International Biodeterioration & Biodegradation 127 (2018) 217–226

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Tolerance strategies in cyanobacterium *Fischerella* sp. under pesticide stress and possible role of a carbohydrate-binding protein in the metabolism of methyl parathion (MP)

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ARTICLE INFO

Keywords:
Organophosphorus
Fischerella sp.
Methyl parathion
Proteomics
Docking

ABSTRACT

The tolerance strategy of cyanobacterium *Fischerella* sp. under methyl parathion (MP) stress was investigated through proteomics analysis using 2-DE technique coupled with MALDI- TOF MS/MS. Proteomic study of the cyanobacterium treated with or without MP for two and eight days exhibited differential expressions of proteins related to photosynthesis, energy and protein metabolism, redox homeostasis, signal transduction and cellular defence. Inhibitory effect of MP on the growth of the test organism was more pronounced after the 2nd day of treatment and the majority of the proteins, except those involved in the protein metabolism (DnaK, Ef-Tu and proteases), were downregulated. However, the growth of the cyanobacterium was significantly less affected after the 8th day of MP treatment and a number of proteins, *viz*., antioxidative enzymes, signalling protein, chaperones, were induced. Transcript analyses of the genes of few upregulated and downregulated proteins, i.e., phycocyanin α subunit (*cpcA)*, ribulose bisphosphate carboxylase (*rbcl)*, F0F1 ATP synthase subunit *α*, F0F1 ATP synthase subunit β, SOD (*sod)*, NifH (*nif H)*, DnaK (*dnaK)* and Peptidase S8 showed similar results after the 8th day of MP treatment. Furthermore, some hypothetical proteins were also found to be upregulated in the cyanobacterium under MP stress. Functional prediction of four of these hypothetical proteins using bioinformatic tools revealed their roles in signalling and carbohydrate metabolism. Interestingly, one hypothetical protein was homologous to lectin and found to possess a binding pocket for MP. Therefore, this lectin-type protein of the cyanobacterium might have a crucial role in the removal and degradation of MP.

1. Introduction

In recent days, agriculture without the application of pesticides has become nearly impossible anywhere in the world. Pesticides are of various types, such as organochlorine, organophosphorus, carbamates, pyrethroids, etc. Among these, organophosphorus pesticides are one of the most commonly used pesticides. These compounds are generally used in agricultural fields and households to prevent the growth, mainly of insects. After use, a large portion of these pesticides remains in the environment and subsequently contaminates soil, water, air, sediments and vegetables. As a result, growth and development of a number of nontarget organisms including birds, fishes, soil microbes and algae are being adversely affected (Singh, 2009; Sonne et al., 2012). Methyl parathion (MP) is one such organophosphorus pesticide and has been classified by WHO as an extremely hazardous compound (Kegley et al., 2014). It causes different types of toxicity, such as chronic toxicity, sub-chronic toxicity, acute toxicity, genotoxicity, neurotoxicity, oncogenicity, reproductive toxicity, developmental toxicity and immune toxicity in humans and other nontarget organisms (Sharma, 2015). Therefore, the removal of this dangerous material from the environment is of utmost importance.

Although various physical and chemical processes have been developed to remove excess pesticide from the environment, but the utilisation of biological organisms has attracted considerable attention. In this context, autotrophic macrophytes, green algae and cyanobacteria are potent candidates for the remediation of pesticide-contaminated environments (Ebtesam et al., 2007). Of these organisms, cyanobacteria are very unique as they have the capacity to perform photosynthesis, fix nitrogen and grow in almost all possible extreme habitats including wastewater and highly polluted environments (Cuellar-Bermudez et al., 2017; Singh and Thakur, 2015; Dubey et al., 2011; Akoijam et al., 2015). In addition, they are distinct from other organisms due to their

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https://doi.org/10.1016/j.ibiod.2017.11.025
Received 13 July 2017; Received in revised form 20 November 2017; Accepted 23 November 2017

extreme ability to sorb and degrade organic contaminants (Tao et al., 2013; Megharaj et al., 1994; Dubey et al., 2011; Subramanian et al., 1994). Hence the use of cyanobacteria for the decontamination of MP will be a cost-effective and an environment-friendly approach in comparison to the use of heterotrophic organisms as well as physical and chemical methods.

Earlier studies showed the biodegradation and removal of MP from the growth medium by the experimental organism *Fischerella* sp. (Tiwari et al., 2017). In this study, molecular mechanisms of the cyanobacteria to tolerate MP toxicity have been examined by analysing the changes in proteome and transcriptome. Proteomics and transcript analysis are used as powerful tools to reflect the changes and to explain the adversity and acclimation strategies of an organism experiencing different kinds of stresses (Castielli et al., 2009; Kosová et al., 2011; Fang et al., 2013). Two-Dimensional Gel Electrophoresis (2DE) coupled with MALDI-TOF MS/MS and Real Time PCR (RT-PCR) are the acclaimed techniques for the assessment of quantitative and qualitative changes in proteome and transcriptome, respectively (Basu and Apte, 2012). Adaptation of cyanobacteria to stress is linked to profound modifications in proteome. Proteomics has been used to examine the response of cyanobacteria to stresses, such as heat, UV-B (Ehling-Schulz et al., 2002; Suzuki et al., 2006; Gao et al., 2009), heavy metals (Surosz and Palinska, 2005), salinity (Fulda et al., 2006; Pandhal et al., 2009), and iron stress (Castielli et al., 2009).

Therefore, the present study has been conducted to elucidate the role of metabolic modulations to enhance the cyanobacterial tolerance to pesticide stress by analysing the changes in the gene-expression patterns.

2. Material and methods

2.1. Experimental condition and growth measurement

Pure culture of cyanobacterium *Fischerella* sp. was maintained in BG 11 medium (Rippka et al., 1979), pH 7.4 under cool white light (intensity 50 $\mu mol^{-1}m^{-2}s^{-1}$) at 27 °C ± 1 °C. To evaluate its MP tolerance, the cyanobacterium was first subjected to different concentrations of MP (5, 10, 20 and 30 mg L^{-1}). It was observed that at 20 mg L^{-1} of MP concentration the biomass, chlorophyll and protein content of the cyanobacterium was less affected (respectively 27%, 33% and 21.6% reduction), and extremely reduced at 30 mg L^{-1} of MP (respectively 56%, 62% and 44% reduction) after 8 days of incubation (Tiwari et al., 2017). Hence, 20 mg L^{-1} was determined as the sub-lethal dose of MP and selected for further experiments. All the experiments were performed in two sets of conditions: (i) cyanobacterial culture without MP (control) and (ii) cultures supplemented with 20 mg L^{-1} of MP. The experiments were conducted in triplicate and the bar in each graph represents the standard deviation from the mean. The growth of cyanobacterium was measured in terms of chlorophyll content up to 16 days. The chlorophyll of the cyanobacterium was extracted in methanol at intervals of 2 days and absorbance was recorded at 663 nm as per MacKinney (1941).

2.2. Measurement of phycobilins and carbohydrate contents

To extract Phycobiliproteins (PBS), cyanobacterial cultures treated with or without MP were harvested by centrifuging at 10,000 rpm for 5 min. The pellets obtained were resuspended in phosphate buffer (50 mM, pH 7.0) containing lysozyme (1 g L^{-1}) and incubated at 37 °C for 1 h. Thereafter, the cyanobacterial cultures were subjected to freeze-thawing cycles until the pigments were completely leached out in the buffer. Cyanobacterial suspension was again centrifuged at 5000 rpm and the absorbance of the supernatant was recorded at 652, 615 and 565 nm (Bennett and Bogorad, 1973; Singh et al., 2013). The total cellular carbohydrate content was determined as per Dubois et al. (1956) using glucose as the standard.

2.3. Measurement of PSII activity

The efficiency of PSII was monitored in terms of Fv/Fm by using pulse amplitude modulated (PAM) fluorimetry as per Schuurmans et al. (2015). Prior to the experiment, cyanobacterium from both control and MP-supplemented flasks were dark-adapted for 30 min.

2.4. Oxidative stress

The intracellular reactive oxygen species generated after the 2nd and 8th days of methyl parathion treatment were detected by adding 5 μM of a fluorescence probe 2', 7′ -dichlorofluorescin diacetate (DCFH-DA) to 5 μl of cyanobacterial culture and kept in dark for 1 h on a shaker (Ling et al., 2011). The resulting fluorescence produced inside the cells was further visualised and analysed using florescent microscope (Deventer).

2.5. Estimation of superoxide dismutase activity

Activity of antoxidative enzyme superoxide dismutase (SOD) was measured by the protocol of Beauchamp and Fridovich (1971) in terms of the % inhibition of reduction in nitro blue tetrazolium chloride (NBT). One unit of enzyme activity was equivalent to the amount of enzyme required to inhibit the reduction of NBT by 50% in the presence of light.

2.6. Protein extraction and two-dimensional gel electrophoresis

To extract the total protein content, cyanobacterial cultures treated with or without MP were harvested after the 2nd and 8th day of treatment by centrifugation at 10,000 rpm for 5 min at 4 °C. The obtained pellet cells were collected and ground into fine powder using mortar and pestle with liquid nitrogen. The powder was mixed with 500 μL of extraction buffer (Tris HCl, $MgCl_2$ and KCl) and incubated for 15 min at 4 °C. Cellular debris was removed by centrifugation at 12,000 rpm for 30 min at 4 °C and the proteins present in the supernatant were further precipitated in 2% pre-chilled TCA acetone for 12 h at −20 °C. The precipitated protein was washed with ice-cold acetone and then dissolved in rehydration buffer (2 M thiourea, 7 M urea, 4% CHAPS). The protein content was estimated by the method of Bradford (1976). After that, 800 μg of protein was loaded on IPG strip (pH 4–6, 11 cm Bio-Rad) for active rehydration using Bio-Rad focussing system. Isoelectric focussing (IEF) was performed as follows: 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h and kept at 8000 V for a total of 30,000 V-hours (Vh) at 20 °C, after which the strips were equilibrated in equilibration buffer and placed on 12% polyacrylamide gels for the second dimension run. The 2D gels were stained in Coomassie Brilliant Blue (CBB) R-250. Protein spots, which appeared on the gels, were analysed using PDQuest software (Version 8.01, Bio-Rad, Hercules, CA). Prominent protein spots were selected for trypsin digestion and MALDI-TOF/MS-MS analysis.

2.7. Trypsin digestion and mass spectrometry analysis

The selected protein spots were manually excised and processed according to Wang et al. (2013). Excised spots were destained using 50% acetonitryl (ACN) prepared in 25 mmol L^{-1} NH_4HCO_3. The destained spots were further dehydrated in 100 μl of 100% ACN and again rehydrated in 5 μl trypsin solution (Promega, Madison, USA) containing 20 μg trypsin ml^{-1} and 25 mmol L^{-1} NH_4HCO_3 for 30 min. An additional 20 μL of 25 mmol L^{-1} NH_4HCO_3 was added to the gel after 30 min and kept overnight for digestion at 37 °C. After incubation, gel pieces were extracted thrice in 20 μL of extraction solution (67% ACN and 5% TFA). Extracts were completely dried using SpeedVac (Eppendorf, USA) and then resuspended in 5 μL 0.1% TFA. Finally, the supernatant was mixed with the matrix and spotted on the sample target