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RESEARCH ARTICLE

EFFECT OF METHOMYL ON PROTEASE ACTIVITY IN *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

In modern agriculture, pesticides are frequently used in the field to increase crop production. Besides combating insect pests, insecticides also affect the population and activity of beneficial microbial communities in soil. Microorganisms are widely applied test-species in different bioassays because of the ease and low costs of their culturing as well as the lack of ethical issues often accompanying the use of higher organisms Proteases are a group of enzymes that belong to one of the four major classes of proteolytic enzymes and are generated by a variety of organisms including viruses, bacteria, protozoa, yeasts, plants, helminthes, insects and mammals. The proteases are widespread group of enzymes that catalyze the hydrolysis of different proteins and perform a pivotal role in the degradation and turnover of intracellular proteins.. In the present study *Pseudomonas aeruginosa* were exposed to methomyl of concentrations ranging from 10^{-7} M to 10^{-3} M for a period of 96 hrs and protease activity was evaluated at regular intervals of 24, 48, 72 and 96 hrs. The results indicated that protease activity increased with the increase in the dose and duration of exposure of methomyl in *Pseudomonas aeruginosa* and that protease is dose and duration dependent.

INTRODUCTION

Methomyl is a carbamate insecticide used for vegetables and fruits and the mode of action is by the inhibition of acetyl cholinesterase (Barakat, 2015). It has been classified by the WHO, EPA (Environmental Protection Agency, USA), and EC (European Commission) as a very toxic and hazardous pesticide (Tamimi *et al.,* 2009). Methomyl is highly soluble in water (57.9 gl-1) has a low sorption affinity to soils and can therefore easily cause ground water contamination in agricultural areas (Stadtman *et al*., 1999). Bonatti *et al.* (2006) have shown Genotoxic effects of methomyl in *in vitro* studies (Bonatti *et al*., 2006). The proteases are a group of enzymes that belong to one of the four major classes of proteolytic enzymes, and are generated by a variety of organisms, including viruses, bacteria, protozoa, yeasts, plants, helminthes, insects, and mammals (Joo, 2007). Proteases are enzymes, which cleave certain proteins to produce low molecular weight products. Such proteases have several functions as destruction of abnormal or foreign proteins, protein excretion, protein turnover during starvation and inactivation of functional or regulatory proteins (Beckwith and Strauch, 1988). The oxidative damage to proteins may be an important factor in triggering their rapid degradation *in vivo*.

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The selective elimination of such damaged polypeptides can be an important protective mechanism for the cell (Young *et al*., 1988). Stadtmann Levine and coworkers have proposed that inactivation by oxidants may be a specific mechanism initiating the breakdown of critical proteins by the enzyme protease, in *Escherichia coli.* Several efforts have been undertaken to search for a proteolytic system that sel ectively attacks the oxidized proteins and moreover, extracts of *Escherichia coli* have been shown to degrade rapidly this damaged enzyme, but not the native protein and several preliminary reports have appeared concerning *Pseudomonas aeruginosa* protease that may be responsible for selective degradation of the modified glutamine synthetase (Berlett *et al*., 1997). Proteases are a group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. They constitute 59% of the global market of industrial enzymes, They have got wide range of commercial usage in detergents, leather, food and pharmaceutical industries. Sources of proteases include all forms of life, that is,plants, animals and microorganisms. Proteases produced from microorganisms play important role in several industries example detergent, tanning, photographic industries, pharmaceutical and waste treatment etc. (Joo, 2007). Proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically

manipulated to generate new enzymes with altered properties that are desirable for their various application. Therefore, methomyl, a carbamate pesticide was evaluated for its toxicity to the soil isolate- *Pseudomonas aeruginosa* cells with emphasis on protease activity.

MATERIALS AND METHODS

Preparation of stock solution of methomyl

The sample of methomyl (Lannate ®) used in the experiment was supplied by E.I. Dupont India Pvt. Ltd., Haryana obtained. The stock solution of 1 M of methomyl was prepared and further diluted to give different required molar concentrations.

Maintenance and propagation of culture

The organism *Pseudomonas aeruginosa* was isolated from the soil and the bacteria was maintained at 4°C on nutrient agar formulated by Lapage and Shelton (Jellouli *et al*., 2009) and sub cultured very fortnight. Synthetic sewage medium (S-medium) formulated by Lackey and White (Lapage *et al*., 1970), was used as the medium for toxicity testing.

Screening for protease production

The soil isolates was Screened for protease prodution by plate assay using protease specific medium containing (g/l) K2HPO4 2.0, glucouse1.0, peptone 5.0, gelatin 15.0, and agars 15. The clear zone diameter were measured after 24h of incubation at 28°C by flooded the plates with mercuric chloride solution, this method was referred as gelatin clear zone method (Babich and Stotzky, 1977).

Innoculum Preparation

Pre-inoculum was prepared by inoculating a loopful of bacteria from the overnight incubated nutrient agar slant cultures on a 100 ml sterilized synthetic sewage medium and incubated for 18-24 hours at 37°C under static conditions depending on the exponential phases of bacteria under test. Five ml of the pre-inoculum was inoculated to 250 ml Erlenmeyer's flask containing 100 ml of sterilized S-medium amended with different molar concentrations of heavy metals. The flasks were incubated at 37°C for 96 hours under shaking conditions at 120 rpm on a rotary shaker (REMI – CIS-24). At regular intervals sample was taken out from each flask aseptically for analysis.

Protease Activity

The soil isolate was inoculated in 50 ml of protease specific medium broth containing (g/L) glucose, 5.0; peptone, 7.5; (MgSO4.7H2O, 5.0; KH2PO4, 5.0; and FeSO4.7H2O, 0. 1, pH-7.0 and were culture in a rotary shaker (180 rpm) at 28°C for 3 days. After the completion of fermentation, the whole fermentation broth was centrifuged at 10,000 rpm at 4°C, and the clear supernatant was recovered. The crude enzyme supernatant was subjected to further studies. The protease activity of the respective samples was measured as per the procedure of Gaddad *et al.,* (1982). Two ml of the sample was incubated with phosphate buffer Ph 7.6 and 1% casein for two hours at 37^{o} C. the reaction was stopped by the addition of 3 ml of 10% TCA solution and filtered through Whatman No 42 filter paper. 2ml of the filtrate was mixed with 3ml of 0.5 N NaOH and 0.5 ml of folin phenol reagant. The optical density was estimated against an appropriate blank with a spectrophotometer at 660 nm. The unit of protease activity is calculated as micrograms of tryptophan released per minute per 100 ml of the sample.

RESULTS AND DISCUSSION

The present investigation was attempted to study the protease activity in the soil isolate *Pseudomonas aeruginosa* qualitatively. The proteolytic activity was assayed using skim milk agar and gelatin agar, and exhibited as diameter of clear zone. Gelatine agar was the best than skim milk agar for qualitative test of protease. As shown in Fig. (1) the soil isolate *Pseudomonas aeruginosa* showed high proteolytic activity, further the effect on the protease enzyme activity in *pseudomonas aeruginosa* when exposed to different concentrations of methomyl ranging from 10^{-7} M to 10^{-3} M of methomyl for a period of 96 hrs was also studied. The activities of the enzyme protease was compared with the dose and duration of exposure of methomyl in *pseudomonas aeruginosa* and the results revealed that the protease activity increased with an increase in the dose and duration of exposure of methomyl when compared with those of the corresponding parameters of the control indicating that the pesticide methomyl induces the protease enzyme. Our results agreed with the observations made by Asghar *et al.,* (2006), who analyzed the stress proteins induced in response to the pesticides cypermethrin, zeta-cypermethrin, carbofuran and bifenthrin. Our Experimental data indicate that the activity of the entire protease enzyme is more in treated cells than its controlled counterpart. Since the microbial cells possess antioxidant enzymes, which are induced in response to oxidative stress and are directly exposed to the pesticide. Protease play a major role in the cellular defense mechanism, they are susceptible to inactivation by reactive oxygen species (Pattanasupong *et al*., 2004).

Fig. 1. The zone of inhibition by *Pseudomonas aeruginosa* on gelatin agar plates

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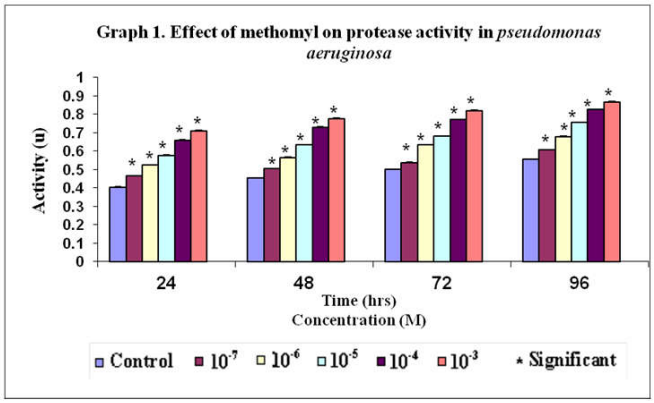

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Further the protease are found in one or more sub cellular compartments in microorganisms, including the cytoplasm and the periplasmic space and preferentially brings about rapid intracellular degradation of the proteins (Han, Sueng, Joo, 2007) and the protein selective proteolytic degradation appears to be rather significant in homeostasis maintaining and metabolism regulation in the cell. Along with short-lived regulatory proteins, the polypeptide chains with disrupted or changed structures are selectively hydrolyzed. Such defects might arise from inaccuracy during protein biosynthesis, chemical or physical damage (Vasilyeva *et al*., 2000) and Moreover, extracts of *E. coli* have been shown to degrade rapidly the damaged enzyme, but not the native protein, and several preliminary reports have appeared concerning and *E. coli* protease that may be responsible for selective degradation of the modified proteins (Young, 1988). The significant decrease in the protease activity of *Pseudomonas aeruginosa* cells on dose and durational exposure of methomyl observed in the present study compared to the control, may be due to expression of intracellular proteins, which require cell lysis for purification which will result in exposure to proteases (Martin *et al*., 1998) hydrolysis of different proteins that perform a pivotal role in the degradation and turnover of intracellular proteins (Joo *et al*., 2007) homeostasis maintenance and metabolism regulation in the cell (Beckwith and Strauch, 1988), protect the cells against effects of toxic peptides (Vasilyeva *et al*., 2000) or selective degradation of the modified proteins (Young, 1988). It was already experimented that the expression of proteins were more conspicuous in *Escherichia coli* cells which was obligatory, since the free *Escherichia coli* cells possess antioxidant enzymes,which are induced in response to the stress and are directly exposed to methomyl (Kulkarni *et al*., 2008). With the development of toxicogenomic approaches, the use of microorganisms for environmental monitoring purposes is expected to become even more extensive because of better knowledge about potential analogies in toxicity mechanisms between higher organisms and microbes (Marinšek Logar and Vodovnik, 2007). It has been suggested that there are many protein synthesized in common with many stress in *Escherichia coli* and some of these proteins may play a major role in the stability of the cells under different stresses (Amritha, 2011). Further the protease are found in one or more sub cellular compartments in *E.coli*, including the cytoplasm and the periplasmic space and preferentially brings about rapid intracellular degradation of the proteins and the protein selective proteolytic degradation appears to be rather significant in homeostasis maintaining and metabolism regulation in the cell (Kulkarni *et al*., 2011). The fact that specific patterns of proteins are expressed for a particular stress has led to the use of stress proteins to monitor environmental samples for the presence of particular pollutants (Amritha, 2011).

Conclusion

The effect of the enzyme protease on exposure to methomyl in *Pseudomonas aeruginosa* was studied and the comparative effect of protease on different dose and durational exposure of methomyl in *pseudomonas aeruginosa* has also been elucidated. The results confirm that protease activity is induced in *pseudomonas aeruginosa* on exposure to methomyl when compared to that of the control. The use of biological methods in environmental monitoring is essential in order to complement chemical analysis with information about actual toxicity or genotoxicity of environmental samples. It is very much clear from the above findings that pesticides interact with different components and enzymes of the target and non-target organisms, thereby impairing the physiological and metabolic activities of the cell. The enzymes and other biochemical indices are providing sensitive index to the changes due to pesticide toxicity. Such parameters can widely be used to rapidly detect and predict early warning of pesticide toxicity.

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