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Cyanobacterial extracellular alkaline phosphatase: detection and ecological function*

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Abstract Dissolved inorganic phosphorus is an important form of directly bioavailable phosphorus for cyanobacteria in natural water. Dissolved organic phosphorus could be used by cyanobacteria via alkaline phosphatase, which is produced mainly by bacteria and also cyanobacteria itself. Herein, we review the current knowledge of extracellular phosphatase excreted by cyanobacteria, highlighting the development of detection method and its ecological roles in regulating phosphorus cycling in freshwater systems, which is based on reports for around 100 species of cyanobacteria. Recommendations are suggested concerning the extracellular phosphatase produced by bloom-forming cyanobacteria in terms of the ecological role, followed by a discussion of the future prospects for the study.

Keyword: alkaline phosphatase; phosphate; dissolved organic phosphorus; cyanobacteria; ecological role

1 INTRODUCTION

Harmful cyanobacterial blooms in lakes are serious aquatic environmental problems. Phosphorus (P) is one of the limiting elements of phytoplankton growth in natural waters (Schindler, 1977; White et al., 2003). Dissolved inorganic P (DIP) is the preferred form of directly bioavailable P for phytoplankton (Cotner and Wetzel, 1992; Björkman and Karl, 1994; Baken et al., 2014). Dissolved organic P (DOP) maintains the supply of DIP via alkaline phosphatase catalyzing (Berman, 1970; Cao et al., 2018; Lim et al., 2018; Feng et al., 2020). Alkaline phosphatase (APase, EC 3.1.3.1) is a type of organophosphate hydrolase, which could catalyze the hydrolysis of phosphate ester bonds containing C-O-P ester bond to release orthophosphate (Pi) (Hoppe, 2003). The substrates of APases include phosphate glycosides, phosphomonoesters, and phosphate diesters, etc. (Bentzen et al., 1992; Yamaguchi et al., 2005), which contributed over 75% of DOP in the water (Kolowith et al., 2001; Young and Ingall, 2010; Yates et al., 2019). In addition to phototrophic and heterotrophic prokaryotes, protozoa and zooplankton, phytoplankton was found to be able to produce extracellular APases (Hoppe, 2003). Despite there have been numerous studies on the eco-physiological responses to P and increasing researches on genomics in bloom-forming algae in the past decades, few attempts to synthesize information in terms of extracellular APases produced by cyanobacteria were reported. Generally, extracellular enzymes are principally located outside the cell membrane and no longer in contact with their producers, while ecto-enzymes were defined as the enzymes located outside the cellular outer membrane, but still in contact with the cell (Chróst, 1991). Here, we consider that extracellular phosphatase includes extracellular (or free) and ecto-phosphatase (Fig.1). Previously, Jansson et al. (1988) and Hoppe (2003) have systematically reviewed the origin, characteristics, and function of phosphatases in lake and ocean. Physiological ecology and molecular mechanisms responding to P status of ecto-APases were subsequently summarized in marine typical bloom-forming eukaryotic algae (Dyhrman, 2005; Lin et al., 2016). In this paper, we give a brief review of the determination method development

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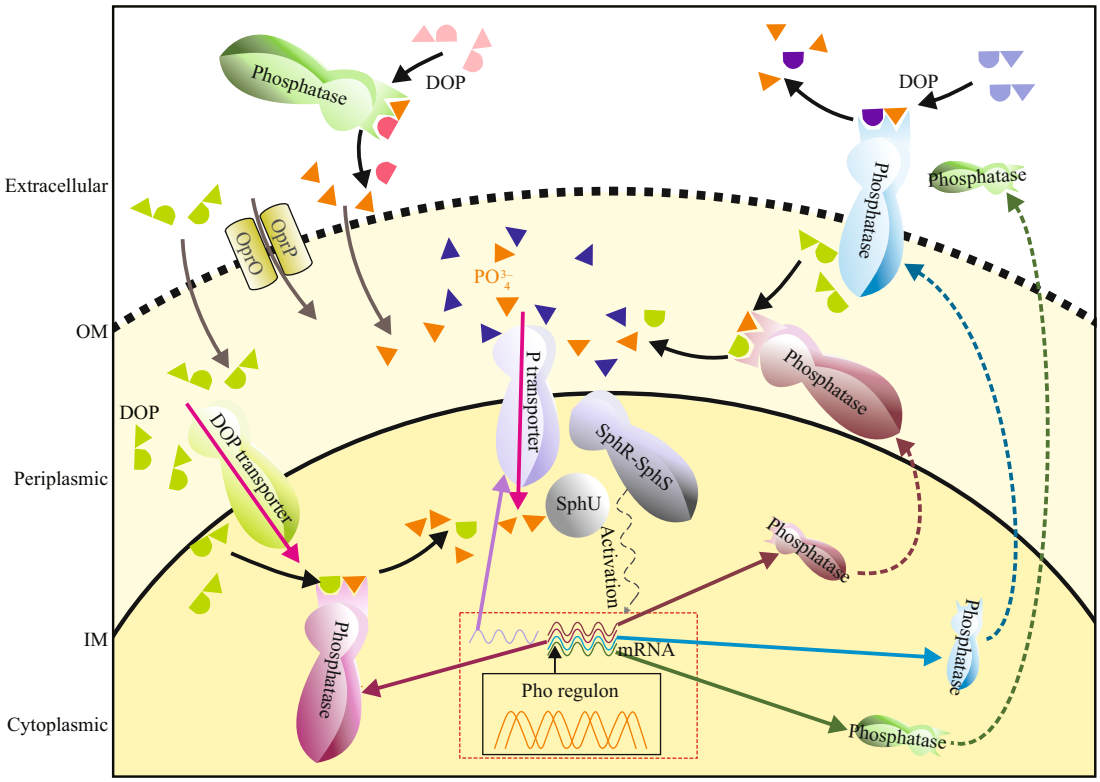

Fig.1 Subcellular localizations of alkaline phosphatase and its regulation by Pho regulons in cyanobacteria

This diagram represents a composite of known cyanobacterial organic phosphate esters acquisition and hydrolysis pathways, which are likely to be present in some cyanobacterial species or bacteria. Grey arrows indicate possible pathways by which inorganic and organic P is transported into periplasm through a phosphate-selective porin OprO-P, which has been identified in bacteria (Rao and Torriani, 1990; Modi et al., 2013). Magenta arrows indicate the transmembrane transport of inorganic and organic P, and these sites of transmembrane transporters reference to Rao and Torriani (1990) and Luo et al. (2009). Dashed arrows (brown, blue, and green) indicate secretion systems of alkaline phosphatases. Subcellular locations of alkaline phosphatases of cyanobacteria reference to Luo et al. (2009). Two component systems regulate the organophosphate metabolism, which is made up with the sensor kinase (SphR) and the reponse regulator (SphS). The specific activation process is: P-limitation conditions imply the unsaturated P transporter, which will prompt the detachment (or conformational changes) of a negative regulator (SphU). Then, Pho regulons will be activated by the phosphorylation of SphS (Juntarajumnong et al., 2007; Su et al., 2007; Hsieh and Wanner, 2010; Tiwari et al., 2015). IM: inner membrane; OM: outer membrane.

in cyanobacterial extracellular phosphatase and its ecological function both in fresh and marine water.

2 DETECTION OF EXTRACELLULAR PHOSPHATASE FROM CYANOBACTERIA

Since Steiner (1938) first discovered that DOP could be enzymatically hydrolyzed in lakes, the research on extracellular phosphatases in water bodies have been a rapid development (Berman, 1970; Zhou and Zhou, 1997; Luo et al., 2009; Zhang et al., 2016; Wang et al., 2021). Tracing the origin or producer of extracellular phosphatase is of great ecological significance. The accuracy mainly depends on the advances in the analytical methods, while extracellular phosphatase from cyanobacteria could be detected by axenic culture and enzyme purification. In-situ detection by enzyme labelled fluoresce (ELF) technology and molecular biological methods could give more solid evidence for the origin of extracellular phosphatase.

2.1 Extracellular phosphatase activity analysis from cyanobacteria

Cyanobacteria can produce APases attached to the cellular outer membrane or dissolved in the medium (Healey and Hendzel, 1979a, b; Smith and Kalff, 1981; Vaitomaa et al., 2002; Luo et al., 2009). Measurements of APase activity (APA) were effective means to quantitatively characterize APases in the field investigations and indoor experiments (Lehman et al., 2013; Yuan et al., 2016; Wan et al., 2019; Zhou et al., 2021). Determination of APA in the lab experiment can further prove the relationship between axenic cultured cyanobacteria and the origins of phosphatases to a certain degree, such as the contribution of cyanobacteria to soluble extracellular APases. For example, in the *Anacystis nidulans* and *Synechococcus* culture medium, APA was detected using para-nitro-pheneye phosphate (pNPP) as substrates (Ihlenfeldt and Gibson, 1975). APA detected using pNPP as substrates were related

to cyanobacterial colonies (*Rivularia*) in the British rivers (Livingstone and Whitton, 1984). The same method was later used to detect APA from *Nostoc linckia*, *Nostoc muscorum*, and *Trichodesmium* in the field survey and lab culture (Kumar et al., 1992; Stihl et al., 2001). Besides pNPP, other organic P compound could also be used as substrates. For example, bis-p-nitrophenyl phosphate (bis-pNPP) was used to determine cell membrane attached and dissolved APA in *Nostoc commune* UTEX 584 culture (Whitton et al., 1990). Later on, 50 species of cyanobacteria were found to secrete extracellular APases using the same assay protocal (Whitton et al., 1991). Since the new century, 4-methylumbelliferyl phosphate (MUFP) has been used as substrate to determine APA in the field survey and lab cyanobacterial culture (Vaitomaa et al., 2002). pNPP and MUFP methods mainly focused on the liberation of Pi hydrolyzed by phosphomonoesterase, while less attentions were paid to phosphodiesterase (Sirová et al., 2013), which is a non-negligible enzyme involved in the organophosphorus hydrolysis pathway (Accoroni et al., 2017; Srivastava et al., 2021). Therefore, bis-pNPP or bis-(4-methylumbelliferyl) phosphate (bis-MUFP) was still suggested to access the phosphodiesterase activity and thus indicate the sources of organic P (Sirová et al., 2013). The accuracy of APA was also constrained by several factors, e.g. assay substrate, incubation time, pH, and sample sources, etc. (Hernández and Whitton, 1996). The peak month of APA of *Rivularia* colonies was observed to be different by using pNPP and MUFP as the sole substrate in intertidal pools at Tyne Sands, Scotland (Yelloly and Whitton, 1996). The comparison experimental results showed that the maximum release of the product (p-nitrophenol) appeared in over 20 min and at slightly acid pH, while the maximum 4-MUF appeared in more than 40 min and at neutral pH (Hernández and Whitton, 1996). Cyanobacterial blooms always occurred in alkalescent water bodies, and higher growth rates of cyanobacteria were also observed in the alkalescent culture mediums (Hong and Lee, 2008; de Souza Santos et al., 2011; Fang et al., 2018; Wei et al., 2022). Therefore, the MUFP method may be more suitable for the detection of APA from cyanobacteria.

Also of note is that, the sources of APases cannot be distinguished by pNPP and MUFP methods both in field investigations and indoor experiments, indicating that the contribution of APA from algae will be overestimated if the contribution of bacteria cannot be ruled out. Reducing the growth of bacteria by adding antibiotic or using UV light is an effective way to exclude its interference to algal APA in the cultivations (Gerloff et al., 1950; Harke et al., 2012; Li et al., 2015b). Purification of APases could relate to the APA and its producers partially (Martland and Robison, 1929; McComb et al., 1979). Jansson (1976) separated and purified the phosphatase in the cell and culture medium. Dissolved extracellular phosphatase in water might be derived from a zooplankton, *Bosmina obtusirostris*, by comparing the physical and chemical characteristics of the enzymes. Phosphatase isolated and purified from cyanobacteria (*Anacystis nidulans*) had different characteristics from other phosphatases described previously (Ihlenfeldt and Gibson, 1975). Doonan and Jensen (1980) extracted attached and free APases from eighteen cyanobacteria and demonstrated the inducibility of eleven of them. APases, purified from cyanobacteria (*Arthrospira platensi*) using Triton X-114, was proven to be a calcium-dependent protein (Asencio et al., 2012). The purification method has not been widely used in the field surveys of phytoplankton ecology or environmental science, since it is complex and inefficient to distinguish between intracellular and extracellular phosphatases attached to the cell (Malherbe et al., 2019). Size fraction measurement of APA was a rough way to evaluate the contributor of particle (including cyanobacteria) APases (Dyhrman and Ruttenberg, 2006; Lim et al., 2018). If the large particle size contributed most to the total APA, extracellular APases were assumed being mainly contributed by the dominant or cultured cyanobacteria (Raoui et al., 2002; Wan et al., 2019).

2.2 Visualization of extracellular phosphatase

Enzyme labelled fluorescence (ELF) method provided an artificial substrate to visually detect extracellular APases in cyanobacteria or algae. The basic principle of this method is as follows: ELF™97 phosphate (ELFP) is a specific substrate of phosphatase, which could be hydrolyzed by APase into Pi and fluorescent organic residues (ELFA). The insolubility of organic residues makes the precipitate attach to the enzymatic reaction, so the fluorescence (or confocal) microscope or flow cytometer can not only determine the presence of extracellular phosphatase of cyanobacteria or algae, but also mark the occurrence site (Dyhrman and Palenik, 1999). Extracellular phosphatase was firstly determined by ELF method in the culture of marine algae (González-Gil et al., 1998).

ELF method was introduced and developed to detect algal and cyanobacterial extracellular phosphatase in Czech reservoirs by Štrojsová et al. (2003, 2005). Nedoma et al. (2007) further proved that the ELF assay could ensure a high saturation of extracellular phosphatases in the marine (>99%) and fresh (>90%) waters. Since then, this method has been widely used in the monitor of extracellular phosphatase of the marine and freshwater cyanobacteria, which has led the utilization of fluorescence microscopy into the visualization of APases in the cyanobacterial cells (Table 1). Significantly, not all tested cyanobacteria displayed fluorescent precipitates of ELFA (Table 1, Dignum et al., 2004b; Rychtecký et al., 2015; Wan et al., 2019). Possible reasons include: firstly, the evoked threshold concentration of P is variable for initiating the secretion of APases by different cyanobacteria. The same P concentration or environmental condition might not trigger the secretion of APases for some cyanobacteria (Nausch, 1998; Dyhrman and Palenik, 1999; Sebastián et al., 2004; Cao et al., 2007; Girault et al., 2013; Zhang et al., 2021). Secondly, some cyanobacteria may secrete dissolved APases, which cannot trace back to the producers by ELF assay (Wang et al., 2021). Overall, there were still some limitations in analyzing cyanobacterial extracellular APA: firstly, absolute axenic conditions were difficult to achieve, as a result, the contribution of bacteria to enzyme activity cannot be ruled out. Secondly, the physiological, biochemical, and external conditions of cyanobacteria grown were not consistent. Therefore, the chemical measurements of APA have many objective disadvantages, and multiple methods must be taken into consideration to comprehensively evaluate the cyanobacterial APases.

2.3 Application of molecular biological methods

Development of molecular biological methods provided deep insights into the origin of extracellular phosphatase, if combined with the quantification and ELF technique. The dominant molecular model, used to describe transformations and assimilations of P, has been the Pho regulon of the gram-negative bacterium *Escherichia coli* (Vershinina and Znamenskaya, 2002). So far, the most important APases in prokaryotes include PhoA, PhoD, PhoV, PhoX, and atypical phosphatases (Luo et al., 2009; Kageyama et al., 2011; Lin et al., 2016). The general elements of the Pho regulon in cyanobacteria and its regulatory mechanisms might be similar to heterotrophic bacteria (Fig.1). The full-length of APase genes were sequenced and expressions of these genes were quantified under different environment in the past two decades. The corresponding complementary DNA and RNA sequencing was coupled to a comprehensive metabolomics survey afterwards. Gene sequence analysis has revealed that *phoD* encodes APase in *Anabaena* (Singh et al., 2015). In addition, the genes encoding APases in *Anabaena* (later named *Dolichospermum*) include *phoA*, *phoD*, and *phoS* (Liu and Wu, 2012). *phoA* and *phoX* have been also reported in pico-cyanobacteria or cyanobacteria (Vershinina and Znamenskaya, 2002; Su et al., 2003; Moore et al., 2005; Sebastian and Ammerman, 2009; Tetu et al., 2009; Kathuria and Martiny, 2011; Harke et al., 2012). Atypical APase genes like other cyanobacterial *phoA* and a classical *phoA* gene are predicted in the genome of *Anabaena* sp. PCC 7120 (Luo et al., 2010).

The development of protein sequencing technology has further revealed the structures and functions of cyanobacterial APases. Ray et al. (1991) found an atypical APase in *Synechococcus* sp. PCC7942, whose size (145 kDa) is larger than the previously reported one (47–87 kDa). The PhoA-type APase has a Zn^{2+} cofactor, while PhoX associated with uncultured *Prochlorococcus* is an active phosphatase with a Ca^{2+} cofactor (Kathuria and Martiny, 2011). PhoX is a monomeric enzyme activated by Ca^{2+} and Fe^{3+} (Majumdar et al., 2005; Monds et al., 2006; Yong et al., 2014) with a lower substrate specificity for C-O-P bonds, such as nucleotides, phosphorylated carbohydrates, and amino acids (Zaheer et al., 2009). PhoX in *Microcystis aeruginosa* FACHB7806 is strongly activated by Mg^{2+}, followed by other divalent ions (like Co^{2+}, Ca^{2+}, Zn^{2+}, and Mn^{2+}), but it is inhibited by Ni^{2+} (Hong et al., 2021). Proteome results suggest that PhoA (Mg^{2+} and Zn^{2+}) and PhoX (Ca^{2+}) has been expressed in *Synechococcus* sp. WH 8102 (Cox and Saito, 2013). PhoD from *Aphanothece halophytica* is a hydrolase activated by Ca^{2+} that can hydrolyze phosphomonoesters and phosphodiesters (Kageyama et al., 2011). PhoD from a unicellular N_2-fixing cyanobacteria (*Halothece* sp. PCC 7418) shows the connection between Ca^{2+} and Fe^{3+}, which harbors eight copies of APase encoding genes (Fernández-Juárez et al., 2019). PhoV is a hydrolase activated by Zn^{2+}, which can hydrolyze phosphate monoesters and has a wide range of adaptation to pH, but it is inhibited by Mn^{2+} (Wagner et al., 1995).

The detection of APase encoding genes and their expression levels enable the interpretation of

Table 1 Extracellular phosphatases of cyanobacteria evidenced by ELF method

Genus/species	ELF	Field or indoor	Reference
Anabaena planctonica	+	Římov Reservoir	Štrojsová et al., 2005
Anabeana sp.	–	Lake Niushan, Lake Yuehu	Cao et al., 2010
Aphanizomenon flos-aquae	+	Indoor	Chen et al., 2020
Aphanizomenon flos-aquae	+	Římov Reservoir	Štrojsová et al., 2005
Aphanizomenon flos-aquae	+	Lake Loosdrecht	Dignum et al., 2004b
Aphanizomenon flos-aquae	–	Lipno Reservoir	Rychtecký et al., 2015
Aphanizomenon flos-aquae	+	Indoor	Rychtecký et al., 2015
Aphanizomenon ovalisporum	–	Lake Kinneret	Bar-Yosef et al., 2010
Aphanizomenon yezoensis	–	Římov Reservoir	Štrojsová et al., 2005
Aphanothece minutissima	–	Římov Reservoir	Štrojsová et al., 2005
Chroococcus minutus	–	Římov Reservoir	Štrojsová et al., 2005
Cuspidothrix issatschenkoi	+	Lipno Reservoir	Rychtecký et al., 2015
Cyanobacterial mat	+	Northern Belize	Sirová et al., 2006
Cylindrospermopsis raciborskii	+	Indoor	Bai et al., 2014
Dolichospermum cf. *flos-aque*	–	Lipno Reservoir	Rychtecký et al., 2015
Dolichospermum cf. *flos-aque*	+	Indoor	Rychtecký et al., 2015
Dolichospermum circinale	+	Lipno Reservoir and indoor	Rychtecký et al., 2015
Dolichospermum crassum	+	Lipno Reservoir and indoor	Rychtecký et al., 2015
Dolichospermum flos-aquaea	+	Lakes Chaohu and Taihu and indoor	Wan et al., 2019
Dolichospermum lemmermannii	+	Lipno Reservoir	Rychtecký et al., 2015
Dolichospermum lemmermannii	–	Indoor	Rychtecký et al., 2015
Dolichospermum spiroides	+	Lipno Reservoir	Rychtecký et al., 2015
Limnothrix sp.	+	Lake Loosdrecht	Dignum et al., 2004a
Limnothrix sp. isolate MR1	+	Indoor	Dignum et al., 2004b
Merismopedia punctata	–	Lipno reservoir	Rychtecký et al., 2015
Merismopedia punctata Mey.	–	Lake Donghu	Cao et al., 2005
Merismopedia sp.	–	Lake Niushan, Lake Yuehu	Cao et al., 2010
Merismopedia sp.	–	Římov Reservoir	Štrojsová et al., 2005
Microcystis aeruginosa	+	Římov Reservoir	Štrojsová et al., 2005
Microcystis aeruginosa Kütz.	–	Lake Donghu	Cao et al., 2005
Microcystis cf. *aeruginosa*	–	Lake Niushan, Lake Yuehu	Cao et al., 2010
Microcystis cf. *aeruginosa*	–	Lipno Reservoir and indoor	Rychtecký et al., 2015
Microcystis ichthyoblabe	–	Lipno Reservoir and indoor	Rychtecký et al., 2015
Microcystis sp.	+	Římov Reservoir	Štrojsová et al., 2005
Microcystis spp.	–	Lakes Chaohu and Taihu and indoor	Wan et al., 2019
Phormidium sp.	–	Lake Donghu	Cao et al.,2005
Phormidium sp.	–	Lake Niushan	Cao et al., 2010
Planktothrix agardhii	+	Indoor	Dignum et al., 2004b
Prochlorothrix hollandica	+	Lake Loosdrecht	Dignum et al., 2004b
Prochlorothrix hollandica PCC 9006	–	Indoor	Dignum et al., 2004b
Pseudanabaena cf. *mucicola*	–	Lipno Reservoir and indoor	Rychtecký et al., 2015
Pseudanabaena mucicola	+	Římov Reservoir	Štrojsová et al., 2005
Snowella lacustris	–	Římov Reservoir	Štrojsová et al., 2005
Synechococcus bacillaris	–	Indoor	Meseck et al., 2009
Synechococcus elongatus PCC 7942	+	Indoor	Dignum et al., 2004b
Trichodesmium	+	North Pacific; west Pacific warm pool; western North Atlantic	Hynes et al., 2009
Trichodesmium erythraeum	+	Taiwan Strait	Ou et al., 2006
Trichodesmium spp.	+	Western subtropical North Pacific	Girault et al., 2013
Woronichinia naegeliana	+	Římov Reservoir	Štrojsová et al., 2005
Woronichinia naegeliana	+	Lipno Reservoir	Rychtecký et al., 2015

–: species are not ELFA-labelled in the study; +: species are ELFA-labelled in the study.

APA and visualization of APases more reasonable. For example, the increased expression of putative APase gene (*phoX*) in *M. aeruginosa* under low P supply evidenced that the extracellular phosphatases originated from *M. aeruginosa*, together with a significantly positive correlation between P concentrations and APA in a monocultural experiment (Harke et al., 2012). Similarly, up-regulated *phoX* gene of the predominant *Microcystis* was observed in low-P regions of Lake Erie (Harke et al., 2016). In respond to the decreasing P concentrations, bloom-forming *Hydrocoleum* sp. elevated the expression level of *phoA* gene (Moisander et al., 2022). The changes in the expression levels of *phoA*-like and *phoD* genes well explained the increase of APA of *Raphidiopsis mediterranea* and *Planktothrix agardhii* in the P-starvation conditions (Aguilera et al., 2019). Increasing the expression level of cyanobacterial APase genes in response to low P supply is species specific in terms of the differences in genes copy numbers and gene types (Harke et al., 2012; Liu and Wu, 2012; Lin et al., 2018; Aguilera et al., 2019; Willis et al., 2019). For example, *phoX* gene was not identified in *Microcystis weisenbergii* but in *M. aeruginosa* (Harke et al., 2012). *PhoA*, *phoD*, and *phoX* genes were identified in *Anabaena cylindrica* FACHB-170 (Lin et al., 2018). Even though *Raphidiopsis raciborskii* CS-505 and CS-506 harbored the same gene type, the copy numbers of their *phoA* gene were also different (Willis et al., 2019). Furthermore, the combined observations in transcriptional changes and metabolic homeostasis in *M. aeruginosa* provide novel and extensive insights into the complex cellular interactions that take place in this important bloom-forming organism (Steffen et al., 2014). Therefore, comprehensively assess molecular aspects of phosphatase encoding gene and its expression, transcriptional patterns in the important bloom-forming cyanobacteria might expand our knowledge in understanding its bloom mechanism.

3 ECOLOGICAL FUNCTIONS OF CYANOBACTERIAL EXTRACELLULAR PHOSPHATASES

3.1 Indicating P deficiency

Phytoplankton will produce extracellular phosphatase when suffering from P deficiency in natural population and lab experiment (Schindler, 1977; Healey, 1978; White et al., 2003; Cao et al., 2018; Lim et al., 2018; Feng et al., 2020). Therefore, algal APA or total APA was inversely proportional to P concentration (Smith and Kalff, 1981; Chróst et al., 1984; Francko, 1984; Pettersson, 1985; Vrba et al., 1993). APA of cyanobacterial mats in the Caribbean showed significantly negative correlation to the P concentrations, as well as the P content of the mat (Rejmánková and Komárková, 2005). Furthermore, APA would be inhibited in the presence of sufficient inorganic P supply (Kuenzler and Perras, 1965). This relationship was summarized as "induction-repression" mechanism (Jansson et al., 1988). As a result, APA has often been recommended as an indicator of P starvation in planktonic systems (Healey and Hendzel, 1980; Istánovics et al., 1992; Rose and Axler, 1997; Thingstad et al., 1998; Jamet et al., 2001) or cyanobacterial mats (Rejmánková and Komárková, 2000).

Normalized APA, such as chlorophyll-specific total APA (Istánovics et al., 1992), surface-area-specific APA (Newman et al., 2003) was more sensitive enough to show changes in the P status of algae than total APA (Zhang et al., 2021). The specific activity divided by algal biomass can substantially reflect the intrinsic catalytic efficiency of the enzyme, and thus serve as a suitable indicator for the P nutrient status of water and algae (Kuenzler and Perras, 1965; Perry, 1972; Fitzgerald and Nelson, 1975; Moegenburg and Vanni, 1991; Istánovics et al., 1992). On the other hand, the reliability of using APase as an indicator of P deficiency in phytoplankton was questioned (Cembella et al., 1982). For example, in Florida Bay, APA was highest during cyanobacterial blooms and particulate APA was related to the bloom, but APA/chlorophyll *a* and dissolved APA showed no correlation to the bloom (Koch et al., 2009). Factors interfering APA as the indicator of P deficiency include variety of extracellular phosphatase producers such as bacteria and zooplankton (Koch et al., 2009), enzyme substrate diversity (Sharma et al., 2014; Zhang et al., 2020b) and its effect on APA (Fonseca-De-Souza et al., 2008; Harke et al., 2012; Li et al., 2015a), extracellular secretions such as extracellular toxins (Bar-Yosef et al., 2010; Dobronoki et al., 2019; Lu et al., 2021), species specific responses (Olsen et al., 1989; Wan et al., 2019), external P threshold concentrations for activated response (Pick, 1987; Ruiz et al., 1997), environmental factors, such as light (Giraudet et al., 1997; Rychtecký et al., 2015; Yadav et al., 2016) and temperature (Bai et al., 2021; Ivančić et al., 2021), and the level of intracellular P

content (Olsson, 1983; Whitton et al., 1990; Zhang et al., 2020a). Furthermore, the activity of endogenous enzymes might be overestimated because of the exogenous input (Stevens and Parr, 1977).

Biochemical components unrelated to P can also cause increase or decrease in APA (Wilkins, 1972; Francko and Wetzel, 1982). In addition to differences in gene homology, the metal ions, located in the active sites of phosphatases, are also different (Luo et al., 2009). Therefore, the lack of external related metal elements will also affect the synthesis and function of cyanobacterial extracellular APA (Singh et al., 2006; Cox and Saito, 2013). APA contributed by dissolved APases could be spontaneously released during cyanobacterial blooms, other than driven by the P limitation. This explained coexistences of high Pi concentrations and high potential APA in water observed previously (Wang et al., 2021). The activity of dissolved phosphatases can be maintained for several weeks (Jansson, 1981; Olsson, 1983), which means that enzymes produced in one place may function in a wider range of waters. Shortly, the relationships between APA and ambient bioavailable P concentration are complicated. There are several limitations to use extracellular APA as a general indicator for describing P deficiency of phytoplankton (Cao et al., 2010). The multiple complex regulatory factors make it cautious when using extracellular APA as indicators of P deficiency.

3.2 Alleviating P stress

Algae could use extracellular phosphatases to obtain nutrients and grow normally with a different organophosphates supply (Ruiz et al., 1997). It was found that 17%–82% of the P absorbed into phytoplankton was from organophosphorus in the Sargasso Sea (McLaughlin et al., 2013). In the eutrophic Lake Nantua, the Pi released from DOP via APases could temporarily maintain the P supply for the algae dominated by *Oscillatoria rubescens* while DIP was deficiency in the summer (Feuillade et al., 1990). Moreover, the growth of cyanobacteria could be mainly supported by hydrolyzing dissolved organophosphate via APase in Lake Taihu and other warm-monomictic lake (Gao et al., 2006; Prentice et al., 2019). While in the absence of inorganic P, organophosphorus, e.g. pesticides, can be used by filamentous-heterocystous cyanobacteria as the only source of P (Subramanian et al., 1994). In the Grangent reservoir, cyanobacteria often relied on the APase to mineralize organic P (Giraudet et al., 1999). The aggregates dominated by *Nodularia spumigena* in the Baltic Sea were the key contributor to P regeneration (Stoecker et al., 2005). The increasing biomass of *Aphanizomenon* in a P-limited summer and autumn was accompanied by high enzymatic activity of APase (Hadas et al., 1999). Filamentous cyanobacteria, *Nodularia* and *Aphanizomenon*, from the Baltic Sea, showed higher APA with the decline of ambient Pi concentrations (Degerholm et al., 2006). Both ELF methods and molecular biology approaches supported the view that *Cylindrospermopsis raciborskii* could use different organophosphates to sustain growth when P was limited (Bai et al., 2014). Besides increasing potential APA, cyanobacteria may compensate for P stress by lowering Michaelis constant (Km) values of APases in the meanwhile (Zhang et al., 2021). During a *Microcystis* bloom outbreak, a large number of P sources was hydrolyzed by extracellular APases and released to sustain the growth of *Microcystis* (Chuai et al., 2011), but the extracellular APases might be excreted mainly by bacteria rather than *Microcystis* (Dai et al., 2018). Therefore, APase plays a crucial role in the process of cyanobacterial growth and blooms, although it might be secreted by microorganisms rather than cyanobacteria themselves (Zhao et al., 2012).

4 FUTURE RESEARCH

4.1 To what extent can extracellular phosphatases alleviate P stress

Researchers, mainly focusing on bloom-forming cyanobacteria, hold a hypothesis that secreting extracellular phosphatase to use DOP is their key competitive advantage, when they realized that APA was regulated by P supply (Kelly et al., 2019; Rabouille et al., 2022; Zhang et al., 2022). Unexpectedly, cells that secrete extracellular phosphatase are often not cyanobacteria even dominated in phytoplankton assemblages (Rengefors et al., 2001; Štrojsová et al., 2003), which makes the above hypothesis difficult to understand. If we can answer the question to what extent extracellular APase can alleviate P stress, the puzzle could be solved. Unfortunately, the current detection method cannot achieve the goal because of obvious deficiencies. Firstly, it is difficult to determine to what extent it can be able to represent a natural substrate for APase when using artificial synthetic organophosphates as the substrate. Therefore, the cyanobacterial extracellular APA, strictly speaking, can only reflect the qualitative rather than quantitative

relationship between APase and environmental variables (Siuda, 1984). To overcome this uncertainty, ecologists have used the radiometric method to determine the availability of P in the organic P pool of water bodies (Bentzen et al., 1992). Hernández et al. (1996) used ^{32}P-G_6P as a substrate to explore a more sensitive method for determining APA while there is still a problem since the radiometric element method might be selectively absorbed by phytoplankton. As a result, the function of phosphatase to alleviate P limitation may be overestimated for cyanobacteria. So, a combination study of cyanobacteria from molecular biology, biochemistry, field investigation, or indoor culture experiments with a more accurate test method is urgently needed.

4.2 Determine ecological function of extracellular phosphatases on species level

Excretion of extracellular APases is species specific among cyanobacteria (Rengefors et al., 2003; Dignum et al., 2004b). Cyanobacteria respond to low P supply by combining with other physiological process. Several perspectives deserve noting, including interspecific response between toxin and non-toxin producing cyanobacteria; the connection between toxin and extracellular APases producing; combining study among the different response mechanisms (e.g., P uptake, storage, regulating P demand, etc.). Since APases can be active, which are sometimes irrelevant to ambient P concentrations, other functions of APases are indeed worthy of attention at this moment, e.g. to constrain pigment biosynthesis, photosynthesis, fatty acid biosynthesis and cell division, which are of great significance in phytoplankton ecology. Although single-cell phosphatase of phytoplankton could be visualized and quantified by the ELF technology (Diaz-de-Quijano et al., 2014, 2020). Relation between transcriptional levels of APase encoding genes and the functional microbial compositions might give deep insight to the enzyme producers or origin (Bai et al., 2014; Dai et al., 2018). Integration of molecular biology and ELF assay is advised to give a comprehensive and reasonable result for the contributors of extracellular phosphatases at the species level. In summary, future research on specific species ecophysiology will benefit from a growing suite of tools available for assessing the activity and subcellular location of APase in field populations or cultures and ultimately the work done with specific species will be useful for studies of other harmful ones.

5 DATA AVAILABILITY STATEMENT

All data generated and/or analyzed during this study are included in this published article.

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