).

SODs are divided into three isoforms according to their active metal-cofactors beside the evolutionary homology; Cu/ZnSOD, MnSOD and FeSOD (Bannister et al. 1987). Consequently, NiSOD and Fe/ZnSOD were revealed in *Streptomyces* spp. (Kim et al. 1996). The Cu/ZnSOD enzyme is found in many organisms including eukaryotes as fungi and plants; while MnSOD is present in bacteria, plants and animals (Salin and Bridges 1982; Wolfe-Simon et al. 2005). FeSOD mainly exist in prokaryotes (Puget and Michelson 1974; Scandalios 1993), cyanobacteria (Dos Santos et al. 2000; Shirkey et al. 2000; Li et al. 2002; Ismaiel et al. 2014), plants and eukaryotic algae (Sakurai et al. 1993; Alscher et al. 2002; Pokora et al. 2003;

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✉ Mostafa M. S. Ismaiel
mostafamsami@yahoo.com

¹ Department of Biological Sciences, Faculty of Science, University of Manitoba, Winnipeg, MB R3T 2N2, Canada

² Botany Department, Faculty of Science, Zagazig University, Zagazig 44519, Egypt

³ School of Science and the Environment, Memorial University of NL (Grenfell Campus), Corner Brook, NL A2H 5G4, Canada

Armbrust et al. 2004). The SOD isoforms could be differentiated on the basis of its sensitivity to some inhibitors, e.g. H_2O_2 and KCN. Both Cu/ZnSOD and FeSOD were inactivated by 5 mM H_2O_2 , yet Cu/ZnSOD was the only isoform affected by 3 mM KCN. Whereas MnSOD was not affected by any of these inhibitors (Rubio et al. 2001). Evolutionary, SOD had been found to be connected with two principal factors. Firstly, the development of oxygen in the atmosphere (hence to cope its potential harmful effect) and secondly, the availability of transition metals in the biosphere. Likely, the first SOD had iron metal in its active site as a result of iron availability in the biosphere at that time (Bannister et al. 1991). However, as the availability of soluble iron decreased in response to the increased oxygen level in the atmosphere, manganese was found to replace iron in the active site of the SOD. The saturation of atmosphere with oxygen led to the introduction of copper in the SOD (Bannister et al. 1991).

Chlorella is a well-known unicellular eukaryotic green algal genus and belongs to the phylum Chlorophyta. It is widespread in diverse habitats throughout the world, and it is known to be abundant in hard environments such as wastewater and high rate oxidation ponds (Mallick 2004). In the past, three ellipsoidal “*Chlorella*” species have been recognized, *C. saccharophila*, *C. pringsheimii*, and *C. ellipsoidea* (Shihira and Krauss 1965). Recently, the classification of the elliptical coccoid and spherical forms was reconsidered due to insufficient morphological differentiation. Physiological, molecular and biochemical investigations were performed to revise the taxonomy of the species belonging to “*Chlorella*” (Darienkov et al. 2010; Görs et al. 2010; Baytut et al. 2014; Heeg and Wolf 2015).

The objectives of the present work were (1) to identify the SOD isoforms, (2) to sequence the entire FeSOD gene from *P. pringsheimii* (Trebouxiophyceae, Chlorophyta), (3) to express and characterize the FeSOD enzyme using a bacterial host, and (4) to examine the relationship among FeSOD sequences from *P. pringsheimii* and other known SOD genes from other species.

Materials and methods

Algal strain and culture conditions

Pseudochlorella pringsheimii MIYA 102 was acquired from the Phycology Lab, Faculty of Science, Zagazig University, Egypt. Voucher specimen has been deposited in the algae division of the University of Manitoba herbarium (WIN), Winnipeg, Manitoba, Canada. The Bold's Basal medium (as described by Bischoff and Bold 1963) was used to support the algal growth. The algal cultures were maintained at 27 ± 0.5 °C, under cool and continued white

fluorescent lights ($85 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) with frequently shaken by hand. After the mid-log phase (10 days), the cells were harvested by centrifugation (at a speed of $6000 \times g$ for 10 min at 4 °C, Sorvall Legend X1R, Thermo Scientific) and cleansed twice with 10 mM $\text{Na}_2\text{-EDTA}$ solution and sterile distilled water.

Isolation and cloning of FeSOD

The total RNA of *P. pringsheimii* was extracted (Trizol[®] method, Invitrogen, USA), treated with DNase I (Invitrogen, USA), and verified by measurement of absorbance ratio (A260/280; Sambrook et al. 1989). Additionally, the RNA was tested by run on 1% agarose gel. The RevertAid[™] H Minus First Strand cDNA synthesis kit (Fermentas, USA) was used to reverse-transcribed the RNA to cDNA. A partial FeSOD cDNA (470 bp, Fig. S1) was isolated by PCR amplification based on the sequence of a related species (*Chlorella pyrenoidosa* strain 211-8b; acc. no. DQ183067). Briefly, a 20- μL PCR mixture consisted of $1 \times$ PCR buffer, 2 mM MgCl_2 , 200 μM of each dNTPs, FeSOD primers (0.4 μM of each FL3-F and CH-R, Table S1), 200 ng cDNA template and 1 U Taq DNA polymerase (Invitrogen). The thermocycler was adjusted to 94 °C for 4 min, followed by 35 cycles of 94 °C for 40 s, 57 °C for 50 s, and 72 °C for 50 s, and a final 1 cycle of 72 °C for 10 min. The amplified product (470 bp; Fig. S1) was gel-purified and then cloned into the pGEM plasmid (pGEM[®]-T Easy Vector System, Promega, USA) as per manufacturer's instruction, for the purpose of DNA sequencing. The inserted cDNA fragment was confirmed by PCR amplification (using specified primers, Table S1, Fig. S2) and agarose gel electrophoresis. The DNA sequence was verified via BigDye v. 3.1 (following the procedures recommended by Doering and Piercey-Normore 2009). The cDNA fragment was later used to isolate the full-length FeSOD using the 5'-RACE and 3'-RACE systems (First Choice RLM-RACE kit, Ambion, USA) as per manufacturer's instructions using specified primers (Table S1). Finally, the full-sequence of *P. pringsheimii* FeSOD gene was amplified in PCR-reaction mixture (20- μL as above) including Chl_SOD_F and Chl_SOD_R as primers (0.4 μM , designed to have the *Bam*HI and *Hind*III sites, respectively; Table S1). The thermocycler was adjusted as mentioned above with the exception of the annealing temperature (to be 58 °C).

Expression of FeSOD

The previous product (644 bp, Fig. 1a) was gel-purified, and cloned into the pET28c expression vector (Novagen, USA). The FeSOD nucleotide sequences in the expression vector were sequenced again for validation, and then the vector was

Fig. 1 **a** Agarose stained gel (1%) showing the amplified FeSOD RT-PCR product (644 bp) of *P. pringsheimii*. Marker, 1 Kb Plus DNA Ladder (0.7 µg, Invitrogen); lane 1, PCR amplification product of the open reading frame (ORF) cDNA of *P. pringsheimii* FeSOD using Chl_SOD_F & Chl_SOD_R; lane 2, negative control (no cDNA template). **b** lane 1, the purified FeSOD recombinant protein (15 µg/lane) by HiTrap™ ion exchange column filtration; M, molecular weight size markers (Invitrogen, kDa). Expression of recombinant *P. pringsheimii* FeSOD in *E. coli* BL21(DE3) pLysS analyzed by SDS-PAGE (12%) and stained with Coomassie® Brilliant Blue R-250

transformed into *E. coli* BL21(DE3)pLysS cells (Novagen, USA). After the *E. coli* cells reached a cell-density of $OD_{600} = 0.6$, the expression of the FeSOD protein was induced by the addition of 25 µM IPTG (isopropyl-β-D-thiogalactopyranoside), followed by incubation at 28 °C for 15 h. Purification of the expressed protein was done via affinity chromatography on a HiTrap HP column (GE Healthcare, USA), quantified by A_{280} and Bradford (1976) method (Biorad, USA) and the purity was validated by 12% SDS-PAGE (SDS–polyacrylamide gel electrophoresis; Laemmli 1970, Fig. 1b). All the procedures were done as recommended by the manufacturer. The manipulations of DNA were done as per the standard procedures (Sambrook et al. 1989). The nucleotide sequence of the FeSOD of *P. pringsheimii* was deposited at the NCBI GenBank database under accession number KX274326.

Characterization of the SOD

Analysis of the FeSOD activity (either of the native or the recombinant enzyme); SOD isoform inspection (sensitivity test); the optimum pH; pH and thermal stabilities of the recombinant FeSOD was done as described by Ismaiel

et al. (2014). Briefly, the sensitivity test of the SOD isoforms was done by incubating the samples in 5 mM H_2O_2 for 30 min to deactivate both of the Cu/ZnSOD and FeSOD, or in 3 mM KCN to deactivate Cu/ZnSOD only (if present). MnSOD is not affected by any of the mentioned treatments. The effect of pH on enzyme activity was performed by changing the assay buffer of the reaction mixture; while the pH stability was done via incubating the enzyme for 1 h after which its activity was measured.

Phylogenetic analysis

The 22 DNA sequences belonging to the FeSOD gene were aligned in MEGA 6.0 (Tamura et al. 2013) using ClustalW and the ambiguous ends of the sequences were removed from the alignment leaving 630 bp in the alignment. A Maximum Likelihood analysis was performed using the Tamura-Nei model, uniform rates among sites, the method of Nearest Neighbour Interchange heuristic, and gaps and missing data were removed from the analysis.

Statistical analysis

All measurements of the enzyme characterization were performed in set of three and the data were represented as mean $\pm$ SD (standard deviation). The data were analyzed statistically using SPSS 10.0 software (SPSS, Richmond, VA, USA). To discriminate between the significant values, we used one-way analysis of variance (ANOVA) beside Duncan's multiple range tests ($P < 0.05$).

Results

An initial investigation was done to specify the type of SOD isoforms in *P. pringsheimii* via sensitivity test. Four SOD activity bands were observed on a gel, which included three isoforms of SOD, Cu/ZnSOD, Mn and FeSOD (Fig. S3A). The first (faster-moving) and the third bands were inhibited by 3 mM cyanide and 5 mM H_2O_2 , and thus were designated as Cu/Zn-superoxide dismutases. The two other bands were cyanide-resistant (Fig. S3B), a characteristic criteria of FeSOD and MnSOD. Specifically, the upper (slower) band was not affected by 5 mM H_2O_2 (Fig. S3C), which indicates that they belong to MnSODs.

The isolated cDNA sequence of *P. pringsheimii* FeSOD gene consisted of 644 bp (Fig. 1A), beginning with a 15-bp long UTR (5'-untranslated region), and ORF region of 612 bp. The 3'-end of the cDNA has a second UTR with a length of 17 bp. The ORF encodes 203 deduced amino acids which form a molecular size of 23 kDa (Fig. 1b) and has a putative isoelectric point (pI) of 5.65.

The *P. pringsheimii* FeSOD was cloned and expressed successfully in *E. coli* BL21 (DE3). The purified FeSOD protein yield was found to be in active form (Fig. 1b). The purified FeSOD has a specific enzyme activity reached 688 U mg⁻¹ protein (in vitro assay). An additional sensitivity test using 5 mM H₂O₂ and 3 mM KCN was done to verify that the expressed protein was the only FeSOD isoform (Fig S4).

The direct pH assay (which reflects the optimum pH for the enzyme activity) showed that the pH of 7.0–8.0 produced the highest percent activity, which represented the best integrity of enzyme activity. However, the enzyme activity decreased by about 50 and 44% at both pH 5.0 and pH 9.0, respectively (Fig. 2). Similarly, the data for pH stability showed that the activity of the enzyme was maintained within the pH range of 6.0–8.0, outside which it was diminished significantly (Fig. 2). The enzyme was totally inactivated at pH 4.0 or below.

Thermostability of the recombinant enzyme was valid up to 50 °C. The activity was lowered by 32% at 50 °C and by 68% at 70 °C, after 1 h, as compared to the maximum. However, the activity was sharply reduced by 91% at 90 °C and completely inactivated at 100 °C, after 20 min (Fig. 3).

Sequence alignment of *P. pringsheimii* FeSOD with 32 additional SOD genes from different species showed that there are several highly conserved domains, specifically two conserved regions, (TQFGSGWAWL) and (DVWEHAYYLD) at the 123th and 172th amino acid of the aligned SOD genes, respectively (Fig. S5). The NCBI identity of the related amino acid FeSOD sequences is shown (Table S2). The amino acid deduced sequence shares the highest similarity with a FeSOD from *Chlorella*

Fig. 2 Effect of pH on activity and stability of the recombinant *P. pringsheimii* FeSOD. The relative activity (%) was calculated referring to the activity at the standard conditions (mean $\pm$ SD, $n = 3$). For each parameter, the different letters represent significant differences at $P < 0.05$ (Duncan's multiple range test)

Fig. 3 Thermostability of the recombinant FeSOD of *P. pringsheimii*. Data are mean $\pm$ SD, $n = 3$

pyrenoidosa, by NCBI BLAST search, followed by *Chlamydomonas reinhardtii* and *Gonium pectoral*.

The phylogenetic analysis (based on the FeSOD sequences) revealed two main clusters, A and B (supported by 56 and 67% bootstrap values, respectively). The FeSODs of bacteria and Cyanophyta are closely related, whereas that of the eukaryotic plant species assembled with those of Chlorophyta and basal to both clusters (Fig. 4). The dinoflagellate, *Gonyaulax polyedra*, was unresolved and fell outside the two clusters. Meanwhile, *P. pringsheimii* was closely grouped with *C. pyrenoidosa* with 100% bootstrap support (cluster A-2); however, other related species (e.g. *C. pyrenoidosa* strain 820 and other chlorophytes) showed a relative disparity forming separate clusters within cluster A.

Discussion

Superoxide radicals are known to be a by-product of aerobic metabolism, which have to be controlled so as to alleviate the possible damage imposed on the cells. The significance of SOD is related to its capability to protect the cells from the ROS by breaking of O_2^- radicals to give a moderately toxic molecule, i.e. hydrogen peroxide beside oxygen molecule (Fridovich 1995). Surprisingly, our data showed that *P. pringsheimii* has three SOD isoforms, which are two Cu/ZnSODs, one MnSOD, and one FeSOD (Fig. S3). Eukaryotic algae are known to have either FeSOD or MnSOD or both isoforms (Grace 1990; Wang et al. 2011). The Cu/ZnSOD gene was undetected in the chlorophyte green alga *Chlamydomonas reinhardtii* which contains two SOD isozymes, one FeSOD and at least two of the MnSOD isoforms (Sakurai et al. 1993; Kitayama et al. 1999). Cu/ZnSOD was also absent from both the red alga *Cyanidioschyzon merolae* (Matsuzaki et al. 2004), and the bacillariophyte *Thalassiosira pseudonana* (Armbrust

Fig. 4 Phylogenetic tree for FeSOD nucleotide sequences. The tree is midpoint rooted and bootstrap values > 50% are shown above the branches points. The accession numbers were followed by species name for the related sequences in the NCBI GenBank database

et al. 2004). The former has three MnSOD isoforms while the latter has two FeSODs, and two MnSODs isoforms.

Plants generally have mitochondrial MnSOD together with cytosolic Cu/ZnSOD isoforms, whereas FeSOD and/or another Cu/ZnSOD isoform may be present in their chloroplasts' stroma (Bowler et al. 1992). For instance, the rice plant has three isoforms of SODs including 2 Cu/ZnSODs, 1 MnSOD, and 1 FeSOD (Kanematsu and Asada 1990; Sakamoto et al. 1993; Kaminaka et al. 1999). Moreover, *Arabidopsis* has 7 SODs that includes 3 Cu/ZnSODs, 3 FeSODs, and 1 MnSOD isoforms (Kliebenstein et al. 1998). Thus, the evolutionary origin and transfer of chloroplastic SOD between plants and algae needs further investigation.

The recombinant FeSOD isoform of *P. pringsheimii* exhibited a molecular mass of about 23 kDa (Fig. 1b), which fits within one of the two groups identified by Alscher et al. (2002). Previously, the FeSODs were separated into two groups; the first has the homodimeric isoforms of two identical 20 kDa subunit proteins; and the second has the tetrameric isoforms, composed of four proteins, each of which is 80–90 kDa subunit. Both groups were found in prokaryotes and plant species (Alscher et al. 2002). Mostly, Cyanobacteria have FeSOD with varying molecular weight of 23 kDa (*Anabaena* sp. and *Arthrospira platensis*) to 41 kDa as recorded in *Plectonema boryanum* (Asada et al. 1975; Li et al. 2002; Ismaiel et al. 2014). For plants, the FeSOD mass range may extend from 40 to 47 kDa. It is usually composed of a homodimer of non-covalently two combined sub-units (Sandalio and del Río 1987; Almansa et al. 1991). However, exception has

been recorded for the *Citrus limonum* FeSOD which has a sole 22.4 kDa FeSOD protein (Almansa et al. 1991). Steinman (1982) reported previously that both Mn- and FeSODs were typically homodimeric or tetrameric proteins of 14 to 30 kDa.

The activity of SOD was mostly favoured by the alkaline conditions (Fig. 2). Previously, the optimum catalytic rates of Fe- and MnSOD were recognized in the pH range 6.0–8.0 in bacterial species like *E. coli* and *Photobacterium leiognathi* (Grace 1990 and references within). The garlic SOD was consistent at pH values of 4.0 to 11.0 (He et al. 2008), while activity of the recombinant Cu/ZnSOD of *Ipomoea batatas* (sweet potato) was stable in alkaline pH 11.2, with a thermostability at 85 °C for 20 min (Lin et al. 1995). Similarly, the FeSOD activity of *P. pringsheimii* in this study was most active between pH 6.0–8.0 and its activity diminished at low pH levels (Fig. 2). One explanation for a decrease in activity at low pH levels was proposed by Lin et al. (1995), who attributed the reduction of the activity of sweet potato Cu/ZnSOD at the low pH levels to the breakage and disassembly of SOD dimers into the lower active subunits.

To date, different SOD isoforms have been reported to resist thermal inactivation (Bueno et al. 1995; Lin et al. 1995; Miszalski, et al. 1998; Ismaiel et al. 2014) including *P. pringsheimii* in this study (Fig. 3). The recombinant FeSOD of *A. platensis* retained 45% of its activity after exposure to 90 °C for 30 min (Ismaiel et al. 2014). Moreover, many of the known archaeal Fe- and MnSODs are extremely thermostable (Bafana et al. 2011). The recombinant FeSOD from the thermophilic bacteria

Rhodothermus sp. XMH10 (RhSOD) was kept stable under wide temperature range of 40–70 °C. Moreover, its activity was maintained at about 40 and 10% of original value upon incubation for 30 min at 80 and 90 °C respectively (Wang et al. 2008). In plants, the Cu/ZnSOD activity of watermelon cotyledons was reduced by 50% after exposure to 70 and 100 °C in 15 and 3 min, respectively (Bueno et al. 1995). The recombinant Cu/ZnSOD activity of sweet potato (which showed two active forms; a monomer and dimer) revealed that the thermal inactivation rate (0.029 min^{-1}) was constant at 85 °C for the monomer. Its inactivation half-life time was about 28 min, while the dimer activity remained the same even after 40 min at 85 °C (Lin et al. 1995). MnSOD was the most durable isoform of the halophyte, *Mesembryanthemum crystallinum*, which remained active after heating to 90 °C for 1 h, followed by the FeSOD (at 70 °C), and Cu/ZnSOD (at 60 °C) for the same time (Miszalski et al. 1998). The protein thermostability may be related to the increased number of charged and hydrophobic amino acid residues, ion-pairs, and buried surface area (Wang et al. 2008).

The pH and high thermal stability reported here emphasizes the dependence of algal cells on SODs as protectants against free radicals under extreme conditions which may raise evolutionary hypotheses. These superior advantages of the SOD also suggest a versatile application in nutraceutical, therapeutic and healthcare industries (Campana et al. 2004; Sarsour et al. 2010).

The divergence of the FeSOD may suggest different evolutionary phenomenon for prokaryotes and eukaryotes. One explanation may rely on the hypothesis that chloroplasts were developed from endosymbiotic cyanobacteria, subsequently; FeSOD transferred during a later stage of evolution, to the nuclear genome (Grace 1990; Van Camp et al. 1990). Finally, FeSOD moved to higher plants (Bridges and Salin 1981; Salin and Bridges 1982). In this study, the similarity between the FeSOD of *P. pringsheimii* and those of other Chlorophyta and Plantae (Fig. 4) suggests a closer correlation with plants rather than with Cyanophyta and bacteria. In addition, the SOD of the dinoflagellate showed a closer relationship with that of the proteobacteria as cited previously (Wolfe-Simon et al. 2005).

Pseudochlorella pringsheimii was shown to have both MnSOD and Cu/ZnSODs (Fig. S3). It was reported that Archaea tend to mainly have FeSODs, whereas bacteria and eukaryotes may have MnSODs in addition to FeSODs. A previous correlation was reported between the presence of MnSOD in organisms, which may also contain FeSOD and/or Cu/ZnSOD (Wolfe-Simon et al. 2005). The primary and tertiary structures of Mn and FeSODs are very similar and may be considered to be more ancient SOD types with a common ancestral origin (Asada et al. 1980; Stallings

et al. 1984; Bannister et al. 1991; Miller 2012), whereas Cu/ZnSOD show different structural features and may have diverged more recently (Asada et al. 1980; Miller 2012).

The polyphyletic nature of the genus “*Chlorella*” (Kessler 1976) may also account for the separation of the FeSOD genes among *Chlorella* species in the phylogeny. Several attempts have been made to distinguish among these species. Baytut et al. (2014) and Hirooka et al. (2014) showed that the type strain *P. pringsheimii* was separated from the closely related *C. vulgaris* and *C. sorokiniana* strains depending on the sequences of 18S rDNA and chloroplast 16S rDNA regions. However, other species await phylogenetic separation until new taxonomic markers can distinguish their classification. In this study, our data showed the high relativeness between *P. pringsheimii* and *C. pyrenoidosa* (based on the FeSOD sequence) rather than other *Chlorella* species such as *C. variabilis*, *C. pyrenoidosa* strain 820 and *Auxenochlorella protothecoides* (Fig. 4).

Additional studies are needed on the occurrence, sequence and type of SOD isoforms of “*Chlorella*” species and other coccoid green species, which may reveal the evolution of FeSOD gene among the polyphyletic *Chlorella* and it could contribute to a better understanding of the taxonomic relationships within *Chlorella*.

In conclusion, the FeSOD gene sequence of the eukaryotic *P. pringsheimii* was revealed which provided an opportunity to examine the phylogenetic position of *P. pringsheimii* among other *Chlorella* species as well as the broader relationship with plants. Phylogenetic analysis also confirms the disparity between the FeSOD of Cyanophyta and Chlorophyta. The cloning and functional expression of FeSOD from *P. pringsheimii* showed activity at a high pH range and thermal stability, which suggests the potential for its production for several industrial fields.

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