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EFFECT OF METHOMYL ON PROTEASE ACTIVITY IN FREE AND IMMOBILIZED *ESCHERICHIA COLI*

KULKARNI A.G. AND KALIWAL B.B.*
P.G. Department of Studies in Biotechnology and Microbiology, Karnatak University, Dharwad, 580003, India
*Corresponding author. E-mail: b_kaliwal@yahoo.com

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Abstract- Immobilized biocatalyst such as enzymes, microbial cells and cellular organelles, seem to be useful for continuous monitoring of the metabolites because of their high sensitivity and substrate specificity. The approach by confining enzymes in a defined space is extremely useful for enzyme reutilization and particularly favorable for applied bioprocess and it is apparent that *Escherichia coli* remains widely accepted as top choice among the bacterial cells. Free and immobilized *Escherichia coli* 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 a close correlation between the toxic effects in free and immobilized cells. The protease activity increased significantly with an increase in the dose and duration of exposure to methomyl in both free and immobilized cells, however, in comparison with its free counterpart the protease activity was less in immobilized *Escherichia coli* cells revealing that immobilized system shows selective proteolysis and is less sensitive to the toxicant.
Key words: methomyl, protease, *Escherichia coli*, free cells, immobilized cells.

Introduction

Methomyl is a carbamate insecticide used for vegetables and fruits and the mode of action is by the inhibition of acetyl cholinesterase [1]. It has been classified by the WHO, EPA (Environmental Protection Agency, USA), and EC (European Commission) as a very toxic and hazardous pesticide [2]. 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 [3]. Genotoxic effects of methomyl in *in-vitro* studies have also been reported [4].

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 [5]. Proteases in *Escherichia coli* 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, and protein turnover during starvation and inactivation of functional or regulatory proteins [6]. Proteases are involved in the degradation of cell envelope proteins in *Escherichia coli* and are highly sensitive to proteolytic degradation in the periplasmic space [7]. The oxidative damage to proteins may be an important factor in triggering their rapid degradation *in vivo*. The selective elimination of such damaged polypeptides can be an important protective mechanism for the cell [8]. It has been proposed that inactivation by oxidants may be a specific mechanism initiating the breakdown of critical proteins by the enzyme protease, in *Escherichia coli* [9]. Several efforts have been undertaken to search for a proteolytic system that selectively attacks the oxidized proteins and moreover, extracts of *Escherichia coli* have been shown to degrade rapidly the damaged enzyme, but not the native protein and several preliminary reports have appeared concerning an *Escherichia coli* protease that may be responsible for selective degradation of the modified proteins [8]. Proteolysis in bacteria is a widely studied phenomenon that ensures correct functioning of the cell. It is involved in essential functions such as native protein turn-over and recycling, protein activation and abnormal protein degradation, as well as nutrient recognition and degradation into peptides and amino acids that can be assimilated. Proteolysis is thereby involved in diverse processes, including nutrition and growth, protein export and secretion, stress resistance and survival, and regulation [10, 11]. Proteolysis is ensured in the different cell compartments by the presence of differentially localized proteases in *Escherichia coli* and *Bacillus subtilis* [11, 12]. The present investigation was therefore, undertaken to elucidate the effect of methomyl on the protease activity in free and immobilized *Escherichia coli.*

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 *Escherichia coli* was procured from NCL, Pune and the bacteria was maintained at 4°C on nutrient agar and sub cultured very fortnight [13].

Medium used for the study

Sterile synthetic medium (S-medium) was used as the medium for toxicity testing [14].

Preparation of inoculum for free cells

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.

Preparation of immobilized cells

Immobilized cells were prepared by mixing pellet of bacteria obtained by centrifuging the 24 hours culture in nutrient broth at 3,000 rpm for 20 min with 4% sodium alginate (prepared in 0.1 N NaCl) at a final concentration of 1.5% wet weight of bacteria. The mixture was dropped into 4% $CaCl_2$ by syringe and kept at 4°C for 12 hours for hardening. The beads were washed twice with normal saline and maintained at 4°C in normal saline until use.

Experimental procedure

Free cells*:* 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 methomyl. 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.

Immobilized cells: Immobilized beads were inoculated to 250 ml Erlenmeyer's flask containing 100 ml of sterilized S-medium amended with different molar concentrations of methomyl. 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 protease activity of the respective samples was measured as per the standard procedure [15]. 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's 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 ml of the sample.

Statistical analysis

Statistical significance between the control and experimental data were subjected to analysis of variance (ANOVA) together with Dunnett's test ($P<0.05$).

Results and discussions

The present investigation was attempted to study the effect of methomyl on the protease enzyme activity in free and immobilized *Escherichia coli cells* that were exposed to different concentrations of methomyl ranging from 10^{-7} M to 10^{-3} M of methomyl for a period of 96 hrs and was compared to their corresponding controls. The activity of protease observed in the control group of free *Escherichia coli* cells was 0.42, 0.48, 0.56 and 0.62 U at duration of 24, 48, 72 and 96 hrs respectively. On treatment with 10^{-7} M of methomyl the protease activity was 0.50, 0.59, 0.69 and 0.79 U at duration of 24, 48, 72 and 96 hrs respectively. Treatment with 10^{-6} M of methomyl showed an activity of 0.55, 0.69, 0.90 and 0.98 U at the duration of 24, 48, 72 and 96 hrs respectively. On treatment with 10^{-5} M of methomyl the protease activity was 0.60, 0.80, 0.99 and 1.14 U at duration of 24, 48, 72 and 96 hrs respectively. Treatment with 10^{-4} M of methomyl showed an activity of 0.69, 0.90, 1.20 and 1.30 U at the duration of 24, 48, 72 and 96 hrs respectively. On treatment with 10^{-3} M of methomyl the protease activity was 0.78, 1.10, 1.30 and 1.40 U at duration of 24, 48, 72 and 96 hrs respectively **"Fig.(1)".**

In the immobilized *Escherichia coli* cells the protease activity observed in the control group was 0.29, 0.36, 0.41 and 0.46 U at a duration of 24, 48, 72 and 96 hrs respectively. On treatment with 10^{-7} M of methomyl the protease activity was 0.37, 0.42, 0.45 and 0.51 U at duration of 24, 48, 72 and 96 hrs respectively. At 10^{-6} M of methomyl the protease activity observed was 0.41, 0.45, 0.48 and 0.54 U at duration of 24, 48, 72 and 96 hrs respectively. On treatment with 10^{-5} M of methomyl the protease activity was 0.47, 0.50, 0.59 and 0.63 U at duration of 24, 48, 72 and 96 hrs respectively. On treatment with 10^{-4} M of methomyl the protease activity was 0.53, 0.57, 0.65 and 0.70 U at duration of 24, 48, 72 and 96 hrs respectively. Treatment with 10^{-3} M of methomyl showed the protease activity of 0.58, 0.65, 0.70 and 0.80 U at duration of 24, 48, 72 and 96 hrs respectively **"Fig.(2)".**

In the present study, the activities of the enzyme protease was compared with the dose and duration of exposure to methomyl in both free and immobilized *Escherichia coli* and the results revealed that the protease activity increased significantly 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. Similar observations were made on the analysis of the stress proteins of *Escherichia coli* that was induced in response to the pesticides cypermethrin, zeta-cypermethrin, carbofuran and bifenthrin [16]. The increase in the protease activity observed in our results could be due to the expression of intracellular proteins, which require cell lysis for

purification which will result in exposure to proteases [17] or since the proteases are a widespread group of enzymes that catalyze, the hydrolysis of different proteins and perform a pivotal role in the degradation and turnover of intracellular proteins [5]. Further, it has also been reported that the protease are found in one or more sub cellular compartments in *Escherichia coli*, including the cytoplasm and the periplasmic space and preferentially brings about rapid intracellular degradation of the proteins [6]. 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 [18] and moreover, extracts of *Escherichia coli* have been shown to degrade rapidly the damaged enzyme, but not the native protein, and several preliminary reports have appeared concerning and *Escherichia coli* protease that may be responsible for selective degradation of the modified proteins [8].

Our experimental data indicate that the protease activity is more in free cells than its immobilized counterpart since the free *Escherichia coli* cells possess antioxidant enzymes, which are induced in response to oxidative stress and are directly exposed to the pesticide. Although antioxidant enzymes play a major role in the cellular defense mechanism, they are susceptible to inactivation by reactive oxygen species in free state than the immobilized state and it has been revealed that the immobilized system exhibited lesser stress and higher tolerance to methomyl when compared to its free counterpart [19]. It has been suggested that the use of immobilized system is more advantageous over the use of free living cells whenever they are applied to bioremediation of soil/water contaminated by the pesticides [20]. It has been reported that the protease activity increased with the increase in the dose and duration of exposure of methomyl in both free and immobilized cells and in comparison with its free counterpart the activity was less in immobilized *Escherichia coli* cells revealing that immobilized system is less sensitive to the toxicant [21]. It is also reported that protease is essential for *Escherichia coli* to survive at elevated time and temperatures, and degrades abnormal and misfolded proteins [22].

It has been suggested that proteases are of vital importance to all bacteria and is one of the bacterial resistant mechanisms against toxic components is mediated by proteases present on the microbial surface [23]. The increase in the protease activity could be due to the expression of intracellular proteins, which require cell lysis for purification which will result in exposure to proteases [17]. Proteases are a widespread group of enzymes that catalyze the hydrolysis of different proteins and perform a pivotal role in the degradation and turnover of intracellular proteins [5]. Further, it is suggested that the protease are found in one or more sub cellular compartments in *Escherichia 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 [6]. It is reported that, protease expression will be induced under stress as seen for the *Escherichia coli* and *Lactobacillus helveticus* and is essential for growth at high temperatures and under normal growth conditions, it is involved in surface proteolysis of abnormal proteins, conditions [24]. It has been suggested that one of the major functions of extracytoplasmic proteases is to protect the cells against effects of toxic peptides [25]. 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 [18] and moreover, extracts of *Escherichia coli* have been shown to degrade rapidly the damaged enzyme, but not the native protein, and several preliminary reports have appeared concerning and *Escherichia coli* protease that may be responsible for selective degradation of the modified proteins [8]. It has been suggested that endogenous proteases may play an important role as a protection mechanism available to organisms against peptides.

The significant decrease in the protease activity in free and immobilized *Escherichia coli* on dose and durational exposure of methomyl observed in the present study may be due to expression of intracellular proteins, which require cell lysis for purification which will result in exposure to proteases [17], hydrolysis of different proteins that perform a pivotal role in the degradation and turnover of intracellular proteins [5], homeostasis maintenance and metabolism regulation in the cell [6], protect the cells against effects of toxic peptides [25], selective degradation of the modified proteins [8].

Conclusion

We have reported the effects of the enzyme protease on exposure to methomyl in free and immobilized *Escherichia coli*. The comparative effect of protease on different dose and durational exposure of methomyl in free and immobilized *Escherichia coli* has also been elucidated. The results confirm that lesser protease activity is induced in immobilized cells than in the free cells. The inert nature of the synthetic matrix gives additional advantage of high rigidity with high resistance to degradation and microbial attack. Thus immobilization seems to be more promising in the development for the selective degradation of proteins enhancing biotransformation and bioremediation of xenobiotics.

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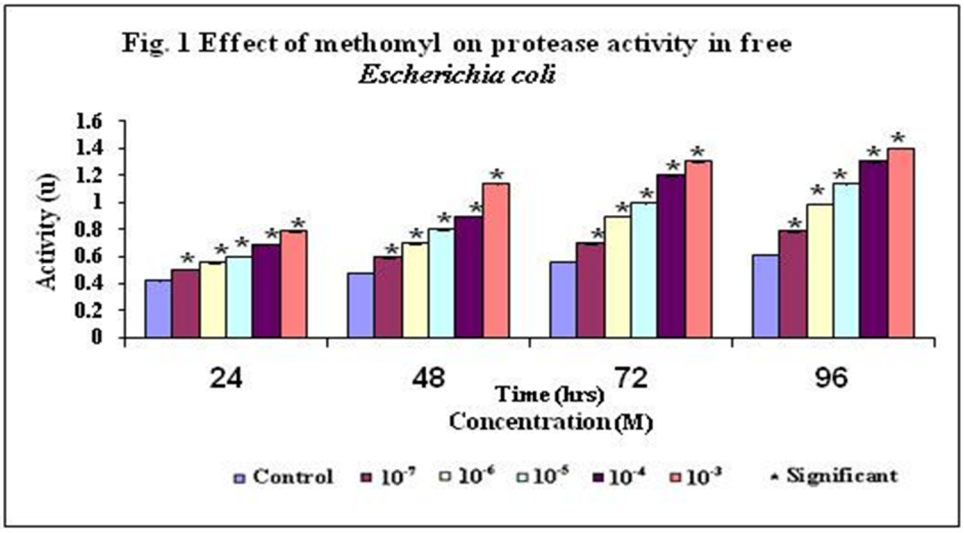

Fig. 1 Effect of methomyl on protease activity in free *Escherichia coli*

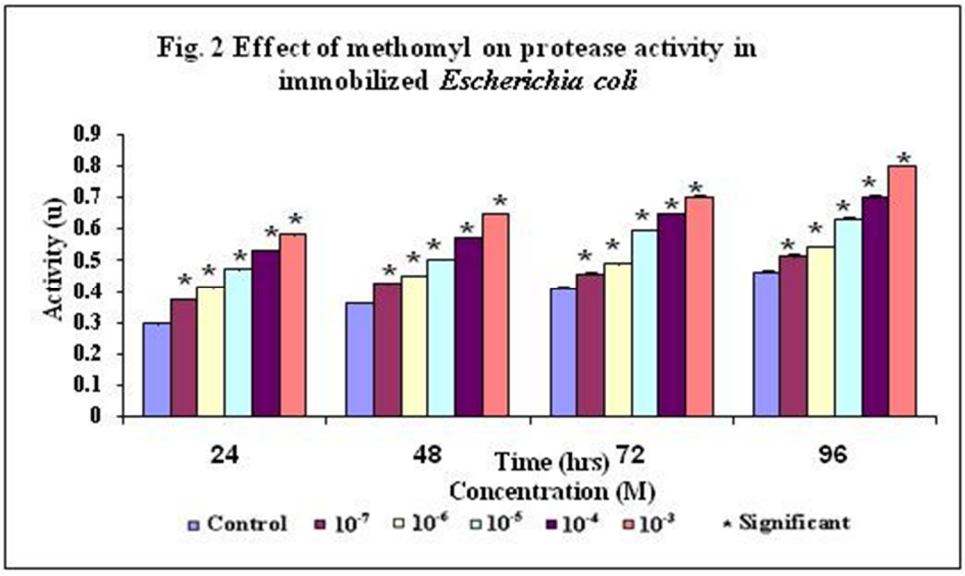

Fig. 2 Effect of methomyl on protease activity in immobilized *Escherichia coli*