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SELECTIVE ISOLATION OF A GRAM NEGATIVE CARBAMATE PESTICIDE DEGRADING BACTERIUM FROM BRINJAL CULTIVATED SOIL

Dilara Islam Sharif and Mithun Mollick

Biotechnology and Genetic Engineering Discipline,
School of Life Sciences, Khulna University, Khulna-9208, Bangladesh

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ABSTRACT

A bacterial species having the ability to grow in the presence of carbosulfan pesticide "Marshall" was isolated from *Solanum melongena* (brinjal) cultivated soil in Khulna region, Bangladeh, having a history of pesticide usage. The strain was morphologically and biochemically identified to belong to the genera *Pseudomonas*. A comparative study of growth of this strain with other isolated bacteria showed its ability to grow in the presence of different concentrations of Marshall. The susceptibility of the stain to Marshall was also assessed through disk diffusion assay which showed the strain to be resistant at concentrations of Marshal commonly used under field conditions. The selected strain also showed its capability to degrade Marshall through observed characteristics on sublimated agar plates. The biodegradation capability of the strain isolated in this study can be valuable for further study towards bioremediation of pesticide contaminated soils.

Keywords: Carbosulfan, Pesticide, Microbial Degradation

1. INTRODUCTION

Bangladesh is an agricultural country. Various kinds of pesticides are commonly applied to agricultural fields to increase crop production and pest control. The use of pesticide in Bangladesh has increased many times during the last 20 years. In 1992 the total consumed pesticide was 7400 metric tons in Bangladesh Bangladesh Rice Research Institute (BRRI), But in 2010 it has increased 6 times in 20 years and the consumed amount was 53460 metric ton Bangladesh Bureau of Statistics (BBS). Four types of pesticides are commonly used in the world which is pyrethrins or pyrethroids, organochlorines, neonicotinoids, bacterial products and organophosphates or carbamates. Although the use of pesticides is considered beneficial in augmenting crop yields, excessive and indiscriminate use can lead to microbial imbalance, environmental pollution and health hazards (Köhler and Triebskorn, 2013; Kalia and Gosal, 2011). The long term persistence of pesticide in soil may directly and indirectly affect soil enzyme activities and physiological characteristics of nontarget soil microflora including plant growth-promoting rhizobacteria, which can reduce the performance of several crop plants (Hussain *et al*., 2009; Angelini *et al*, 2013; Hernandez *et al*., 2011). On the other hand growth of various bacteria is promoted by pesticides such as in populations of *Azospirillum* and aerobic nitrogen fixers, growth is stimulated in the presence of pesticide (Kanungo *et al*., 1995).

Various biological strategies are generally devised to remove such harmful pesticides from the environment through a process known as "bioremediation". The biological strategies where microorganisms are used to act upon these pesticides remains the most efficient and cost-effective option to clean up pesticide-contaminated sites. Such types of microorganisms generally have diverse ability to act upon the pesticides including their ability to degrade them in to non toxic compounds. According to Singh and Walker (2006), Isolation of bacteria

Corresponding Author: Dilara Islam Sharif, Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna-9208, Bangladesh

capable of degrading pesticides can be considered important for three main reasons: (1) To determine the mechanism of the intrinsic process of microbial metabolism. (2) To understand the mechanisms of gene/enzyme evolution. (3) To use these microbes for the detoxification and decontamination of polluted aquatic and terrestrial environments (bioremediation). Carbosulfan [2,3-dihydro-2,2-dimethyl-7-benzofuranyl[(dibutylamino)thio] methylcarbamate; CAS Registry no. 55285-14-8; chemical formula: C_20H_{32} N_2O_3S] belongs to the benzofuranyl methyl carbamate group. It is active against caterpillars, green leafhopper, white-backed plant hopper, brown plant hopper, gall midge, stem borer and leaf folder of paddy and white aphids of chilies. Carbamate pesticides have been used extensively in Khulna region, Bangladesh and carbosulfan (trade name 'Marshal') is one of the most commonly used carbamate pesticide in this region. Repeated application of this pesticide can stimulate growth of carbosulfan degrading microorganisms (Sahoo *et al*., 1998). Various bacteria having the ability to degrade carbamate group of pesticides have been isolated from agricultural lands having a history of pesticide usage (Omolo *et al*., 2012). This study focused on the selective isolation and characterization of a gram negative bacterial strain from brinjal (*Solanum melongena*) cultivated lands having a history of the usage of the pesticide "Marshal".

2. MATERIALS AND METHODS

2.1. Pesticide

Carbosulfan pesticide commercial name Marshal-250EC was obtained from the market. According to instruction the applied concentration is 5 mL L^{-1} (5 µL mL^{-1}) of water. Different concentrations (500, 50, 5, 1uL mL^{-1} of pesticide containing broth) were prepared according to study. When mixed with EMB for isolation 1µL mL^{-1} was added to the media. Marshal is slightly soluble in water (0.3 ppm at 25°C).

2.2. Collection of Soil Sample

Soil samples were collected from different brinjal cultivated agricultural lands in Chuknogor, Khulna which had a history of Marshall Pesticide usage. Soils were collected from the 2-5 mm surface and processed to remove lumps and debris. The samples were collected in sterile autoclaved glass bottles; screw capped, labeled and readily brought to the laboratory for analysis.

2.3. Isolation and Identification of Test Organisms

For isolation of insecticide degrading bacteria, one gram of each soil sample was suspended in 9 mL of distilled water. 500 µL of the supernatant was spread on insecticide containing EMB media. A single colony was picked and further purified by streaking on EMB medium. For comparative study the different colony types were selected from pesticide containing EMB plates and pure culture was obtained by repetitive subculture. The isolated strains were then tentatively identified by cultural and biochemical analysis.

2.4. Passive Disk Diffusion Assay

To indirectly measure the toxicity of the pesticide passive disk diffusion assay was performed. Different concentrations (10^{-1}, 10^{-2} and 10^{-3}) of pesticide containing discs were prepared and placed on nutrient agar seeded with lawns of different test organisms. The plates were then incubated at 37°C overnight. Next day the plates were observed for zone of inhibition around the discs.

2.5. Growth of Test Organisms in Presence of Pesticide (Marshal)

Abuot 100 µL of an overnight broth culture of different test organisms were each inoculated in to 100 mL nutrient media containing different concentrations of pesticide. The amounts of pesticide were 500, 50, 5 and 1 µL mL^{-1}. The test was duplicated to reduce error. The optical density of each was recorded at 600 nm at different time intervals. The OD_{600} was plotted against time to obtain growth curve of each test organism at different pesticide concentrations.

2.6. Pesticide Degradation Test or Sublimation Test

Sublimation method was used to deposit a thin layer of pesticide onto the surface of nutrient agar media. The pesticide was sublimed from a heated aluminum dish containing the compound onto the surface of an inverted ice cooled inoculated agar Petri dish. The method resulted in the deposition of a thin even layer in the agar surface. Then all the plates were incubated at 37°C overnight (Huang *et al*., 2008; Alley and Brown, 2000).

3. RESULTS

3.1. Biochemical and Morphological Identification of Test Isolates

Three morphologically different colonies of bacteria were selected from EMB agar plates

containing pesticide. The bacterial colonies were then isolated in pure culture form by repetitive sub culturing on EMB agar plates. The bacterial strains were Termed as test Organism 1 (TO1), Test Organism 2 (TO2) and Test Organism 3 (TO3). The colonies of different isolates are shown in **Fig. 1** and results of morphological and biochemical identification of the isolates are summarized in **Table 1 and 2**. Results indicate TO1 as *Pseudomonas* sp. TO2 as *E. Coli* and TO3 as *Shigella* Sp.

3.2. Analysis of Pesticide Tolerance Through Passive Disc Diffusion Assay

Passive disc diffusion assay was used for susceptibility profiling of the isolated bacteria against Marshal. Different dilutions of Marshall were used and their zone of inhibition was measured in millimeter. Results from passive disc diffusion assay (**Fig. 2**) shows that TO 1 (identified as *Pseudomonas* sp) to be more resistant to the toxic effect of Marshall forming a small zone of inhibition compared to TO2 (*E.Coli*) and TO 3 (*Shigella* sp) at 10^{-2} dilution. No toxicity was observed with TO1 at 10^{-3} and 10^{-4} dilutions indicating TO1 to be more resistant to the toxic effect of Marshal compared to TO2 and TO3. However, for TO2 and TO3 the zone of inhibition increased with higher concentrations of pesticide indicating these isolates to be susceptible to increased concentrations of the pesticide. Results indicate that TO1 is resistant to the toxic effect of Marshal (carbosulfan) pesticide at low dilutions commonly used under field conditions.

3.3. Growth of Test Isolates in the Presence of Marshall

The growth TO1 and TO2 was measured at OD_{600} nm over 14 hrs periods and at different time intervals using different concentrations of pesticide Marshal. Both TO1 and TO2 were compared to respective controls of pure cultures without Marshal (**Fig. 3 and 4**). Since TO2 and TO3 showed a similar response in disc diffusion assay only the result of the comparison between TO2 and TO1 is presented here (**Fig. 5**). Results indicate TO1 to have a similar growth response at different concentrations of pesticide over a 14 h period (**Fig. 3**). However, a decrease in OD was observed initially with pesticide supplemented cultures which decreased with increasing concentrations of pesticide, which may indicate the initial effect of Marshal on microbes. The effect was more pronounced in TO2 compared to TO1 indicating the isolate to be more susceptible to the pesticide than TO1. Results indicate that TO1 can adapt and grow in the presence of the pesticide and has less effect on the overall growth compared to TO2 (**Fig. 5**). In TO2 the pesticide has a marked effect on growth making it more susceptible at increasing pesticide concentration. Since no marked increase in growth was observed in TO1 in the presence of pesticide it could be possible that the organism has the capability to degrade the pesticide instead of using it as growth.

3.4. Sublimated Agar Test

Sublimated agar test is used as an indirect method to measure microbial degradation of the sublimated product on agar plates by the ability of the microbe to grow and form colonies. The test result shows that TO1 has the ability to degrade Marshal through colony formation (**Fig. 6**). However, TO2 showed no growth on sublimated media indicating that TO2 was unable to degrade marshal or the toxic effect of Marshal inhibited the growth of TO2. In this test the concentration of the pesticide used was 5 µL mL^{-1} since marshal pesticide is being sprayed on the field by suspending 100 mL Marshal with 20L water (5 µL mL^{-1}), it has been found that TO 1 has the capacity to degrade and grow at this concentration at which it is being deposited in field.

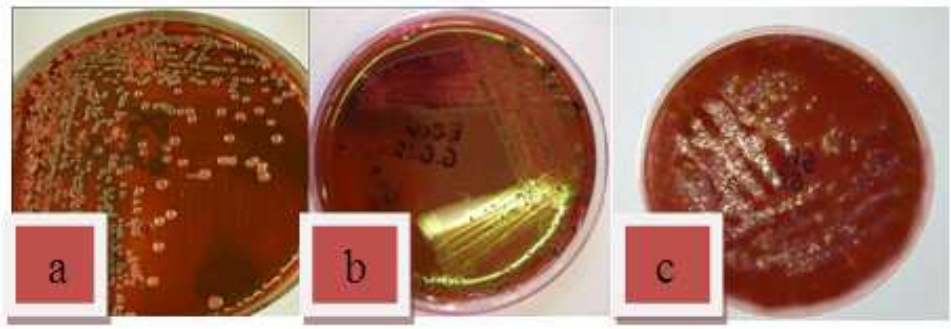

Fig. 1. Pure culture of different isolates on EMB agar (a) TO 1 (b) TO2 (c) TO3

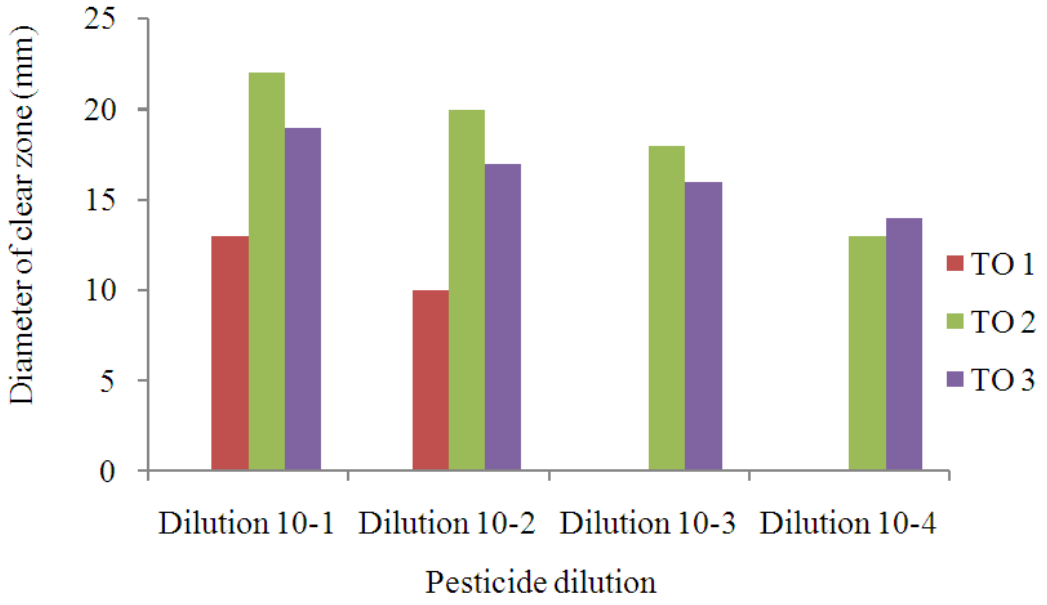

Fig. 2. Zone of inhibition (mm) of different dilutions (10^{-1}, 10^{-2}, 10^{-3}) of Marshall of three test organisms (TO1, TO2, TO3)

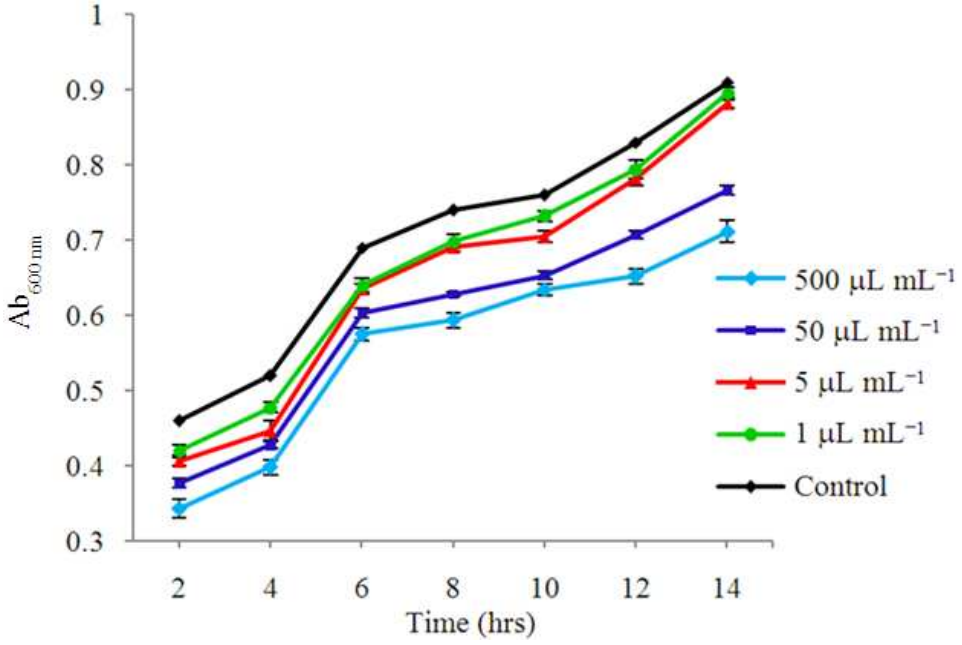

Fig. 3. Growth of TO1 in the presence and absence of Marshall. Cultures with different amounts of Marshall (1, 5, 50, 500 µL) and control cultures (without Marshal) were grown over 14 h time period and OD_{600nm} taken as a measure of growth. Bars represent standard error of triplicate cultures

Table 1. Colony morphology of different isolates on EMB agar and their tentative identification based on biochemical tests (**Table 2**)

Test Organism (TO)	Colors on EMB	Ferfement laclactose	Colony morphology (from EMB agar plates				
			Shape	*Elevation*	*Edge*	*Surface*	Assumed bacteria
TO1	Pinkish	Small amount	Small	Elevated	Flat edges	Rough/mucoid	*Pseudomonas* sp.
TO2	Metal green	Large amount	Small circular	Slightly raised	Smooth	Smooth	*E.coli*
TO3	Colorless	No	small	Raised	Smooth	Slightly rough	*Shigella* sp.

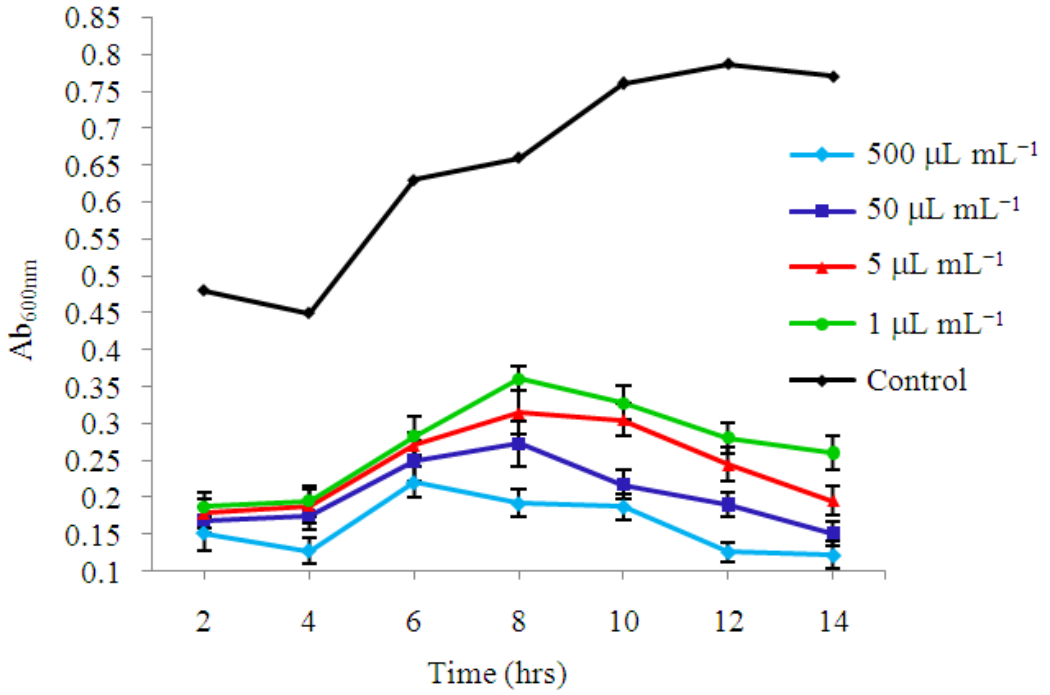

Fig. 4. Growth of TO2 in the presence and absence of Marshall. Cultures with different amounts of Marshall (1, 5, 50, 500 µL) and control cultures (without Marshal) were grown over 14 h time period and OD_{600nm} taken as a measure of growth. Bars represent standard error of triplicate cultures

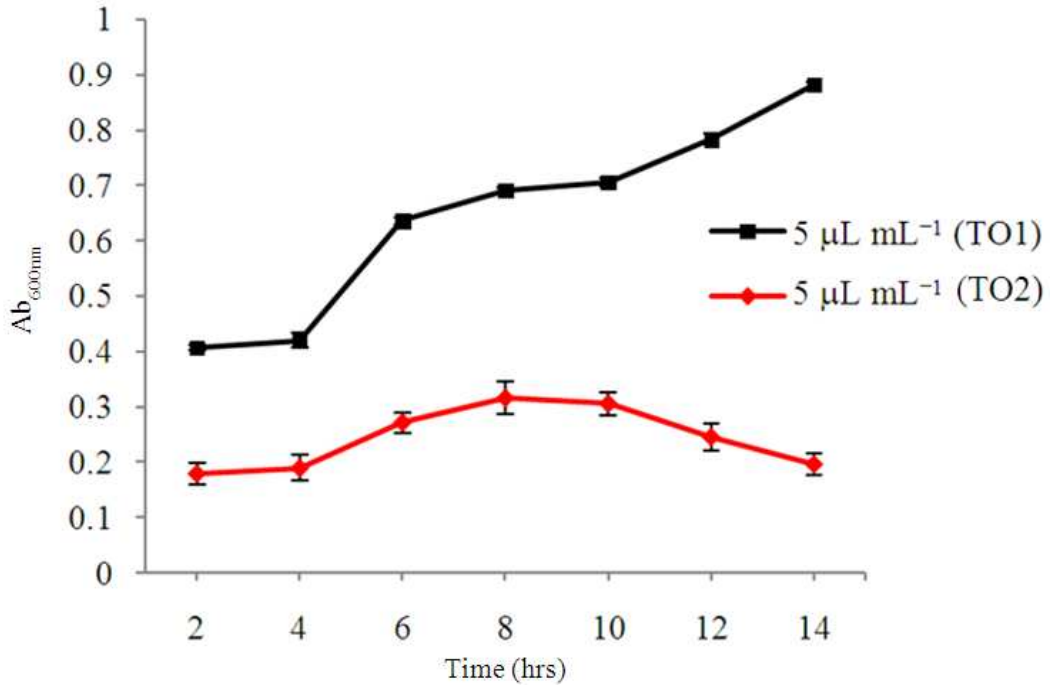

Fig. 5. Comparison of growth of TO1 and TO2 in the presence of Marshall. Growth in the presence of 5 µL of Marshal and control cultures (without Marshal) was grown over 14 h time period and OD_{600nm} taken as a measure of growth. Bars represent standard error of triplicate cultures

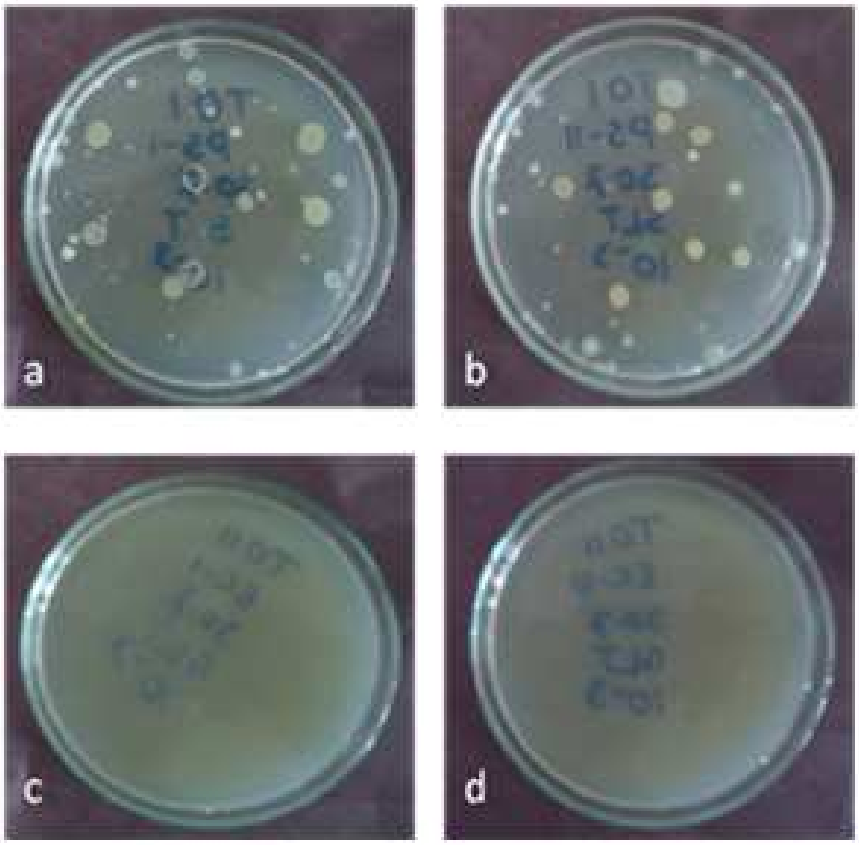

Fig. 6. Marshal Degradation observed through growth on sublimated agar plates: a,b, Showing growth of TO 1 (top figure) and c, d, showing no growth of TO2 (bottom figure) in pesticide sublimed media after 3 days incubation

Table 2. Summary of Biochemical characterization of test organisms (TO 1, TO2, TO3)

Biochemical test	TO1	TO2	TO3
Gelatinase	+	–	–
Nitrate reduction	+	+	+
Methyl red	+	+	+
Voges-proskeaur	–	–	–
Catalase	+	+	+
Oxidase	+	–	–
Urease	+	–	–
Citrate	–	–	–
H2S	–	–	–
Indole	–	+	–

4. DISCUSSION

Brinjal is an important ingenious vegetable crop in Bangladesh. Various pesticides or combination of pesticides are sprayed in controlling pests in brinjal fields. Carbosulfan pesticides commonly used in controlling pests can have toxic effects on indigenous soil microflora as shown in this study. However, the effect can be temporary and last for a short term up to 4 days (Latif *et al*., 2008). The dissipation of carbosulfan can follow from 2-4 days and its residues can persist up to 7 days (Gupta *et al*., 2007). However, injudicious use of pesticide can allow its accumulation in soil and vegetables at much higher concentrations (Bhattacherjee, 2013) which may have profound effect on the environment and health. In Bioremediation, the use of bacteria to degrade and detoxify pesticides when isolated from an indigenous source of natural microbial consortia can offer the advantage of being highly attenuated to the existing ecosystem (Paliwal *et al*., 2012). There have been no reports so far on the isolation of an indigenous natural strain from brinjal fields capable of degrading carbosulfan pesticide which can potentially be used in situ bioremediation. Such technique can provide lower cost and fewer disturbances and can become suited to the country's need. *Psedomonas* is known to be a versatile oraganism having the ability to degrade various kinds of pollutants which has been isolated from various agricultural lands (Jilani, 2013; Abo-

Amer, 2012). In this study a strain identified as *Pseudomonas* showed its tolerance and degradation capability to a carbosulfan pesticide. The study has several implications in bioremidation of pesticides since it is highly adapted to the environment and can overcome some of the drawbacks of bioaugmentation, where non indigenous microbes are imported to contaminated sites which have to compete with the natural microbial community. However, it may be necessary to study the effect of other pesticides against the isolated strain for its efficiency to be used against mixed pesticides. Also the end products of degradation needs to be non toxic and studied through HPLC or Mass spectrometry for further evaluation. For use in bioremediation it will also be essential to determine factors that will enrich the strain in soil (biostimulation) and other factors that will increase the efficiency of pesticide degradation.

5. CONCLUTION

The repeated use of pesticides in field is known to have various effects on soil microbial community (Wang *et al*., 2012) (In many instances pesticides may kill more than just their intended targets which include necessary micro-organisms in the soil. The detrimental effect of pesticides on soil microorganisms may last for years taking several years before microorganisms can once again live in soil that has had toxic chemicals applied to it. In bioremediation, microbes that can degrade pesticides in situ are used. For a successful bioremediation technique an efficient bacterial strain that can degrade largest pollutant to minimum level is required. The finding in this study suggests a possible tool of constructing a bacterial strain that could be useful in bioremediation of pesticides.

6. REFERENCES

Abo-Amer, A.E., 2012. Characterization of a strain of *Pseudomonas* putida isolated from agricultural soil that degrades cadusafos (an organophosphorus pesticide). World J. Microb. Biotechnol., 28: 805-814. DOI: 10.1007/s11274-011-0873-5

Alley, J.F. and L.R. Brown, 2000. Use of sublimation to prepare solid microbial media with water-insoluble substrates. Appl. Environ. Microbiol., 66: 439-442. DOI: 10.1128/AEM.66.1.439-442.2000

Angelini, R., R.J. De Morais, A.C. Catella, E.K. Resende and S. Libralato, 2013. Aquatic food webs of the oxbow lakes in the Pantanal: A new site for fisheries guaranteed by alternated control? Ecol. Modell., 253: 82-96. DOI: 10.1016/j.ecolmodel.2013.01.001

Bhattacherjee, A.K., 2013. Persistence behavior of imidacloprid and carbosulfan in mango (*Mangifera indica* L.). Bull. Environ. Contam. Toxicol., 90: 233-7. DOI: 10.1007/s00128-012-0902-6

Gupta, S., R.K. Sharma, S.R. Sinha, R.K. Gupta and V.T. Gajbhiye, 2007. Persistence of some new insecticides in brinjal and their efficacy against brinjal leafhopper and borer. Pesticide Res. J., 19: 205-209.

Hernandez, P.P., C. Undurraga, V.E. Gallardo, N. Mackenzie and M.L. Allende et al., 2011. Sublethal concentrations of waterborne copper induce cellular stress and cell death in zebrafish embryos and larvae. Biol. Res., 44: 7-15.

Huang, X., Y. Tian, Y.R. Luo, H.J. Liu and W. Zheng *et al*., 2008. Modified sublimation to isolate phenanthrene-degrading bacteria of the genera *Sphingomonas* and *Burkholderia* from Xiamen oil port. Marine Pollut. Bull., 57: 538-543. DOI: 10.1016/j.marpolbul.2007.12.013

Hussain, S., T. Siddique, M. Saleem, M. Arshad and A. Khalid, 2009. Impact of pesticides on soil microbial diversity, enzymes and biochemical reactions. Adv. Agronomy, 102: 159-200. DOI: 10.1016/S0065-2113(09)01005-0

Jilani, S., 2013. Comparative assessment of growth and biodegradation potential of soil isolate in the presence of pesticides. Saudi J. Biol. Sci., 20: 257-264. DOI: 10.1016/j.sjbs.2013.02.007

Kalia, A. and S.K. Gosal, 2011. Effect of pesticide application on soil microorganisms. Arch. Agronomy Soil Sci., 57: 569-956. DOI: 10.1080/03650341003787582

Kanungo, P.K., T.K. Adhya and V. Rajaranamohan Rao, 1995. Influence of repeated applications of carbofuran on nitrogenase activity and nitrogen-fixing bacteria associated with rhizosphere of tropical rice. Chemosphere, 31: 3249-3257. DOI: 10.1016/0045-6535(95)00186-C

Köhler, H.R. and R. Triebskorn, 2013. Wildlife Ecotoxicology of Pesticides: Can we track effects to the population level and beyond? Science, 341: 759-765. DOI: 10.1126/science.1237591

Latif, M.A., M.A. Razzaque and M.M. Rahman, 2008. Impact of some selected insecticides application on soil microbial respiration. Pak. J. Biol. Sci., 11: 2018-2022. DOI: 10.3923/pjbs.2008.2018.2022

Omolo, K.M., G. Magoma, K. Ngamau and M. Tsanuo, 2012. Characterization of methomyl and carbofuran degrading-bacteria from soils of horticultural farms in Rift Valley and Central Kenya. Afr. J. Environ. Sci. Technol., 6: 104-114. DOI: DOI: 10.5897/AJEST11.067

Paliwal, V., S. Puranik and H.J. Purohit, 2012. Integrated perspective for effective bioremediation. Applied Biochem. Biotechnol., 166: 903-924. DOI: 10.1007/s12010-011-9479-5

Sahoo, A., N. Sethunathan and P.K. Sahoo, 1998. Microbial degradation of carbosulfan by carbosulfan--and carbofuran-retreated rice soil suspension. J. Environ. Sci. Health, Part B, 33: 369-379. PMID: 9674149

Singh, B.K. and A. Walker, 2006. Microbial degradation of organophosphorus compounds. FEMS Microbiol. Soc., 30: 428-471. PMID: 16594965

Wang, H.H., P.Y. Huang, G. Xu, W. Haas and A. Marblestone et al., 2012. Multiplexed *in vivo* his-tagging of enzyme pathways for in vitro single-pot multienzyme catalysis. ACS Synth. Biol., 1: 43-52. DOI: 10.1021/sb3000029