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Original Article

Isolation and partial characterization of organophosphate pesticide degrading bacteria from soil sample of Noakhali, Bangladesh

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Extensive use of organophosphate pesticides particularly malathion can result in pollution of soil, surface water and ground water, and thus disrupts ecosystem byexposing non-target species to its toxicity. Bioremediation with indigenous microorganisms having pesticide utilizing abilities is considered to be a viable solution regarding decontamination of organophosphate residues from pesticide contaminated soil. In this study, we isolated five malathion degrading bacter ial strains designated as S-1, S-2, S-3, S-4 and S-5 from paddy fields of Noakhali, Bangladesh by observing visible growth in malathion supplemented mineral salts medium (MSM) agar following selective enrichment technique. The isolates were provisionally identified based on their morphological and biochemical characteristics as *Pseudomonas* spp. (S-1), *Bacillus subtilis* (S-2), *Staphylococcus aureus* (S-3), *Pseudomonas aeruginosa* (S-4) and *Pseudomonas* spp. (S-5) respectively. To determine their malathion utilization potential, the isolates were inoculated in MSM containing 50 mg/l malathion as sole source of carbon. When compared with control, the turbidometric growth study with the isolates revealed that all the isolates showed a significant increase of growth, indicating utilization of malathion (conc. 50 mg/l) in MSM at 37ºC. The rate of growth varied for all the isolates when this growth study was done using different temperature schemes (25ºC, 35ºC and 45ºC).

Keywords: Pesticides, organophosphates, Malathion, Biodegradation, Bioremediation

Introduction

As the demand for agricultural crops increases, so unsurprisingly does the need for pesticides. Bangladesh is no exception in its excessive and recurrent use of pesticides including malathion[1]. Malathion, S-(1,2-dicarbethoxyethyl)-O, O-dimethyl dithiophosphate, also known as carbophos, maldison and mercaptothion is a nonsystemic, wide-spectrum organophosphate insecticide used extensively to control a wide range of chewing and sucking insects in paddy fields of Bangladesh[2]. It is classified as a toxicity class III pesticide with a maximum permitted amount of 8 parts per million (ppm) of malathion to be present as a residue in specific crops used as foods by the U.S. Environmental Protection Agency, EPA[3].

Malathion irreversibly inactivates acetylcholine esterase (AChE) at various sites resulting in an accumulation and continued action of neurotransmitter acetylcholine at postsynaptic sites and may cause spasms, in coordination, convulsions, paralysis and ultimately death to non-target species of soil and surface water[4, 5].It has been estimated that approximately less than1% of the total applied pesticides reach to their target pests[6].This result in accumulation of high levels of its residues and toxic intermediates in soil, surface water and ground water by bulk handling in the farmyard and the rinsing of containers[6- 7]. These health and environmental concerns have prompted researches focusing on detoxification and removal of residual malathion from soil and water.

Bioremediation by microorganisms capable of degrading organophosphate compounds (OPs), including malathion, has emerged as a way of decontamination of these pesticide residues as they possess a wide variety of enzymes to break down the active molecules to produce less toxic products. Bioremediation is a potential cost effective solution that is considered to be a viable, environment-friendly approach for removal of organophosphate molecules. It is also an attractive alternative to other conventional techniques as it does not produce harmful toxic intermediates[8-10]. Numerous bacterial and fungal species have been isolated and characterized that can degrade malathion. Goda*et al.* isolated and characterized five bacterial species (*Pseudomonas spp., Pseudomonas putida, Micrococcus lylae, Pseudomonas aureofaciens,* and *Acetobacter liquefaciens)* capable of degrading malathion to malathion monocarboxylic and dicarboxylic acids, which formed as a result of carboxylesterase activity[7]. Kim *et al.* characterized a fungal cutinase from *Fusarium oxysporum* that was able to remove 60% of malathion (500 mg/l) [11]. Researches based on malathion degrading bacteria demonstrates that indigenous bacteria isolated from contaminated sites have the genetic and metabolic make up for malathion degradation as they possess catabolic genes responsible for production of a number of malathion degrading enzymes such as carboxyl esterase, malathion esterase and malathion dicarboxy lateoxi reductase that convert malathion to more simpler products such as thiophosphate and phosphate[12].

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Since malathion degrading bacteria are often prevalent at contaminated sites, studying these bacteria as well as their growth kinetics in presence of malathion is necessary to aid in the development of effective bioremediation strategies. This study was designed to isolate and identify new bacterial isolates from pesticide contaminated soil from Noakhali, Bangladesh. The bacterial isolates were comparatively characterized based on their ability to utilize malathion as sole carbon source. Their malathion utilization potential under different temperature schemes was also monitored.

Materials and methods

Collection and preparation of soil sample

With the help of sterile spatula, approximately 10gsoil sample was collected from each of the several sites of different paddy field of Noakhali, Bangladesh that have undergone repeated treatment with chemical pesticides. The soil sample mainly constituted top soil layer. All the samples were then transported to the laboratory for analysis within a short period of time.

Selective enrichment for pesticide degrading baceteria

For selective enrichment, analytical grade malathion (PESTANAL, Fluka chemical, Sigma) was used as a as a sole source of carbon. Sterilized liquid minimal salt medium (MSM) was used for selective enrichment which contained following ingredients with their concentration in the parentheses: NH_4Cl (0.05%), $Na_2HPO_4.2H_2O$ (0.64%), KH_2PO_4 (0.025%), $MgSO_4.7H_2O$ (0.02%), NaCl (0.05%). About 250ml sterilized MSM broth was taken in an Erlenmeyer flask and malathion (50mg/l) was added to it as sole source of carbon. A control flask was maintained without the pesticide to compare the pesticide degradation ability of the bacteria. An amount of 2.5g soil sample was measured and added to the pesticide containing enrichment media and the control flask. Both the flasks were incubated at 37ÚC for 7 days. The flasks were observed periodically for change of colour or formation of turbidity due to pesticide degradation.

Isolation of pesticide degraders on solid media

Sterilized MSM agar media with 2% agar supplemented with malathion (50mg/l) was poured onto sterile petri plates and allowed to solidify. A loopful of enrichment culture from the pesticide supplemented flask was streaked onto the surfaces of the pesticide supplemented solidified MSM agar media. The plates were then incubated at 37ºC for 7 days. A control plate containing MSM media without pesticide supplementation was maintained for each of the treated plates. After incubation, both control and treated plates were observed for bacterial growth. The colonies that had grown in pesticide supplemented plates were selected for further investigation. Selected colonies were presumed initially as pesticide degraders and were repeatedly streaked on nutrient agar plates to obtain pure, isolated colonies. The isolates were preserved as stock culture in nutrient agar slants at 4ºC.

Morphological and cultural studies of the selected isolates

The selected isolates were streaked on nutrient agar medium to distinguish their morphological characters such as size, shape (rhizoidal, irregular, circular, undulate, spindle, filamentous, punctiform etc.), edge (curled, lobate, entire, crenate, dentate, filamentation, rhizoidal, etc.) elevation (raised, flat, convex, concave, papillate, umbonate, etc.), opacity, surface (smooth, rough, glistering, crispy etc.) and color of the colony (various shades of color). For the study of size and shapes of the bacterial cells, two types of differential staining techniques, gram staining and endospore staining was performed to observe the shape and arrangement of vegetative cells as well as differentiate the isolates into different broad groups such as Gram positive, Gram negative, spore or non-spore formers.

Screening of the isolates based on their growth rate

Growth of the different organisms was tested by growing each isolate in a 150 ml conical flask containing 50 ml of the screening medium (MSM) supplemented with 50mg/mlstock solution of malathion. To make sure all the inoculums had same density of bacteria; 1mlinoculum culture in LB broth was taken in a micro-centrifuge tube and centrifuged at 5000 rpm for 10 minutes to separate the cells from the growth medium. The supernatant was poured off and the pellet containing bacterial cells was washed twice with sterilized 0.9N NaCl solution. The cells were then diluted with 0.9N NaCl solution to adjust their optical density (OD) value to 1.0 measured at 600 nm with an UV-Visible spectrophotometer. The ability of each isolate to utilize pesticide was assessed by periodical measurement of turbidity at 600 nm using a UV-Visible spectrophotometer. For each isolate, a control flask was maintained without the addition of any pesticides. The OD_{600} value of the control was also measured and recorded following aforementioned procedure. To select the most potent degrading strains paired t-test was performed with OD_{600} values of control and pesticide supplemented flasks using Graph Pad Prism software[13]. The islolates, which showed significant difference ($P<0.05$) in growth by utilizing malathion compared to control in the growth curve, were selected for further analysis.

Growth comparison of the isolates based on temperature

The isolates were assessed for their pesticide degrading capabilities in different temperature schemes. For this experiment, the isolates were inoculated in 50 ml MSM media supplemented with 50 mg/l of malathion and incubated at 25ºC, 35ºC and 45ºC respectively. The inocula were prepared by following the previously mentioned method mentioned in above section.

Identification of the isolates based on their morphological and biochemical properties

The isolates that showed significant pesticide degrading capabilities were selected for identification based on their morphological and biochemical characteristics. By following

Bergey's Manual of Systematic Bacteriology[14] the following important physiological and biochemical tests of the isolated bacteria were carried out *viz.,* Gram staining, endospore staining, catalase test, mannitol fermentation, oxidase test, glucose fermentation, fluorescent pigment formation test, methyl red test, voges-proskaur test, h_2s production, citrate utilization test, starch hydrolysis, gelatin hydrolysis, motility test, indole test, and urease test.

Statistical analyses

All the statistical analyses were done using Graph Pad Prism software[13]. Paired t-test was performed to obtain P-value to determine the significance level ($P<0.5$) of an experiment when two sets of data were compared. Two-way ANOVA test was performed when a set of grouped data was analyzed to obtain the significance of the results.

Results

Selective enrichment

In this study, indigenous pesticide degrading bacteria present in the soil sample of pesticide treated paddy fields were selectively enriched by allowing them to grow in a malathion supplemented MSM medium at 37ºC. We found that after 96 h of incubation, the treated flask shows a change of colour to a yellowish-orange hue and develops turbidity, indicating possible utilization of malathion. The control flask without pesticide showed no change in colour and turbidity after complete incubation period.

Isolation of pesticide degrading bacteria

Pesticide degrading bacteria was isolated by growing the selectively enriched culture at 37ºC for 7 days in malathion supplemented MSM agar plates following streak plate method. Numerous colonies having wide variety of colony characteristics were found on the malathion supplemented plates. Morphological analysis showed that the isolates comprised of a variety of gram positive, gram negative, spore former and non-spore former bacteria having different types of cell shape and arrangements. Among the isolates, 5 were selected for further degradation analysis based on their morphological variation by eliminating similar isolates according to results of Gram staining, endospore staining and cultural properties on nutrient agar. The selected isolates were coded as S-1 (Gram negative non-spore forming rods), S-2 (Gram positive spore forming rods), S-3 (Gram positive non-spore forming cocci), S-4 (Gram negative non-spore forming rods) and S-5 (Gram negative non-spore forming rods).

Screening of isolates pesticide degradability

In order to obtain a scenario of comparative degradation potential of the isolates, they were grown separately on malathion supplemented liquid MSM media at 37ºC for a period of 5 days. Out of 5 isolates, all of them degraded malathion as evident by the gradual increase in turbidity (OD_{600}) over the period of 5 days (Figure 2). When compared to control, the growth of the isolates significantly increased ($P<0.05$) when malathion was used as sole source of carbon (Table 1). Isolate S-4 showed the most increase in growth utilizing malathion as evident by 19% increase of OD_{600} after 5 days incubation at 37ºC, followed by 13% increase by isolate S-5 and 11% increase in OD_{600} by isolate S-3 (Figure 1). The isolates varied in their rate of growth using malathion as sole source of carbon. Isolate S-1, S-3, S-4 and S-5 took almost 4 days to reach highest OD_{600} values, while S-2 required 3 days (Figure 2).

Growth comparison of the isolates based on temperature

This experiment was performed to compare the isolates among each other on the basis of their pesticide degrading capabilities under different temperature scheme. For this reason, pesticide supplemented MSM liquid medium was inoculated with same amount of inocula prepared from the selected isolates followed by incubation at 25ºC, 35ºC and 45ºC respectively. After periodic measurement of optical density at 600nm in a UV-Visible spectrophotometer, it was found that in case of almost all the isolates, maximum pesticide utilization occurred at 35ºC (Figure 5). Isolate S-4 showed maximum growth at 35ÚC (21%), followed by isolate S-1 (18.3%) and S-5 (14%) respectively. Change of temperature had a significant effect ($P<0.05$) on growth rate of the isolates (Figure 3).

Identification of the isolates

The biochemical tests used for identification were done based on the algorithm for identifying gram negative rods and Gram

Table 1: *Result of paired t-test between the OD_{600} values of isolates grown in malathion supplemented media and unsupplemented media(control). Only mean OD_{600} were analyzed to obtain significance level.*

Sample analyzed	Number of pairs	P-value	P-value summery	Significantly different (P< .05)
Control (NM) vs S-1 (MS)	5	.0397	*	Yes
Control (NM) vs S-2 (MS)	5	.0393	*	Yes
Control (NM) vs S-3 (MS)	5	.0425	*	Yes
Control (NM) vs S-4 (MS)	5	.0410	*	Yes
Control (NM) vs S-5 (MS)	5	.0414	*	Yes

NM= No Malathion; MS= Malathion supplemented; *= Significant, ns= Not significant

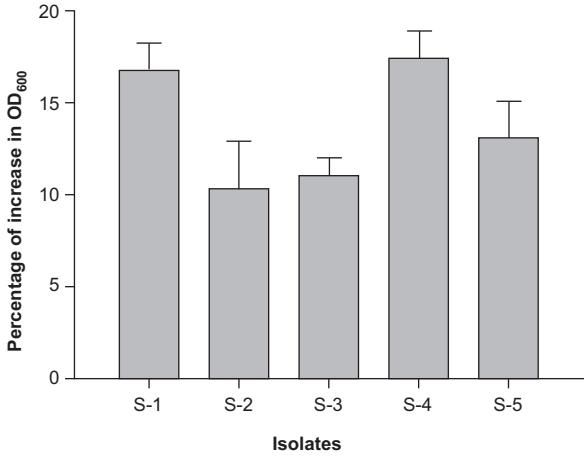

Figure 1. *Comparison among isolates based on their pesticide degrading abilities demonstrated by increase in optical density at 600 nm at 37°C in malathion supplemented MSM liquid media. The percentage in Y-axis was obtained by comparing between OD_{600} values of the initial day (Day-1) and final day (Day-5) of a 5 day incubation period. All the data sets maintained a triplicate to obtain mean and standard deviation.*

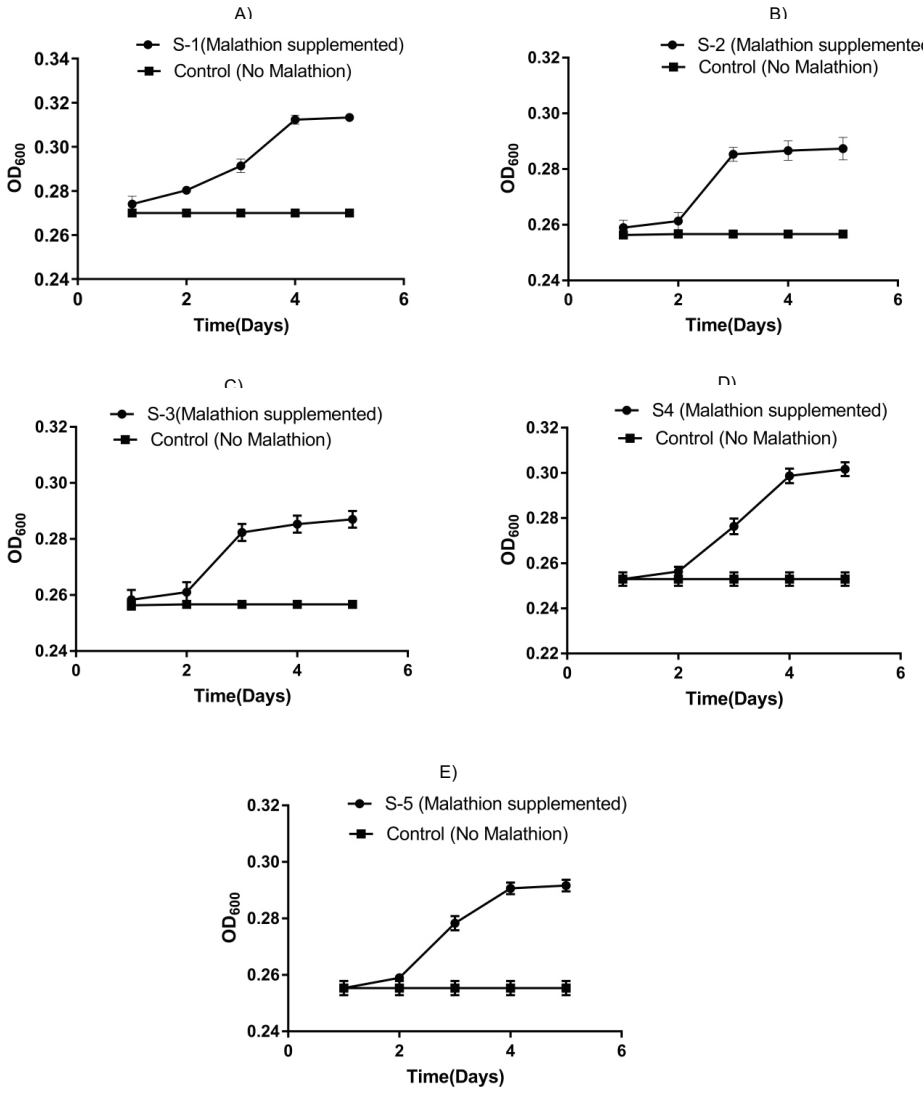

Figure 2. *Demonstration of pesticide degradation by isolates in malathion supplemented MSM media at 37°C represented by increase in OD_{600} as compared to control with no malathion. The experiment was done in triplicates to obtain mean and standard deviation. A) Isolate: S-1, B) Isolate: S-2, C) Isolate: S-3, D) Isolate: S-4, E) Isolate: S-5.*

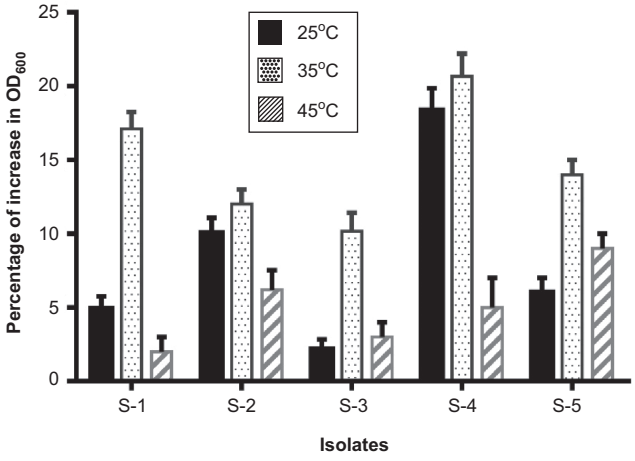

Figure 3. *Comparison among isolates based on their pesticide degrading abilities in different temperature scheme demonstrated by increase in optical density at 600 nm at 25°C, 35°C and 45°C in malathion supplemented MSM liquid media. The percentage in Y-axis was obtained by comparing between OD_{600} values of the initial day (Day-1) and final day (Day-5) of a 5 day incubation period. All the data sets maintained a triplicate to obtain mean and standard deviation. Two-way ANOVA was done in Graphpad prism to analyze the significance level among the growth rates of isolates.*

positive rods and cocci provided in the Bergeys Manual of Systematic Bacteriology[14]. According to the biochemical tests result 3 out of 5 isolates belongs to the genus *Pseudomonas* (Isolate S-1, S-4 and S-5) being Gram negative non-spore forming rods, oxidase and catalase positive. One of the *Pseudomonas* spp. isolate (Isolate S-4) was later confirmed as *Pseudomonas aeruginosa* by observation of formation of fluorescent pigment and growth in cetrimide agar. Other two isolates were provisionally identified based on biochemical tests results as *Bacillus subtilis* (S2) being Gram positive spore forming catalase positive rods and *Staphylococcus aureus* (S3) being Gram positive non-spore forming catalase positive cocci respectively.

Discussion

Chemical pesticides, particularly organophosphates have adverse effects on both terrestrial and aquatic ecosystem due to their toxicity and resistance to microbial degradation. Bioremediation with indigenous microbes can provide a natural solution to the ever increasing toxic effects of pesticides[8].

We used soil sample from paddy fields heavily sprayed with pesticides as a source of indigenous bacteria having pesticide degrading abilities, and found that the pesticide sprayed soil are colonized by a variety of bacteria that can tolerate very high concentration of malathion.

In this study, 5 bacteria were isolated from pesticide contaminated soil that showed visible growth at 37°C in malathion supplemented MSM media. According to morphological and biochemical analysis, three out of 5 isolates belonged to *Pseudomonas* sp. (Isolate S-1, S-4 and S-5). Jilani *et al.* isolated *Pseudomonas* strains capable of growth using 35-200 mg/l of malathion[15]. Characterization studies on these isolates in this study and other researches indicate that these bacteria are gram negative non-spore forming rods capable of degrading petroleum oils, aromatic hydrocarbons and pesticides[16-18]. Other two isolates were provisionally identified as *Bacillus subtilis* (S-2) and *Staphycoccusaureus* (S-3). Salunkhe *et al.* isolated four *Bacillus subtilis* strains from grape rhizospherecapable of proliferating in organophosphate spiked nutrient media[19]. One of the *Pseudomonas* spp. isolates was confirmed as *Pseudomonas aeruginosa* by observation of growth in cetrimide agar plate and fluorescent pigment formation. Involvement of *Pseudomonas aeruginosa* in organophosphate degradation is well documented in previous researches as they possess both genetic and metabolic machinery for organophosphate degradation[20-22]. The variety of bacteria isolated in this study, belonging to both Gram positive and Gram negative genera indicates that pesticide degradation in soil occurs through the activity of a consortium of indigenous microorganisms.

We used selective enrichment technique using malathion as sole carbon source to isolate the pesticide degraders and assessed their degradability at 37°C. All 5 of the selected isolates showed steady increase in OD_{600} values using malathion as sole source of carbon. Malathion had a significant effect on the growth of the isolates as the unsupplemented control flasks didn't show any noticable increase in turbidity (Table 1, Figure 2). The isolates varied in their growth rate as different bacteria peaked in growth (OD_{600}) at different incubation period (Figure 4).Out of the five isolates under study, *Pseudomonas* sp. (S-1), *Bacillus subtilis* and *Staphylococcus aureus* showed significant difference in growth in malathion compared to unsupplemented control media ($P < .05$). These results are similar to the findings of Thabit *et al.* where three isolates (*Pseudomonas aeruginosa, Bacillus pseudomycoides, Bacillus licheniformis*) showed significant increase in malathion degradation rate compared to control at 37°C[23].

Temperature had a prominent effect on the rate of degradation as observed by significant variation in the growth at different temperature. Highest rate of growth in case of all the isolates were seen at 35°C, whereas slowest growth at 25°C was observed in case of *Pseudomonas* sp. (S-5) and *Staphycoccus aureus* (S-3) (Figure 3). This indicates that the preference for optimum growth temperature during pesticide degradation varies with different types of organisms. Further investigation regarding the degradative enzymes could elucidate the metabolic pathways and optimum temperature necessary for bacterial organophosphates degradation.

Conclusion

The present study reports the isolation and provisional identification of five strains of bacteria capable of utilizing malathion as sole source of carbon. Utilization of xenobiotic compounds by soil microorganisms is a crucial criterion by which these compounds are removed from the environment, thus preventing environmental pollution. Results from the present study indicates that the isolated *Pseudomonas* spp.(S-1)*, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa* and *Pseudomonas* spp.(S-5) strains are able to grow in medium in the presence of added organophosphate pesticide (50 mg/l malathion) and may therefore be used for bioremediation of organophosphate pesticide contaminated soil. Further investigation may reveal the genetic and enzymatic basis of pesticide degradation by these bacteria.

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