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Research Article

Impact of Phosphate on Mat Formation, Stress Biomarkers, Antioxidants, Osmoprotectants, and Intracellular Ionic Composition in *Tolypothrix* sp. KJE1

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Abstract

Increase phosphate availability enhances oxidative stress management and metabolic efficiency in *Tolypothrix* sp. KJE1, promoting cellular stability and growth. Under 100 mM phosphate, the organism exhibits reduced levels of H_2O_2 and malondialdehyde (MDA), indicative of improved antioxidant defenses, energy metabolism, and membrane integrity. This regulation minimizes reactive oxygen species (ROS) production and lipid peroxidation, reallocating resources from stress-associated compounds like proline and sucrose to biosynthetic pathways, thus optimizing growth. Decreased total phenol and flavonoid content under high phosphate levels further reflects reduced oxidative stress and diminished ROS production. Initially, antioxidant enzyme activities (SOD, CAT, APX, POD) peak at 25 mM phosphate to mitigate oxidative stress but decline at 100 mM due to excessive ROS accumulation and metabolic imbalances, revealing a threshold for stress tolerance. Enhance phosphate levels also modulate ion regulation, reducing Na^+ and Ca^{2+} while increasing K^+ concentrations to maintain osmotic balance and support metabolic processes. Enhanced carbon, nitrogen, and hydrogen assimilation under high phosphate conditions drives photosynthesis, nitrogen metabolism, and biomass production, highlighting the organism's adaptive biochemical strategies for survival in phosphate-rich environments.

Keywords: Phosphate; Sucrose, Malondialdehyde; Hydrogen peroxide; Superoxide dismutase.

Introduction

Phosphate is a crucial nutrient for the growth and development of all living organisms, including cyanobacteria. It plays a significant role in various cellular processes such as energy transfer, nucleic acid synthesis, and membrane structure. The availability of phosphate in the environment can profoundly influence the formation and functioning of cyanobacterial mats. This essay explores the impact of phosphate on cyanobacterial mat formation, focusing on its effects on growth, structure, ecological interactions, and potential applications (Xiao *et al*., 2022).

Phosphate is a key component of adenosine triphosphate (ATP), nucleic acids, and phospholipids, making it essential for energy metabolism, genetic information storage and transfer, and cell membrane integrity. In cyanobacteria, adequate phosphate availability supports robust cellular growth and division, thereby promoting the formation of dense and stable mats (Tiwari *et al*., 2019). When phosphate is plentiful, cyanobacteria can efficiently carry out photosynthesis and other metabolic processes, leading to increased biomass production and mat development. Phosphate availability can significantly affect the structural

characteristics of cyanobacterial mats. High phosphate concentrations often lead to enhanced growth rates and higher biomass accumulation, resulting in thicker and more cohesive mats. The increased biomass can support a greater diversity of microbial communities within the mat, including heterotrophic bacteria that thrive on organic compounds produced by cyanobacteria (Kramer *et al*., 2022). Conversely, phosphate limitation can restrict cyanobacterial growth, leading to thinner and less stable mats. Under low phosphate conditions, cyanobacteria may enter a state of nutrient stress, altering their metabolic activities and potentially triggering the production of extracellular polymeric substances (EPS). These EPS can help cells adhere to surfaces and to each other, partially compensating for reduced growth by enhancing mat cohesion and stability.

Phosphate availability influences the interactions between cyanobacteria and other microorganisms within the mat. In environments with high phosphate levels, cyanobacterial mats can outcompete other photosynthetic organisms, such as algae, due to their efficient nutrient uptake and utilization (Li *et al*., 2016). This competitive advantage allows cyanobacteria to dominate and shape the microbial community structure. In contrast, phosphate limitation can lead to increased competition and cooperation within the mat. Cyanobacteria may engage in mutualistic relationships with other microorganisms, such as phosphate-solubilizing bacteria, which can help mobilize phosphate from insoluble sources (Nawaz *et al*., 2024). These interactions can enhance nutrient availability and support the overall stability and resilience of the mat ecosystem. Understanding the impact of phosphate on cyanobacterial mat formation has important implications for biotechnological applications. In biofuel production, optimizing phosphate levels can maximize cyanobacterial biomass yield, thereby improving the efficiency of biofuel extraction processes. Similarly, in bioremediation, manipulating phosphate concentrations can enhance the growth and pollutant-degradation capabilities of cyanobacterial mats (Touliabah *et al*., 2022). In wastewater treatment, cyanobacterial mats can be used to remove excess phosphate from contaminated water bodies. By promoting the growth of cyanobacteria in engineered systems, phosphate can be sequestered within the biomass, preventing eutrophication and harmful algal blooms in natural aquatic environments. While phosphate is essential for cyanobacterial growth, its overabundance can lead to environmental issues such as eutrophication, which can cause harmful algal blooms and hypoxia in water bodies (Paerl and Otten, 2013). Balancing phosphate levels to promote beneficial cyanobacterial mat formation without causing ecological harm is a significant challenge. Future research should focus on understanding the complex interactions between phosphate availability, cyanobacterial metabolism, and microbial community dynamics. Optimizing phosphate levels for targeted biotechnological applications, while reducing environmental impacts, will be essential for the sustainable utilization of cyanobacterial mats. In conclusion, phosphate plays a vital role in cyanobacterial mat formation, influencing growth, structure, and ecological interactions. By understanding and managing phosphate availability, we can harness the potential of cyanobacterial mats for various applications, from biofuel production to environmental remediation, while safeguarding ecosystem health.

Materials and Methods

Experimental Design

For phosphate (K_2HPO_4) dose selection, different concentrations of K_2HPO_4 (0, 25, 50, 75, and 100 mM) were freshly prepared. The solution was sterilized by filtering it via a sterile Millipore membrane filter with a pore size of 0.22 µm. The cyanobacterial culture grown in BG-11N$^-$ medium without K_2HPO_4 served as the control culture (0 mM K_2HPO_4), while cultures grown in BG-11N$^-$ medium supplemented with 25, 50, 75, and 100 mM K_2HPO_4 were classified as K_2HPO_4-treated cultures.

Sucrose

Sucrose content in cyanobacterial cells was assessed following the procedure of Lillie and Pringle (1980). Briefly, 20 mg of dried algal mats was combined with 1 ml of 0.5 M TCA and left undisturbed at ambient temperature for 1 hour. The mixture was then mashed and centrifuged at 12,000 g for 15 minutes. The later steps were similar to those used for carbohydrate assessment. The optical density of the supernatant was measured at 625 nm, and the sucrose concentration was estimated as micromoles per milligram of dry weight, using a trehalose calibration curve.

Proline

Proline measurement was determined utilising the method described by Bates *et al*., (1973). A mortar and pestle were used to homogenize 20 mg of dried mats in 3% (w/v) sulfosalicylic acid, which was then kept at ambient temperature for 24 hours. The homogenised sample was centrifuged for 20 minutes at 12,000 g, and the supernatant was then processed with acetic acid and ninhydrin. After boiling the mixture for two hours, the reaction was stopped by immersing the tubes in freezing water. Proline was extracted using toluene (µmole/mg DW), with a corresponding reference where an equal volume of toluene was mixed with the cooled solution to facilitate layer separation. The lower pink layer of toluene was collected in a separate test tube, and the optical density was determined at 520 nm.

Malondialdehyde

Malondialdehyde was measured using 2-Thiobarbituric acid as an indicator of lipid peroxidation, according to the approach of Heath and Packer (1968). Cyanobacterial cells were homogenised in a 5% trichloroacetic acid solution make use of mortar and pestle. The homogenate was

centrifuged for 10 minutes at 12,000 g. After heating the supernatant to boiling for 20 minutes, 1 ml was mixed with 1 ml of 0.65 M 2-Thiobarbituric acid (prepared in a 20 M trichloroacetic acid solution). The compound was then cooled in ice-cold water and centrifuged again at 12,000 g for 10 minutes. Absorbance of the supernatant was recorded at wavelengths of 450, 532, and 600 nm. The MDA concentration was determined using the formula provided by Chokshi *et al*. (2015).

MDA (µ mol/mg DW) = [6.45 × (A_{532}nm - A_{600}nm) - [0.56 ×A_{450}nm] /DW (mg)

Hydrogen Peroxide

To measure hydrogen peroxide (H_2O_2) levels, cyanobacterial mats were homogenised in a 0.1 M TCA solution. Subsequently, 0.5 ml of the supernatant was mixed with 0.5 ml of 0.1 M phosphate buffer saline (pH 7.0) and 1 ml of 1 M KI solution. Absorbance was then measured at 390 nm, and hydrogen peroxide content was expressed as µmol/mg dry weight (DW), following the method of Velikova *et al*., (2000).

Total Flavonoid Content

To assess total flavonoid content (TFC) and total phenolic content (TPC), samples were homogenised in a 90% acetone solution. The TFC of the dehydrated mats was determined utilizing aluminum chloride ($AlCl_3$) according to the method of Ordonez *et al*., (2006). One milliliter of the extract was combined with 1 milliliter of 2 M $AlCl_3$, and the mixture was gently stirred before being left at room temperature for 2 hours. The color change of the mixture was observed, and the optical density of the samples were measured at 420 nm. TFC was reported as µg quercetin equivalents (QE) per mg of dry weight (DW).

Total Phenolic Content

Total phenolic content (TPC) of the cyanobacterial cells were assessed colorimetrically using the Folin-Ciocalteu reagent (FCR) based on the protocol by Singleton *et al*., (1999). A total volume of 5 ml was prepared using deionized distilled water (DDW) by mixing 0.5 ml of supernatant with 1 ml of 2 M Na_2CO_3 and 0.5 ml of 1 N FCR. The mixture was heated until the development of a blue coloration was observed. After cooling to room temperature, absorbance was measured at 760 nm. TPC was reported as µg of gallic acid equivalents per mg of dry weight (DW).

Superoxide Dismutase (SOD)

SOD activity was determined by assessing the photochemical reduction of nitro blue tetrazolium (NBT) using the specified method of Beauchamp and Fridovich (1971). Cyanobacterial mats were quickly preserved using liquid nitrogen, ground, and homogenized in 1.0 ml of extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA). The homogenate was spun at 12,000 rpm for 15 minutes, and the supernatant obtained was utilized as the crude enzyme extract. The reaction mixture, totaling 3 ml, included 1.5 ml of phosphate buffer (50 mM, pH 7-8), 0.2 ml of methionine (13 mM), 0.1 ml of NBT (75 µM), 0.1 ml of EDTA (0.1 mM), and 100 µl of the enzyme extract. Finally, 0.1 ml of riboflavin (2 µM) was added, and the tubes were stirred before being positioned 30 cm below a lighting setup equipped with two 15 W fluorescent bulbs. The light was turned on to initiate the reaction, which lasted for 10 minutes. After that, the light was turned off, and the tubes were covered with black cloth. Absorbance of the reaction mixture was measured at 560 nm. A non-irradiated mixture that did not develop color served as the blank, while a mixture without enzyme exhibited maximum color development. Percent inhibition was calculated as

A560 (blank) – A560 (sample) % inhibition = x 100 A560 (blank)

Fifty percent inhibition of the reaction was recorded as one enzyme unit.

Catalase (CAT)

Catalase (CAT) activity was assessed using the protocol described by Aebi (1984). Cyanobacterial mats were ground in extraction buffer (0.1 M phosphate buffer, pH 7.5, with 0.5 mM EDTA) using liquid nitrogen, then centrifuged for 15 minutes at 12,000 rpm. The resulting crude extract was used for the catalase assay. The reaction mixture was prepared using 0.5 ml of 30 mM H_2O_2, 1.5 ml of 50 mM phosphate buffer (pH 7.0), and 0.1 ml of enzyme extract, with DDW added to adjust the total volume to 3.0 ml. Absorbance was measured at 240 nm, and CAT activity was reported as nmol of H_2O_2 decomposed per mg of protein per minute.

Ascorbate Peroxidase (APX)

Ascorbate peroxidase (APX) activity was measured following the method described by Nakano and Asada (1981). Cyanobacterial mats were pulverized in a medium containing 0.1 M phosphate buffer (pH 7.5), 0.5 mM EDTA, and 1 mM ascorbic acid using liquid nitrogen. The homogenate was then centrifuged at 12,000 rpm for 15 minutes at 4°C, and the supernatant, serving as the enzyme extract, was used for the APX assay. The reaction mixture was prepared with 1.5 ml of 50 mM phosphate buffer (pH 7.0), 0.1 ml of 1 mM EDTA, 0.5 ml of 0.5 mM ascorbic acid, 0.1 ml of 0.1 mM H_2O_2, and 0.7 ml of deionized water, resulting in a total volume of 3.0 ml. To initiate the reaction, 0.1 ml of the enzyme extract was added, and the reduction in absorbance at 290 nm, indicating the oxidation of ascorbic acid, was recorded. The APX activity was expressed as the amount of ascorbic acid oxidized per mg of protein per minute.

Peroxidase (POD)

Peroxidase activity was measured using 4-methylcatechol as the substrate. The reaction mixture, with a total volume

of 3.0 ml at room temperature, comprised 100 mM sodium phosphate buffer (pH 7.0), 5 mM 4-methylcatechol, 5 mM hydrogen peroxide, and 500 µl of crude extract. The increase in absorbance resulting from the oxidation of 4-methylcatechol by H_2O_2 was measured spectrophotometrically at 420 nm.

Measurement of Cations

Cation content was measured using the tri-acid technique with minor modifications, as outlined by Sahrawat *et al*., (2002). A total of 50 mg of lyophilized cyanobacterial mat was combined with concentrated HNO_3, H_2SO_4, and $HClO_4$ in a ratio of 5:1:1 (v/v/v) and left overnight. The mixture was heated on a hot plate for digestion until it turned colorless. After cooling to room temperature, the digest was diluted to the suitable volume was taken and passed through Whatman filter paper. Cation analysis was performed using inductively coupled plasma-mass spectrometry (ICP-MS) on a Perkin Elmer Optima 7000 DV instrument from the United States.

Measurement of Elements

The carbon, hydrogen, and nitrogen content in the cyanobacterial mat was measured using a EuroVector Elemental Analyzer (EuroEA 3000, Italy). Helium and oxygen (99.995% purity) served as transport medium for combustion gases. A 1 mg sample was placed in a combustion capsule, which was heated to 980°C. Sulfanilamide (C 44.85%, H 4.70%, N 13.08%, S 14.97%, and O 22.40%) was used as the standard. The elemental ratios and heating values were calculated based on the CHN content.

Results and Discussions

Effect of Phosphate on Stress Biomarkers and Osmoprotectants of Tolypothrix sp. KJE1

The observed decrease in H_2O_2, MDA, proline, and sucrose levels in *Tolypothrix* sp. KJE1 under increasing phosphate concentrations from 0 to 100 mM can be attributed to its enhanced stress tolerance and efficient metabolic regulation in response to elevated phosphate availability (Fig. 1.). H_2O_2 is a reactive oxygen species (ROS) commonly produced as a byproduct of photosynthesis, particularly under stress conditions such as nutrient limitation or oxidative stress (Banerjee and Roychoudhury, 2017). High levels of H_2O_2 indicate oxidative stress, which can damage cellular components like proteins, lipids, RNA and DNA (Garcia-Caparros *et al*., 2021). The decrease in H_2O_2 levels with rising phosphate concentrations suggests that the cells are under less oxidative stress (Fig. 1.a). Phosphate plays a crucial role in ATP synthesis and energy metabolism, which helps the cells efficiently manage energy, thereby reducing ROS production. Enhanced phosphate availability improves cellular antioxidant defense mechanisms, such as the activity of enzymes like catalase and peroxidase, which scavenge H_2O_2, leading to reduced ROS accumulation (Rajput *et al*., 2021).

MDA is a marker of lipid peroxidation, which occurs when ROS, such as H_2O_2, attack membrane lipids, leading to oxidative damage (Almeida *et al*., 2017). The decrease in MDA levels with increasing phosphate concentrations further supports the idea that *Tolypothrix* sp. KJE1 is experiencing reduced oxidative stress (Fig. 1.b). Lower MDA levels indicate less damage to membrane lipids, suggesting that the enhanced phosphate availability helps maintain membrane integrity and function (Li *et al*., 2021). This reduction in oxidative stress could be due to improved energy management and the efficient utilization of absorbed light energy for photochemistry, minimizing excess ROS generation and lipid peroxidation (Zhao *et al*., 2021).

Proline is an amino acid commonly accumulated in cells under stress conditions, such as drought, salinity, or nutrient limitation. It acts as an osmoprotectant, stabilizing proteins and membranes and scavenging ROS (Shafi *et al*., 2019). The decreased proline levels with increasing phosphate concentrations suggest that *Tolypothrix* sp. KJE1 is under less environmental stress and does not require the protective role of proline (Fig. 1.c). This can be attributed to the improved metabolic state under high phosphate availability, where enhanced ATP synthesis supports overall cellular functioning, reducing the need for stress-related compounds like proline. Sucrose is often produced as a compatible solute to help cells cope with osmotic stress (Hagemann, 2016). Its accumulation is a common response to stress conditions, where it plays a role in osmoprotection and energy storage. The decrease in sucrose levels with increasing phosphate concentrations suggests that the cells are experiencing reduced osmotic stress (Fig. 1.d). In high-phosphate environments, the improved metabolic activity, including enhanced energy production and efficient resource utilization, reduces the need for sucrose as an osmoprotectant. Additionally, the test cyanobacteria may be redirecting resources away from sucrose synthesis towards other biosynthetic pathways, such as exopolysaccharide and lipid production, which are more favorable under nutrient-rich conditions. In conclusion, the decrease in H_2O_2, MDA, proline, and sucrose levels in *Tolypothrix* sp. KJE1 under 100 mM phosphate concentrations reflects reduced oxidative and osmotic stress in the test organism. Enhanced phosphate availability improves metabolic efficiency, energy management, and antioxidant defense mechanisms, reducing the need for stress-related compounds and promoting cellular stability and growth. The organism adapts to high-phosphate environments by reallocating resources away from stress responses and towards biosynthetic processes that support its optimal function and survival.

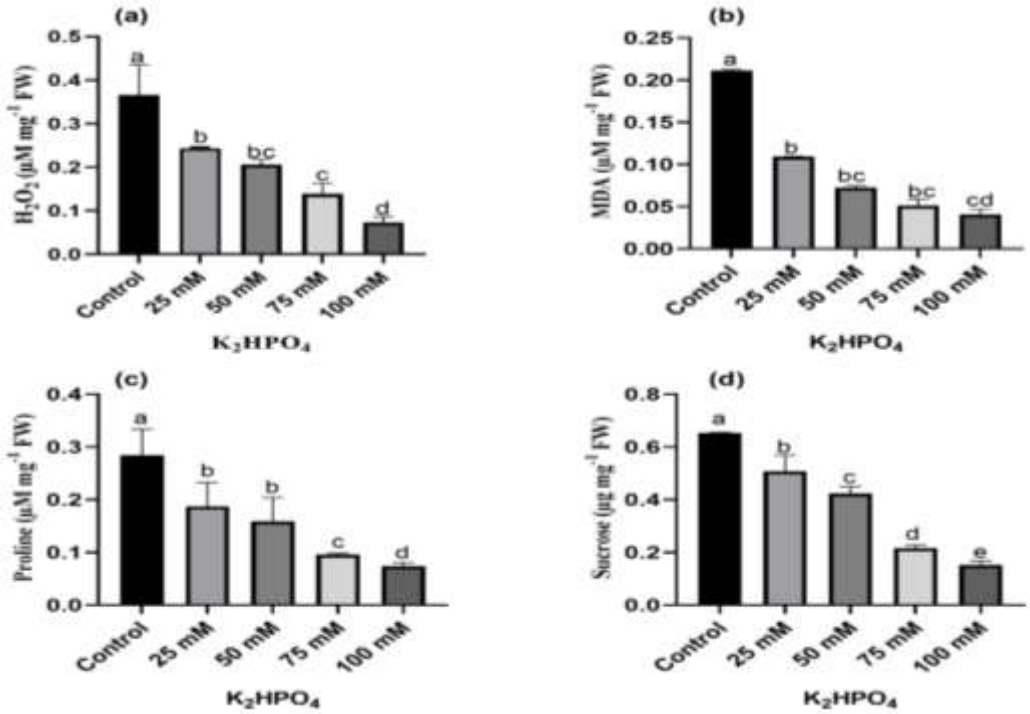

Fig. 1: Effect of phosphate on stress biomarkers and osmoprotectants of *Tolypothrix* sp. KJE1.

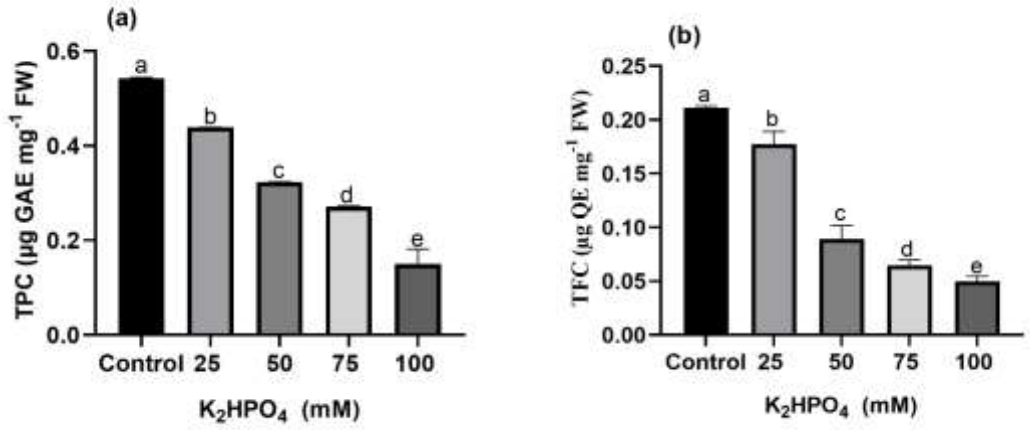

Fig. 2: Effect of phosphate on non-enzymatic antioxidant molecules of *Tolypothrix* sp. KJE1.

Effect of Phosphate on Non-Enzymatic Antioxidant Molecules of Tolypothrix sp. KJE1

The observed decrease in total phenol content (TPC) and total flavonoid content (TFC) in *Tolypothrix* sp. KJE1 under increasing phosphate concentrations from 0 to 100 mM can be directly linked to reduced oxidative stress and a lower demand for antioxidant compounds, driven by elevated phosphate availability. Phenols and flavonoids play acrucial role as antioxidant molecules, scavenging reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2) to protect cellular components from oxidative damage (Ugya *et al*., 2020). Under stress conditions such as nutrient limitations or environmental fluctuations, cyanobacteria often ramp up the production of these secondary metabolites to combat ROS accumulation and mitigate oxidative stress (Shilpi *et al*., 2015). In the case of *Tolypothrix* sp. KJE1, the decrease in TPC and TFC levels as phosphate concentrations increase suggests that the organism is experiencing less oxidative stress (Fig. 2.a, 2.b). This is supported by the previously observed reduction in H_2O_2 levels under similar conditions (Wang *et al*., 2021). Since phosphate is integral to enhancing energy metabolism, particularly through ATP synthesis, improved energy management in the cells leads to reduced ROS production, subsequently lowering the need for high levels of antioxidant defense. As oxidative stress diminishes, the cell has less requirement for phenols and flavonoids, explaining the observed decrease in their synthesis (Akbari *et al*., 2022).

In summary, the decrease in total phenol and flavonoid content under elevated phosphate conditions reflects a shift in *Tolypothrix* sp. KJE1 metabolic priorities. With reduced oxidative stress due to better energy balance and lower ROS levels, the organism reallocates resources away from producing antioxidant compounds like phenols and flavonoids. This adjustment illustrates the cyanobacteria ability to fine-tune its biochemical pathways to optimize growth and energy use in high-phosphate environments, further supporting its adaptive response.

Effect of Phosphate on Enzymatic Antioxidant Molecules of Tolypothrix sp. KJE1

The observation of initially increased and later decreased content of antioxidant enzymes (SOD, CAT, APX, and POD) under elevated phosphate concentrations (0 to 100 mM) in *Tolypothrix* sp. KJE1 can be explained by considering the following biological and biochemical responses. We have found maximum SOD, CAT, APX, and POD at 25 mM phosphate content which was 1.2, 1.5, 1.3, and 1.6 fold higher than 100 mM phosphate content (Fig. 3.abcd). At lower to moderate phosphate concentrations (e.g., below 50 mM), *Tolypothrix* sp. KJE1 experienced moderate oxidative stress due to an increase in metabolic activity. Phosphates play a critical role in cellular processes such as ATP production, nucleotide synthesis, and energy metabolism. Increased availability of phosphates enhances these metabolic activities, which inadvertently lead to higher production of reactive oxygen species (ROS). ROS, if not controlled, can damage cellular structures via proteins, lipids, RNA, and DNA. Superoxide dismutase (SOD) converts superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2), which is less harmful but still needs detoxification. Catalase (CAT) and ascorbate peroxidase (APX) are key enzymes that break down hydrogen peroxide into water and oxygen, preventing oxidative damage to cells. Peroxidase (POD) further assists in the removal of peroxides by reducing them with the help of electron donors. The initial increase in the levels of these enzymes is a protective response, where *Tolypothrix* sp. KJE1 elevates the production of antioxidant enzymes to manage the increased ROS, ensuring cellular protection and maintaining redox homeostasis. As the phosphate concentration continues to increase (closer to 100 mM), the metabolic and oxidative load on the cells may become excessive, or the cells may enter a state of nutrient saturation. This phase could lead to two potential outcomes that contribute to the observed decline in enzyme content. At very high phosphate levels (100mM), the persistent oxidative stress might surpass the ability of the *Tolypothrix* sp. KJE1 to maintain elevated levels of antioxidant defense. Prolonged overproduction of ROS could result in oxidative damage to proteins, carbohydrate and lipid, including the antioxidant enzymes themselves, leading to decreased enzyme activity and content (Rezayian *et al*., 2019). The inhibition of SOD, CAT, APX, and POD under severe stress conditions could explain the reduction in their levels. Excessive phosphate concentrations may trigger a shift in the physiological state of the cyanobacteria. High phosphate levels can slow down cellular growth due to imbalances in nutrient uptake and metabolism (Brembu *et al*., 2017). When growth slows or enters a stationary phase, the need for high antioxidant activity diminishes, causing a natural downregulation of enzyme production. In this state, the cells may also rely on other stress responses, such as the accumulation of osmolytes or the formation of thicker extracellular matrices, reducing the emphasis on enzymatic detoxification of ROS. In cyanobacteria, phosphate homeostasis is tightly regulated. While low to moderate phosphate levels (0 to 100 mM) are essential for growth, high concentrations can result in metabolic imbalances that lead to cellular stress. The initial increase in antioxidant enzyme levels reflects the cells capacity to adapt and protect against phosphate-induced ROS. However, as stress continues or intensifies at higher concentrations, the protective mechanisms might no longer be sufficient or sustainable, leading to the observed decline in enzyme content. In summary, the initially increased levels of SOD, CAT, APX, and POD are part of the cyanobacteria's defense mechanism to cope with phosphate-induced oxidative stress. However, as phosphate levels continue to rise, the declining trend suggests that prolonged or excessive stress overwhelms these systems, leading to enzyme depletion, inhibition, or metabolic shifts that reduce the need for high antioxidant activity.

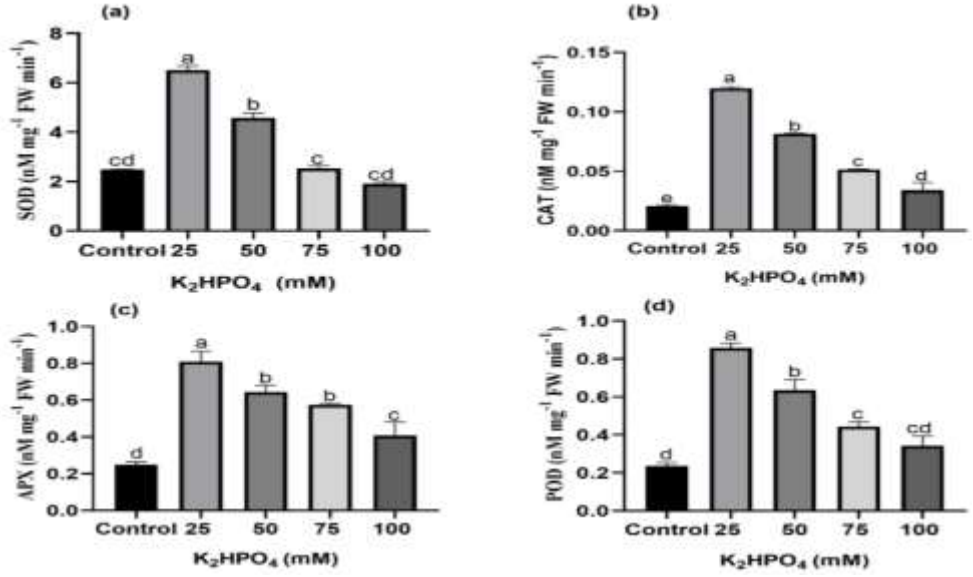

Fig. 3: Effect of phosphate on enzymatic antioxidant molecules of *Tolypothrix* sp. KJE1.

Effect of Phosphate on Intracellular Cations of Tolypothrix sp. KJE1

The observation of decreased sodium (Na^+) and calcium (Ca^{2+}) ions, along with increased potassium (K^+) ions under increased phosphate concentrations (0 to 100 mM) in *Tolypothrix* sp. KJE1 cyanobacteria can be understood by considering the role of ion homeostasis, stress adaptation, and nutrient uptake mechanisms. The reduction in Na^+ and Ca^{2+} levels under increasing phosphate concentrations could be linked to a shift in the cyanobacteria regulatory mechanisms aimed at balancing internal ion concentrations and maintaining homeostasis under nutrient stress. Sodium ions are often involved in osmotic regulation and ion homeostasis (Kumari and Rathore, 2020). However, in this case, the decrease in Na^+ under elevated phosphate levels may indicate a reduced need for sodium to maintain osmotic balance (Fig. 4.a). The cells may be regulating sodium efflux to prevent excess Na^+ accumulation, which can be toxic under certain stress conditions. Elevated phosphate could activate sodium pumps or antiporters (such as Na^+/H^+ antiporters), expelling Na^+ from the cell to maintain ionic stability and minimize stress-related damage (Magda *et al*., 2021). Calcium is a key secondary messenger in various stress signaling pathways. However, under prolonged or high phosphate concentrations, the decrease in Ca^{2+} levels could suggest that the cells have downregulated calcium signaling pathways once the initial adaptive responses are complete (Fig. 4.b). Another possibility is that the elevated phosphate may interfere with calcium uptake, possibly through competition for transporters or by altering membrane permeability (Kahil *et al*., 2021). Additionally, high phosphate concentrations could reduce the need for calcium as a signaling molecule, leading to its decreased concentration within the cells.

Potassium is essential for many cellular functions, including enzyme activation, osmotic regulation, and maintaining cell turgor (Hasanuzzaman *et al*., 2018; Ahmad and Maathuis, 2014). The observed increase in K^+ concentration under elevated phosphate levels can be linked to several factors: Potassium plays a crucial role in maintaining osmotic pressure and internal charge balance. The rise in K^+ levels may be part of the cyanobacteria strategy to cope up with osmotic pressure in response to decreased Na^+ and Ca^{2+} (Checchetto, 2016). By increasing K^+ levels, the cells can compensate for the loss of sodium and calcium ions and stabilize internal osmotic conditions under elevated phosphate concentrations (Clausen and Poulsen, 2013). Phosphate and potassium often interact through co-transport mechanisms. High phosphate availability might enhance potassium uptake via co-transport systems, leading to increased K^+ accumulation. The higher K^+ concentration may also reflect an increased demand for potassium to support growth, enzymatic activity, and phosphate metabolism under high phosphate conditions (Spijkerman *et al*., 2007).

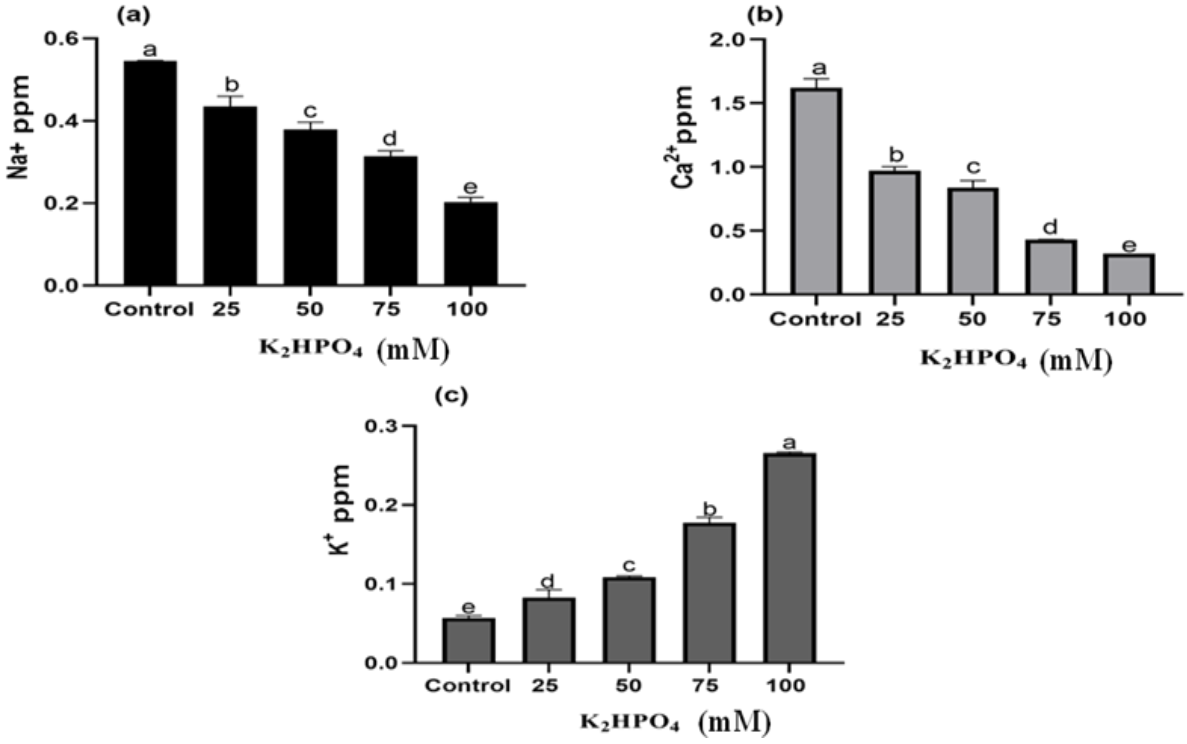

Fig. 4: Effect of phosphate on intracellular cations of *Tolypothrix* sp. KJE1.

The shift in ion concentrations decreased Na^+ and Ca^{2+}, increased K^+ which reflects an adaptive response by *Tolypothrix* sp. KJE1 to optimize nutrient uptake and metabolic processes under phosphate-rich conditions. High phosphate availability may reduce the need for sodium and calcium ions in certain cellular processes while promoting potassium uptake to support growth and metabolic activity (Zafar *et al*., 2021). Potassium is critical for cellular metabolism and biosynthetic processes, especially in the presence of 100 mM, which can enhance ATP production and other energy-demanding processes. Increased K^+ concentrations might support these metabolic pathways, allowing the cyanobacteria to optimize growth and phosphate assimilation (Cuellar-Bermudez *et al*., 2017). This could indicate reduced dependence on Na^+ and Ca^{2+} for osmotic regulation and signaling under elevated phosphate levels. The cells may expel Na^+ and reduce Ca^{2+} uptake as part of their adaptation to high-phosphate conditions. Potassium accumulation may serve as a compensatory mechanism to maintain osmotic balance and support metabolic processes, particularly those linked to phosphate metabolism (Sanz-Luque *et al*., 2020). These ion concentration shifts reflect the adaptive strategies of *Tolypothrix* sp. KJE1 to maintain homeostasis and optimize growth under varying phosphate concentrations.

Effect of Phosphate on Intracellular Elements of Tolypothrix sp. KJE1

The increase in carbon, nitrogen, and hydrogen percentages under elevated phosphate concentrations in *Tolypothrix* sp. KJE1 indicates an enhanced metabolic response to nutrient availability. The maximum percentage of carbon, nitrogen and hydrogen were found in the cultures with 100 mM K_2HPO_4 (Fig. 5.). Phosphate plays a crucial role in many biochemical processes, via energy transfer (ATP synthesis), nucleic acid synthesis, and membrane stability. Increased phosphate levels can improve photosynthetic efficiency, leading to greater carbon fixation via the Calvin cycle (Yang *et al*., 2017). This results in enhanced carbon incorporation into cellular biomass, contributing to the observed increase in carbon percentage. Phosphate can boost the synthesis of nitrogen-containing compounds such as proteins, nucleotides, and pigments like chlorophyll (Alipanah *et al*., 2018). A more efficient nitrogen uptake system may be activated under elevated phosphate conditions, resulting in higher nitrogen percentages within the cells. Hydrogen is a key component of organic molecules, including carbohydrates, lipids, and proteins. Increased phosphate could enhance overall biosynthetic activity, increasing the synthesis of macromolecules and thus raising the hydrogen content. Together, these changes reflect a more active and possibly more efficient metabolic profile under phosphate enrichment, potentially aiding in growth, mat formation, and stress tolerance.

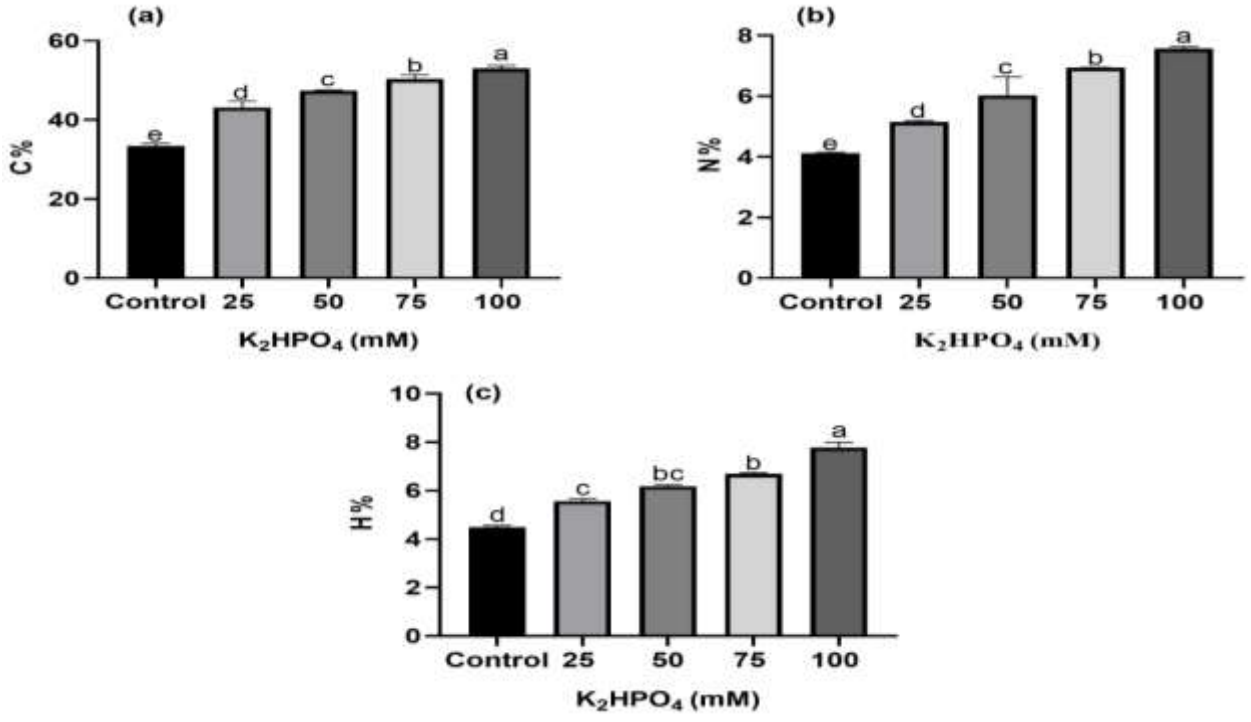

Fig. 5: Effect of phosphate on intracellular elements of *Tolypothrix* sp. KJE1.

Conclusion

Enhanced phosphate availability reduces oxidative stress in *Tolypothrix* sp. KJE1 by lowering H_2O_2 and MDA levels, indicating improved antioxidant defense, energy management, and membrane integrity. This metabolic regulation minimizes ROS production and lipid peroxidation, promoting stress tolerance under elevated phosphate conditions. Under 100 mM phosphate, *Tolypothrix sp.* KJE1 experiences reduced oxidative and osmotic stress due to enhanced metabolic efficiency, energy production, and antioxidant defenses. This promotes cellular stability and growth, reallocating resources from stress-related compounds like proline and sucrose to biosynthetic pathways for optimal function and survival. The decrease in total phenol and flavonoid content in *Tolypothrix* sp. KJE1 under increased phosphate conditions reflects reduced oxidative stress and lower reactive oxygen species (ROS) production. Improved energy metabolism, driven by higher phosphate availability, diminishes the need for antioxidant defense. This adaptive response allows the organism to reallocate resources, optimizing growth and metabolic efficiency in high-phosphate environments, highlighting its ability to fine-tune biochemical pathways for better survival and energy use. The study shows that *Tolypothrix* sp. KJE1 initially increases antioxidant enzymes (SOD, CAT, APX, POD) at moderate phosphate levels (25 mM) to manage oxidative stress caused by elevated metabolic activity. This response peaks at 25 mM and declines at higher phosphate levels (100 mM) due to excessive ROS production, oxidative damage to enzymes, and metabolic imbalances. Prolonged stress and nutrient saturation reduce enzyme activity, indicating that the cyanobacteria's protective mechanisms are insufficient to cope with extreme conditions. This highlights the dual role of phosphate in enhancing metabolism and inducing stress in cyanobacteria.

Under heightened phosphate concentrations (0–100 mM), *Tolypothrix* sp. KJE1 exhibits adaptive ion regulation, with decreased Na^+ and Ca^{2+} levels and increased K^+ concentrations. The decline in Na^+ and Ca^{2+} reflects reduced reliance on these ions for osmotic balance and signaling, possibly through efflux mechanisms and competition for uptake. Simultaneously, K^+ accumulation compensates for osmotic pressure and supports metabolic processes like enzyme activation and phosphate metabolism. These shifts highlight the cyanobacteria's ability to optimize ion homeostasis, nutrient uptake, and metabolic efficiency in response to phosphate-rich conditions, ensuring growth and survival. Elevated phosphate concentrations enhance the metabolic activity of *Tolypothrix sp.* KJE1, increasing carbon, nitrogen, and hydrogen incorporation into biomass. This is driven by improved photosynthesis, nitrogen assimilation, and biosynthesis of macromolecules, leading to better growth, mat formation, and stress tolerance.

Authors' Contribution

Mr. Jalaluddin is responsible for conducting fieldwork, collecting cyanobacterial samples, performing morphological and molecular analyses, and drafting the manuscript. Prof. Rajan Kumar Gupta, provided guidance throughout the study, reviewed the results, and contributed to the finalization of the manuscript. Both the authors have read and approved the final version of the research article.

Conflict of Interest

No conflict of interest is interest is there.

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