Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2017, 9(5):96-103

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Effect of Propiconazole on the Nucleic Acid and Protein Composition of Pesticide Resistant *Pseudomonas aeruginosa* PS-4 Strain

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ABSTRACT

Propiconazole is one of the major triazole fungicide, use to treat the standing crops from the disease like leaf spot, powdery mildew and leaf and stem rust. The present work evaluates the impact of propiconazole on the DNA, RNA and protein contents of propiconazole resistant Pseudomonas aeruginosa PS-4 strain in different concentrations (10, 20 and 30 µg/l) and durations. Additionally, the expression of protein under the stress condition was studied by the SDS PAGE analysis. The results indicate that, the higher concentrations (20 and 30 µg/l) of propiconazole were found to be toxic for nucleic acid and protein composition of PS-4 strain and the induction of different protein spots were observed in a pesticide treated group. Moreover, the DNA, RNA and protein contents in the PS-4 strain was found maximum in 10 µg/l of concentration at 24 hrs, it was further decreased after 72 hrs of exposure to propiconazole. Furthermore, the protein banding pattern was observed to be similar in all the propiconazole treated groups and maximum protein spots were observed at 66 to 44 kDa. Results of present investigation exhibits that, the higher dose of toxicant may cause damage to bacteria. Proteins, however, which were expressed under the stress conditions may have the adverse impact on the bacterial strain to resist under certain stress conditions and helps to gain the resistance over toxicants.

Keywords: Propiconazole; *Pseudomonas aeruginosa* PS-4 strain; DNA; RNA; Protein

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INTRODUCTION

The protein examination study is generally called as proteomics; it specifically implemented to understand the protein structures and functions [1,2]. Proteins are the most essential components for physiological and biological activities including the metabolic pathways of living organism's cells and these are commonly formed by the organism, production of proteins will vary with time and distinct necessaries or stress that an organism undergoes [3]. Moreover, Bacterial cells respond to environmental stress by inducing exact locations of proteins characteristic to each stress. Stress proteins are expressed in response to a wide range of stress conditions in various bacteria [4]. Each coding genes of proteins constitute stimulon, for instance, heat shock, SOS response and oxidative stress. Similarly, proteins related to any stimulant, will be induced through additional stresses, for instance many heat shock proteins in *E. coli*. This type of proteins can also synthesized once the cells are exposed to hydrogen peroxide, ethanol, UV light and lack of amino acid. In other stimulants, exposed to non-lethal concentrations of toxic agents can deliberate defense in contradiction of follow exposure to toxic concentrations of the identical stress agents [5,6]. This kind of state proposes that despite the parameter of a unique protein being organized, major change in the regulatory pathways regulating the activity of different stress proteins [7]. Investigations on stress enzymes and existing approaches of enteric bacteria have advanced a variety of multipart mechanisms, which use diverse regulatory structures and genetic mechanisms for their existence and virulence [8]. Asghar et al. [9] have reported that, the induction of stress protein in response to different insecticides for instance cypermethrin, zeta-cypermethrin, carbofuran and bifenthrin were evaluated by protein profiling of *E. coli*. It has been reported that, against diverse classes of toxic metals, the cellular adaptation and compensation of bacteria have been proposed. Though, the response to the different pesticides by the molecular mechanisms and responses of cells of microbes are not diversely studied [10]. Propiconazole is one of the major triazole fungicide, use to treat the standing crops from the disease like leaf spot, powdery

mildew and leaf and stem rust. The fate of propiconazole became an increasing concern because of its persistence in soil as well as because of its Eco toxicological effects on fish, invertebrates, soil microbes and algae. The half- life of Propiconazole ranged from 96 to 575 days [11]. Hence, the present study was undertaken to examine the effect of propiconazole on the DNA, RNA and protein of *Pseudomonas aeruginosa* PS-4 strain.

MATERIALS AND METHODS

Chemical, bacterial strain and media

Chemical:

The stock solution of propiconazole was prepared by concentration of 1 mg/ml. Further, the stock solution was diluted to require concentrations.

Bacterial strains:

The propiconazole resistant bacterial soil isolates *Pseudomonas aeruginosa* PS-4 strain which was isolated and identified in our laboratory was used for the present study [11]

Medium:

The optimized dextrose medium was used for the protein profiling study. The media contain dextrose - 0.65 g/l, Yeast extract - 1.05 g/l, KHPO - 0.30 g/l, and NaCl - 0.25 g/l and pH (7.0).

Microbial inoculum

Primarily the inoculum was prepared by the inoculating the trace of bacterial culture into the nutrient broth and incubated at 30^{o}C in 140 RPM shaking conditions for overnight and after the incubation 1 ml of liquid culture from the overnight incubated flasks was aseptically transferred to the 100 ml sterile dextrose medium. Further, at the regular time of interval the bacterial culture was withdrawn from the flasks for the protein profiling.

Treatment of bacterial strain with propiconazole

The bacterial isolate *Pseudomonas aeruginosa* PS-4 was treated with 10, 20 and 30 µg/l of propiconazole and grown at 30^{o}C. A fraction of culture was drawn at 24, 48 and 72 h of growth. A control without propiconazole treatment was maintained.

Effect of propiconazole on biochemical contents of *pseudomonas aeruginosa* PS-4 strains

From the above mentioned medium 5 ml of the inoculum was aseptically inoculated to 250 ml Erlenmeyer's flasks containing 100 ml of sterilized optimized medium amended with different concentrations of propiconazole. Further, all the flasks were incubated at 35°C for 72 hrs for *Pseudomonas aeruginosa* PS-4 strain under shaking conditions at 120 RPM on a rotary shaker. At regular intervals, fraction cultures were aseptically withdrawn from the each flask for examination of DNA, RNA and protein contents.

Nucleic acids isolation and estimation

Perchloric acid of 0.5 N strength was added to the bacterial pellet of 10 ml culture and preparation was allowed to incubate in the water bath at 70°C for 10 mins with constant shaking and further tubes were centrifuged at 3000 rpm for 20 min in 4°C. The obtained extracts were again treated with the 3 ml of 0.5 N Perchloric acid. Further, the supernatant was used for the both DNA and RNA estimation by the diphenylamine (DPA) and orcinol method respectively.

DNA estimation

DNA was estimated by the diphenylamine method (DPA) which was previously described by the Waterborg and Matthews (Burton assay) [12]. Briefly, Once DNA is treated with diphenyl amine with acid environments, a bluish green colored complex was formed which has an absorption at 595 nm and this reaction was formed of deoxypentose. In acidic solution deoxypentose was converted into a very sensitive β-hydroxylevulinaldehyde further which responds with diphenylamine forms bluish green colored complex. Four ml of diphenylamine reagent was added to 2 ml of the bacterial extracts and mix thoroughly. Further, the mouth of the tubes was covered and incubated in boiling water bath for 10 mins and the tubes were cooled to room temperature and the optical density was taken at 595 nm against water blank and to find the concentration of DNA in bacterial extracts standard calibration graph was referred. All the tests were performed in three independent replicates (Figure 1).

RNA estimation

RNA was estimated by the orcinol method which was previously described by the Roe and Rice [13]. Briefly, RNA was treated with the orcinol reagent in the presence of ferric chloride and Hydrochloric acid (HCL) which can be estimated calorimetrically. This reaction of pentoses forms furfural rings when treated with concentrated HCL. Further, orcinol reacts with furfural in the presence of ferric chloride as a catalyst to form the green color which can be measured at 665 nm. Two ml of cell filtrates of *Pseudomonas aeruginosa* PS-4 strains was supplemented with the 3 ml of orcinol reagent and heated on a boiling water bath for 20 min, and optical density (OD) was determined at 665 nm against water blank. The amount of RNA was calculated by referring to the standard graph. All the tests were performed in three independent replicates (Figure 2).

Protein isolation

The bacterial fractions of drawn were centrifuged at 6500 rpm for 8 mins and the pellet was treated with 200 µl of lysozyme prepared in 50 mM sodium phosphate buffer (pH 7.4) and incubated at room temperature for 20 min. The tubes were centrifuged at 10,000 RPM for 10 min and the supernatant was used for further analysis [11].

Protein estimation

The protein content of samples at every stage of purification was determined by the method of Bradford [14]. 0.1 ml of protein sample was mixed with 5 ml of 1X Bradford reagent, incubated at room temperature for 5 min and absorbance was measured at 595 nm. Bovine serum albumin (BSA) was used as a standard.

Analysis of protein by using SDS-PAGE

The SDS-PAGE analysis was done according to the method described by Laemmli [15]. Clean glass plates were used for gel loading and the underside of the assembly was sealed using agar. Ten percentages separating and stalking gel was prepared with distilled water, with the components of 30% Acrylamide, 1.5 M Tris-Cl Buffer, SDS, APS and TEMED. Stacking gel mixture was poured onto resolving gel and comb was placed, further, which was carefully removed from the polymerized gel. The cast gel was placed in Poly Acrylamide Gel Electrophoresis Unit and run buffer (Tris- 3 g, Glysine- 14.3 g, SDS- 2.0 g for 1 litre) was poured into upper and lower buffer tank. The samples were treated with sample buffer (1.5 M Tris-Cl (pH 8.0) 0.625 ml, 20% SDS 1.0 ml, Glycerol 1.0 ml, 2-mercaptoethanol 0.5 ml, 0.2% Bromophenol blue) in 1:1 ratio and heated in boiling water bath for 10 min. The sample was carefully loaded onto the gel and ran at constant voltage of 100 V for 3 hrs. The gel was carefully removed and placed in Staining solution (Methanol 4 ml, distilled water 5 ml, glacial acetic acid 1 ml, 0.2% Coomassie Brilliant Blue) under constant Shaking for 2 hrs. Excess of stain was destained using destaining solution (Methanol 4 ml, distilled water 5 ml, Glacial acetic acid 1 ml).

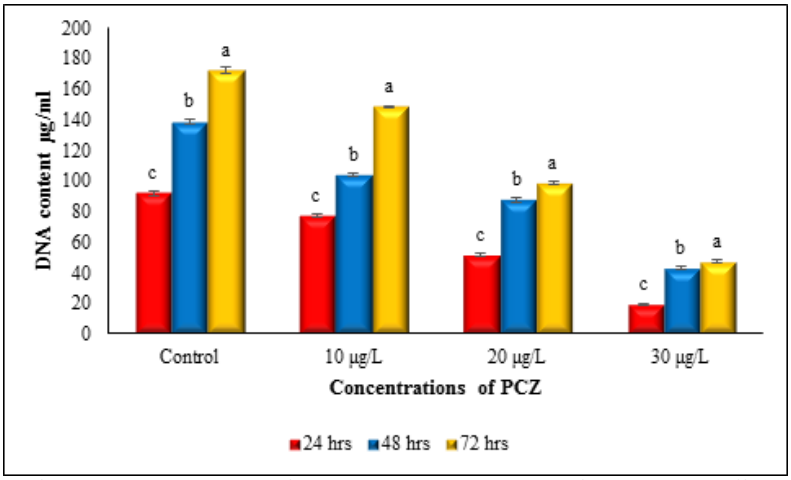
RESULTS

Effect of propiconazole on DNA content in *pseudomonas aeruginosa* PS-4 strain

The impact of propiconazole on the DNA content in *Pseudomonas aeruginosa* PS-4 strain, treated with the propiconazole concentration of 10, 20 and 30 µg/l and strain without treated with propiconazole was maintained as a control. The DNA content in the *Pseudomonas aeruginosa* PS-4 strain in control was found to be 91.92, 138.43 and 172.46 µg/ml after 24, 48 and 72 hrs of incubation respectively. Similarly, the DNA content in PS-4 strain at 10 µg/l of propiconazole was found to be 77.16, 104 and 148.33 µg/ml after 24, 48 and 72 hrs of incubation respectively. In the meantime, bacterial cells of PS-4 strain treated with the 20 µg/l of propiconazole was showed the similar trend of DNA content and it was found to be 51.66, 87.77 and 98. 45 µg/ml after 24, 48 and 72 hrs of incubation respectively. The DNA concentrations in the PS-4 strain treated with 30 µg/l were found to be 19.32, 43.42 and 47.34 µg/ml after 24, 48 and 72 hrs of incubation respectively (Figure 1).

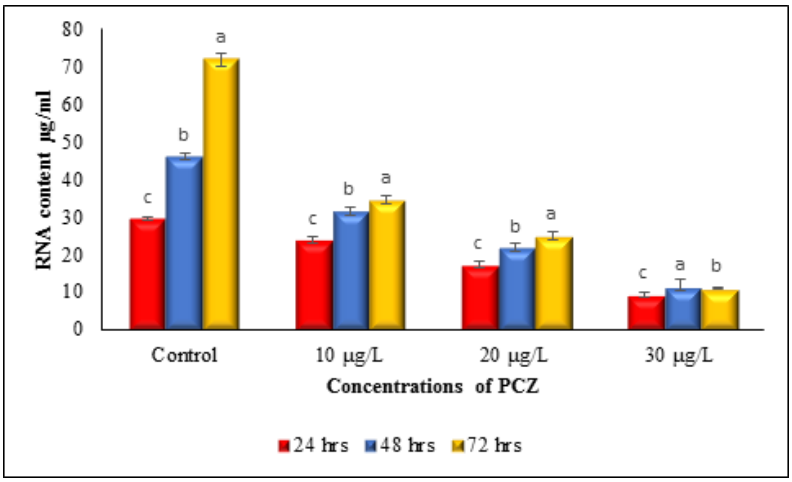
Effect of propiconazole on RNA content in *pseudomonas aeruginosa* PS-4 strain

The bacterial cells (PS-4) which was not treated with the propiconazole (control) was found to be 29.49, 46.0 µg/ml and 71.96 at 24, 48 and 72 hrs respectively. The DNA concentration the cells treated with the 10 µg/l was found 23.7, 31.53 and 34.64 µg/ml after the incubation of 24, 48 and 72 hrs respectively. Conversely, bacterium treated with 20 µg/l of propiconazole showed the increasing trend in the RNA concentrations and it was found to be 17.24, 21.8 and 24.93 µg/ml in 24, 48 and 72 hrs respectively. On the other hand, cells treated with the 30 µg/l were found lethal and as a result the RNA was found to be 9.32, 11.75 and 11.0 µg/ml after 24, 48 and 72 hrs of incubation period respectively (Figure 2).



Data are the means ±S.E of three independent replicate for each incubation period.Means followed by the different letter are significantly different from each other according toTukey's test (P<0.05)

Figure 1: Effect of propiconazole treatment on DNA content in *Pseudomonas aeruginosa* PS-4 strain



Data are the means ±S.E of three independent replicate for each incubation period.Means followed by the different letter are significantly different from each other according toTukey's test (P<0.05)

Figure 2: Effect of propiconazole on the RNA content in *Pseudomonas aeruginosa* PS-4 strain

Effect of propiconazole on protein content in *pseudomonas aeruginosa* PS-4 strain

The effect of propiconazole on protein content of *Pseudomonas aeruginosa* PS-4 strain is given in the Table 1. In the controls the protein concentration was found to be 1.21, 1.25 and 1.12 mg/ml in the given incubation period of 24, 48 and 72 hrs respectively. On the exposure with the 10 µg/l of propiconazole, the protein concentration was 1.18, 1.13 and 1.04 mg/ml at the given incubation time of 24, 48 and 72 hrs respectively. Simultaneously, bacterial strain treated with 20 µg/l of propiconazole showed 1.16, 1.27 and 1.0 mg/ml after the 24, 48 and 72 hrs of incubation. On exposure with 30 µg/l of propiconazole to strain PS-4 exhibits 1.19, 1.07 and 0.89 0 mg/ml of protein concentration at 24, 48 and 72 hrs respectively.

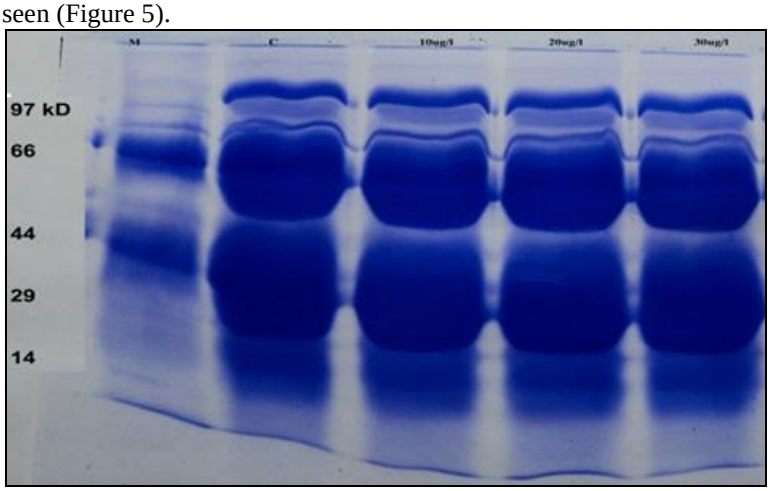
Table 1: Effect of propiconazole on the protein content in *Pseudomonas aeruginosa* PS-4 strain

Group	Treatment concentration (µg/l)	Protein Content (mg/ml)		
		Duration (hrs)		
		24	48	72
I	Control	1.2	1.3	1.1
II	10	1.2	1.1	1
III	20	1.2	1.3	1
IV	30	1.2	1.1	0.9

Protein profiling of *pseudomonas aeruginosa* PS-4 strain on exposure to propiconazole

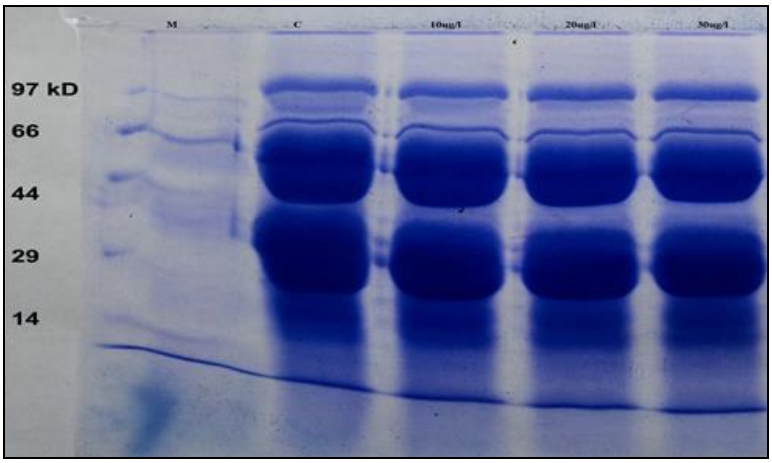
The present study was conducted with the goal of investigating the protein profiling in *Pseudomonas aeruginosa* PS-4 strain that was treated with the various concentrations of propiconazole ranging from 10, 20. 30 µg/l and experimental control samples were maintained throughout the study. It was observed that, in the period 24 hrs sample the banding pattern seems to be similar in all the concentrations. The SDS-PAGE ran with the 24 hrs

protein sample showed that the protein having more than 97 KDa the protein was expressed in all the concentration including control. Similarly, approximately at 76 KDa in all the recommended concentration the similar banding pattern was seen. Moreover, the over expression of protein was observed in all the concentrations of propiconazole at approximately 74 to 50 KDa. Further, the similar trend of banding pattern was observed in 44 to 20 KDa in 24 hrs. In addition, at 14 KDa the single and was appearing in the protein samples of 10 and 20 µg/l and it was not present in control and protein sample of propiconazole concentration 30 µg/l (Figure 3). In the 48 hrs samples, protein having a 97 KDa band was observed in all the doses of propiconazole, interestingly, approximately at 85 KDa the protein was expressed only in the control. In the same way, approximately at 76 KDa similar protein was expressed in all the lanes. Furthermore, the same protein was expressed approximately at 60 KDa in all the treated groups and also in the control. Likewise, approximately at 20 KDa at same protein was seen in the all the concentrations of propiconazole including the control and also at 14 KDa the banding pattern was same (Figure 4). In the same way the culture filtrate of *Pseudomonas aeruginosa* PS-4 extracted at 72 hrs for the protein profiling showed that, a similar protein was appeared at more than 97 KDa in all the concentrations and approximately at 76 KDa the same protein was expressed. In all the treated and control samples the similar banding trend was observed approximately at 29 KDa. Additionally, approximately at 20 KDa again a similar type of protein was expressed and also at 14 KDa the same kind banding patter were seen (Figure 5).



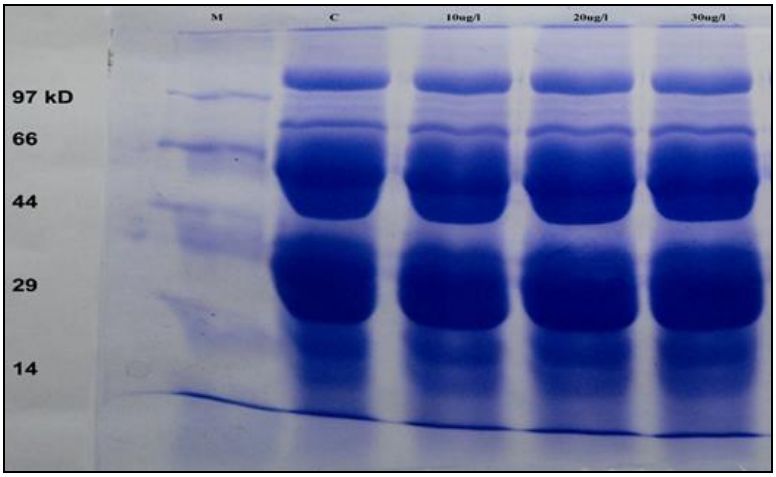
Lane 1 –M- Marker, Lane 2- C- Control, Lane 3- 10 µg/l, Lane 4- 20 µg/l, Lane 5- 30 µg/l

Figure 3: SDS gel analysis of 24 h old *Pseudomonas aeruginosa* PS-4 strain



Lane 1 –M- Marker, Lane 2- C- Control, Lane 3- 10 µg/l, Lane 4- 20 µg/l, Lane 5- 30 µg/l

Figure 4: SDS gel analysis of 48 h old *Pseudomonas aeruginosa* PS-4 strain



Lane 1 –M- Marker, Lane 2- C- Control, Lane 3- 10 µg/l, Lane 4- 20 µg/l, Lane 5- 30 µg/l

Figure 5: SDS gel analysis of 72 h old *Pseudomonas aeruginosa* PS-4 strain

DISCUSSION

The present study was undertaken to elucidate the protein profiling of *Pseudomonas aeruginosa* PS-4 strain that was treated with different doses of propiconazole ranging from 10, 20 and 30 µg/l for a period of 72 hrs. Protein profile of *Pseudomonas aeruginosa* PS-4 strain treated with propiconazole exhibited a synthesis of regularly expressed proteins, different stress proteins and over expression of some of the proteins. It was also noticed the protein band were seen as bulged and this type of protein expression may be because of more quantity of whole protein and over expression of proteins. It was observed that the content of DNA and RNA in the *Pseudomonas aeruginosa* PS-4 strains was affected by the exposure of propiconazole at 24, 48 and 72 hrs. It was also noticed that, DNA and RNA concentrations in the control groups were increased as the incubation time significantly increased and similar trend was also seen in the propiconazole treated groups. However, the highest concentration of propiconazole was doing the damage on the production of DNA and RNA from *Pseudomonas aeruginosa* PS-4 strain. In evidence to our experimental results Singh et al. [16] have also reported the similar type of observation by saying the pesticides chlorpyrifos and cypermethrin have the toxic effect on the biochemical parameters of microorganisms. Similarly, pesticide butachlor will have the capacity to reduce the population and biochemical parameters of Azospirillum. Contradictorily, pesticide carbofuran will have the ability to stimulate the growth and enhance the biochemical contents in Azospirillum. This type of mode of actions of pesticides on the microorganisms will witnessed for two kind of possibilities like some pesticide (beneficial) will have the ability to stimulate the growth and better biochemical mechanisms in microorganisms and other pesticides (toxic) will affect/limit the growth and biochemical parameters in microbes [17], it is clear clue that our experimental results are falls under the category that, pesticides will have the ability to reduce the growth and biochemical parameters significantly. In addition, the Significativity of our results assures that the propiconazole will have the greater influence over the inhibition of DNA and RNA concentration in *Pseudomonas aeruginosa* PS-4 strain. The previous reports on the protein profiling suggests that, when the *E. coli* was treated with the methomyl and the molecular weight of different protein expressed were purely based on the dose and durational exposure to the toxic compound and also it was reported that, examining the stress protein is a promising method for the analysis of pesticides toxic levels on the microorganism. However, the protein extracts which were not treated with methomyl were rarely expressed a limited number of protein [18] though, in our study protein samples which were not treated with the propiconazole (control) were also expressed proteins. In support to our experimental results, Shetti and Kaliwal [19] have reported proteomic profile of *Brevundimonas* sp. MJ 15 on exposure with the imidacloprid and it was observed that the control groups were also shown the similar kind of protein banding pattern when comparisons with treated groups. In addition, it was reported that the some specific protein were induced in response to stress caused by the imidacloprid and it was also described that the stress induced by the toxic compounds may be applied for the remediation purpose of contaminanted sites.

Previously, 2,4-D, Pencycuron, Endosulfan and Tebuconazole are toxic to the *Anabaena fertilissima*, *Aulosira fertilissima* and *Westiellopsis prolifica* and proteins of these organisms showed sensitivity to above mentioned pesticides. It was also reported that, the toxicity of above mentioned pesticides will have their adverse effect if they are used in heavily [20]. Similarly, it was stated that, if microorganisms were treated with certain harmful physical and chemical agents, the microorganisms obtain an induced resistance against the toxic impacts of chemicals [21]. The minimum iron content in microorganims will lead to the leads to the amplified mixture of

virulence factors in numerous bacteria with the shiga pollutant of enteropathogenic *E. coli* [22], the shiga pollutant of *S. dysenterie*, diphtheria toxin of *Cornybacterium diptheriae* [23], and exotoxin of *P. aeruginosa* [24]. The major reason of stress protein is, they regulated at different stages of σ^S [25] and CspA [26,27] in *E. coli.* The antecedent reports stated that, regulatory proteins, the polypeptide chains with interrupted or altered structures are selectively hydrolyzed. These effects may arise because of erroneousness in the protein biosynthesis, physiological and biological harm [28].

The heavy metal resistant bacterial strains were isolated from the garden soil and industrial effluents were used for the testing of impact of mercury, cadmium, arsenic and lead on the protein profiling and it was observed that, microbes treated toxicants showed varied banding pattern with an expression of some stress proteins [29]. However, earlier literature reported that the outer membrane proteins are indispensable components of bacterial cells and participate in several relevant functions of the microorganisms. Changes in the outermost membrane protein structure might alter antibiotic sensitivity and pathogenicity. Moreover, the adverse effects of various influences on outermost membrane protein expression, such as antibiotic treatment, mutation, changes in the environment, lipopolysaccharide modification and biofilm formation [30].

The over expression of some of the proteins observed in our study in *Pseudomonas aeruginosa* PS-4 strain might due to bacteria ability to succeed in diverse environments [31], another proteins that were induced in common to each stress and might be the cause for fractious defense in bacterial cells. In addition the exposure of bacteria to the toxic compounds will induces the stress proteins in microorganisms and this condition will lead to little disturbance in the protein concentrations. For instance, suppression of protein that are expressed regularly, increased levels of expression of proteins, decreased level of expression of some regularly produced proteins and expression of some unique proteins [20] and these difference may directly associate to the response of propiconazole resistant bacterial strains under pesticides induced stress environments. Our results have a tendency to reach agreement with researchers who conveyed that the expression of stress proteins or the increased yields of already existed proteins, which are only produced during stress environments due to stress response in microorganisms [32].

CONCLUSION

The higher doses of propiconazole was effected the protein, DNA, RNA and protein content of *Pseudomonas aeruginosa* PS-4 strain. Moreover, the PS-4 strain showed the expression of stress proteins during the concentration and duration treatment. The expressed proteins were commonly produced and play an important part in the metabolism of cells under the stress conditions and it was also observed that the stress proteins were induced in all the treated concentrations. Moreover induction of stress proteins may helpful for the microorganism to counter the toxic effects of pollutants. The proteins expressed were induced by treatment of higher doses of propiconazole and they can be applied for the environmental monitoring.

ACKNOWLEDGMENT

The authors are thankful to the Bioinformatics infrastructure facility. Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India, Delhi, (BT/PR/4555/INF/22/126/2010 dated 30-09-2010). Authors also thankful to the UGC-UPE fellowships and Post Graduate Department of Studies in Microbiology and Biotechnology, Karnatak University Dharwad for providing the laboratory facilities.

Conflict of Interest

The authors do not have any conflict of interest connected to the manuscript.

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