Isolation and characterization of *Bacillus cereus* strain JY9 and *Methylobacterium* sp. HJM27 and their growth kinetics studies in presence of pesticides

Nazia. S. Sultan, Bharti. S. Raipat and M. P. Sinha

ABSTRACT

Beneficial soil bacteria contributes a good share in maintaining the fertility of agronomical lands naturally .This work was undertaken with the aim to isolate, identify, characterize and study growth pattern of these non targeted beneficial soil bacteria under the influence of organophosphates and Biopesticides. In this regard the morphological examination followed by biochemical characterization of isolated bacterial culture was done .Further species level identification was carried out based on 16S rDNA sequence method. The results of BLAST and phylogenetic analyses revealed 1318nt and 1347nt contig region of sample F and H which were characterized as *Bacillus cereus strain JY9* and *Methylobacterium* sp. *HJM27* respectively, found homologous with sequence with Genbank accession no. HQ833026.1 and HM243761.1. Growth of *Bacillus cereus* was most adversely affected by Dimethoate followed by Phorate. The effect of Malathion and Chloripyrifos were not significant on its growth. Similarly *Methylobacterium sp* under the influence of Dimethoate, Chloripyriphos and Phorate showed an increased generation time whereas in presence of Malathion, it showed a normal growth curve similar to that of control. The growth pattern of both the species were not much affected by biopesticides. Our results emphasizes on less use of organophosphates in agroecosystem to reduce biotic stress on normal beneficial soil bacteria, that would ultimately be helpful in better crop yield, thereby maintaining natural ecological balance.

Key words: Biopesticides, biotic stress, contig region, Malathion, 16S rDNA.

INTRODUCTION

Increasing food productivity for food security is very essential in many tropical countries (FAO, 2005) and one direct consequence of the booming food production is the threat to natural ecosystems. A significant proportion of the world's biodiversity is recorded in agroecosystems (Pimentel *et al*., 1992). The increasing use of agrochemicals in such systems affects non-target organisms in soil and water (Abdullah *et al*., 1997; Reinecke and Reinecke, 2007) and is often a major factor contributing to declining biodiversity (Barr, 1993; Chamberlain *et al.*, 2000; Robinson and Sutherland, 2002; Benton *et al*., 2003; Bianchi *et al*., 2006). The use of synthetic pesticides as crop protection chemicals have become the most accepted ecological weapons for assure crop production.

With the restricted use of most of the organochlorine insecticides organophosphorus compounds are taking the major share of insecticide consumption in India (Dutta *et al*., 2010) It is of importance to know whether these alternative pesticides have any pronounced influence on the activity of soil microorganism (Aditya *et al*., 1997). From the aspect of environmental pollution, extensive use of pesticides and other agrochemicals not only limits plant growth but also induce mutagenic and carcinogenic effects on non target microorganism (Ajaz *et al*., 2005). Although pesticides may not be universally toxic to all species of microorganism, they have the potential of disturbing microbial events/activities in the environment, polluted by these chemicals (Pimental, 1971). The soil microorganisms like bacteria, fungi, algae and nematodes play important role in soil nutrition through their role in decay of plant and other organic matter in soil as nitrifiers. Anything that disrupts their activity could be expected to affect the nutritional quality of soils and would thus have serious consequences.

Bacillus cereus is a species of Gram-positive bacteria inhabiting numerous environments including soil, plant materials and many foods (Floristean *et al*., 2007). Bacteria associated with roots and the rhizosphere of many plant species known to benefit the plants through growth promotion and biological protection against diseases and pests(Vetrivelkalai *et al.,* 2010). The abundance of cultivable

Methylobacterium clearly correlated with the abundance of other phyllosphere bacteria, suggesting that methylobacteria constitute a considerable and rather stable fraction of the phyllosphere microbiota under varying environmental conditions (Knief *et al*., 2010) The present study was initiated to isolate, identify and characterize and study growth kinetics of the isolated bacteria under the influence of various organophosphates and biopesticides. It is being hypothesized that the growth of the above two bacteria may be disrupted in the culture medium in presence of organophosphates, whereas negligible influence or no hinderance by the biopesticides used under the study.

MATERIALS AND METHODS

Biochemical Characterization

Soil samples were collected as per the method of (Dutta *et al*., 2010) from the surface layer (0-15cm) of the agricultural land of ICAR, Research Complex, Eastern Region, Palandu, Ranchi. Bacteria were isolated from this by spread plate technique (Ahmed and Ahmed, 2006) pure colonies streaked out and sub-cultured on Nutrient Agar. Morphological characteristics were studied and cultures were gram stained. Different biochemical tests were performed by methodologies as described (Green and Bousfield, 1982) for further identification.

Molecular identification/characterization

DNA was isolated from the pure culture (marked as F and H) as per the protocol of (Van Elsas *et al*., 1997), which included mechanical lysis of cells, phenol and chloroform extractions, a potassium acetate precipitation. Its quality was evaluated on 1.2% Agarose gel (Peixoto *et al*., 2002). The 16S rDNA gene was amplified as per the method of (Li *et al*., 2004). Colony PCR was performed with universal primers complementary to phylogenetically conserved portions of the 5' and 3' ends of the 16s rDNAs of *Bacillus cereus* as carried out for *Bacillus thuringiensis.* Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16sF and 16sR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The 16S rDNA gene sequence was analyzed using BLAST with nrdatabase of NCBI GenBank database to find closely related bacterial 16S rDNA sequences. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed according to Neighbour-joining method (Saitou and Nei, 1987) using MEGA 4 (Molecular Evolutionary Genetics Analysis software version 4) (Tamura *et al*., 2007) for studying evolutionary relationship. The bootstrap consensus tree was inferred from 500 replicates (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and were in the units of the number of base substitutions per site. A similar protocol was followed by (Reddy *et al*., 2009) for identification and characterization of *Bacillus cereus*.

Bacterial growth Study

1 ml of overnight culture of each of the two pure isolated bacteria were inoculated in different test tubes containing 20 ml nutrient broth.Two sets (one for *Bacillus* and other for *Methylobacterium*) of ten test tubes were made, to which 100µL of various organophosphates and biopesticides were added in eight different test tubes with one test tube as control (no pesticide) *and one test tube left as blank with only nutrient broth media.* Organophosphates / biopesticides used were50%EC of century (Malathion), BR Agrotech ltd , Dhanwan-20 (Chloropyrifos) Dhanuka Agritech ltd,ROGOR insecticide (Dimethoate), Isagro (Asia) Agrochemicals pvt. ltd, DHAN-10G (Phorate) Agritech pvt.ltd, Biosanjeevani (Trichoderma viride and Pseudomonas fluorescence) Nirmal seeds pvt. ltd, Biosoft (*Beauveria bassiana*) Agriland biotech limited, aqueous extract of karanj leaves and Neemta 2100 (Neem) A.J. Bio-tech. The test tubes were incubated in a incubator shaker at 370C (150rpm) and O.D. at 530 nm, was recorded periodically at a regular interval of 2 hours until the growth reached the stationary phase (Madigan *et al*., 1997).

RESULTS AND DISCUSSION

Colony marked, "H" was circular convex, with entire margin, pink opaque colony and gram negative bacteria ,and was found to be related as *Methylobacterium* as supported by (Kang *et al*., 2007). Similar results were obtained by (Floristean *et al*., 2007) for characterization of *Bacillus cereus* for "F" colonies, which were irregular, raised, undulate, white opaque colony and gram positive bacteria. Further biochemical characteristics were studied and it was observed that bacterial isolates were identified as *Bacillus* and *Methylobacterium* as shown in (Table 1). In biochemical characterization, *Methylobacterium* showed positive result for oxidase test which is favoured by similar observations of (Weon *et al*., 2008) who stated that *Methylobacterium* produces oxidase and catalase. *Bacillus cereus* showed negative result for oxidase, supported by the results of (Chatterjee *et al*.,2010) who justified in his finding that *Bacillus* sp. is oxidase negative.

On quality evaluation of all isolated DNA, a single band of high molecular weight DNA was observed on 1.2% Agarose Gel. A single discrete PCR amplicon band of 1500bp of 16S rDNA was observed when resolved on Agarose Gel as in

Table 1. Biochemical characteristics of the isolates *Bacillus cereus JY9* and *Methylobacterium sp. HJM27*

Isolated organism	Nitrate reduction	Voges Proskauer	Glucose fermantation	Catalase	Oxidase	Motility
Bacillus cereus JY9	+	+	+	+	-	+
Methylobacterium sp. HJM27	-	-	-	+	+	+

(Fig. 1). Similar results were obtained by (Orengo *et al.,* 2010) on amplification of 16S rDNA using universal primer. The consensus sequence (Fig. 2 and 3) of "F" and "H" bacterium were homologous to sequences with GenBank Accession No:- HQ833026.1 and HM243761.1 respectively. Based on nucleotide homology and phylogenetic analysis (Gurtner *et al*.,2001) it was found to be *Bacillus cereus* strain *JY9* and *Methylobacterium sp. HJM27* respectively as in Fig. 4.

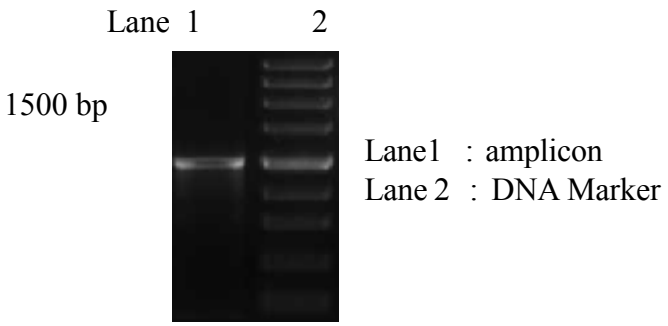

Figure 1. Gel Image of 16SrDNA amplicon

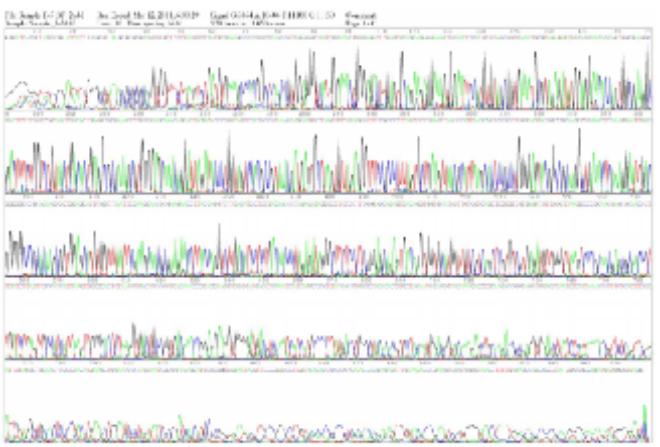

Figure 2. Chromatogram of sample –F (*Bacillus Cereus strain* JY9)

Growth curves of *Bacillus cereus* is most adversely affected by Dimethoate (0.18 O.D, 16 Hour) followed by Phorate (0.25 O.D, 16 Hour) as can be seen in Fig.5 . In comparison to the control (0.62 O.D, 16 Hour) these curves have a longer lag phase and show greater generation time. Chlorpyrifos and Malathion did not affect the growth pattern and the curves were similar to that of the control. This can be explained by the fact that *Bacillus cereus* is capable of degrading Malathion (Singh *et al*., 2009; Shan *et al*., 2009). Tolerance of soil microflora to chlorpyrifos could be due to the effects such as degradation or to the microflora's developing the tolerance to the organophosphate insecticide (Pozo *et al*., 1995). Biopesticides do not show any inhibitory effect on the growth kinetics of *bacillus cereus*. Similar results were obtained by Weller *et al*. (2002); Guo *et al*. (2004) and Huang *et al*. (2005) while studying growth kinetics of bacillus under the influence of *Trichoderma viridae*.

By comparing the mean of all organophosphate treatments with control, it was found that F- value (F=9.90; df=4, 95; P=0.005) is not statistically significant for *Bacillus cereus* JY9 growth, which indicated that the effect of organophosphate treatment is non-significant justifying our hypothesis that organophosphates does not favour the normal growth of *Bacillus cereus* JY9, where as the f -value in case of biopesticides is found to be statistically significant (F=3.53; df=4, 95; P=0.010) indicating that biopesticides

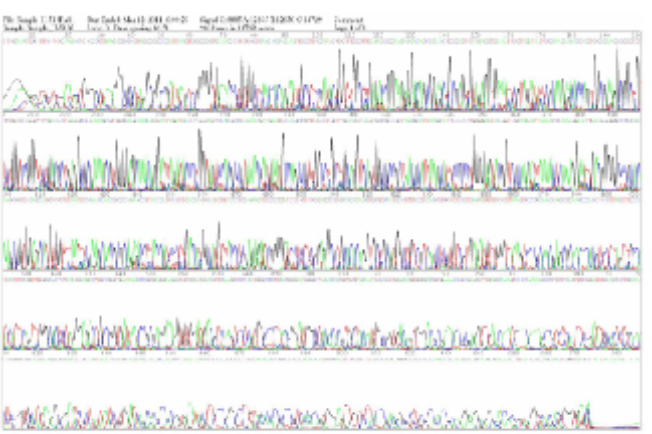

Figure 3. Chromatogram of Sample –H (*Methylobacterium* sp. HJM27)

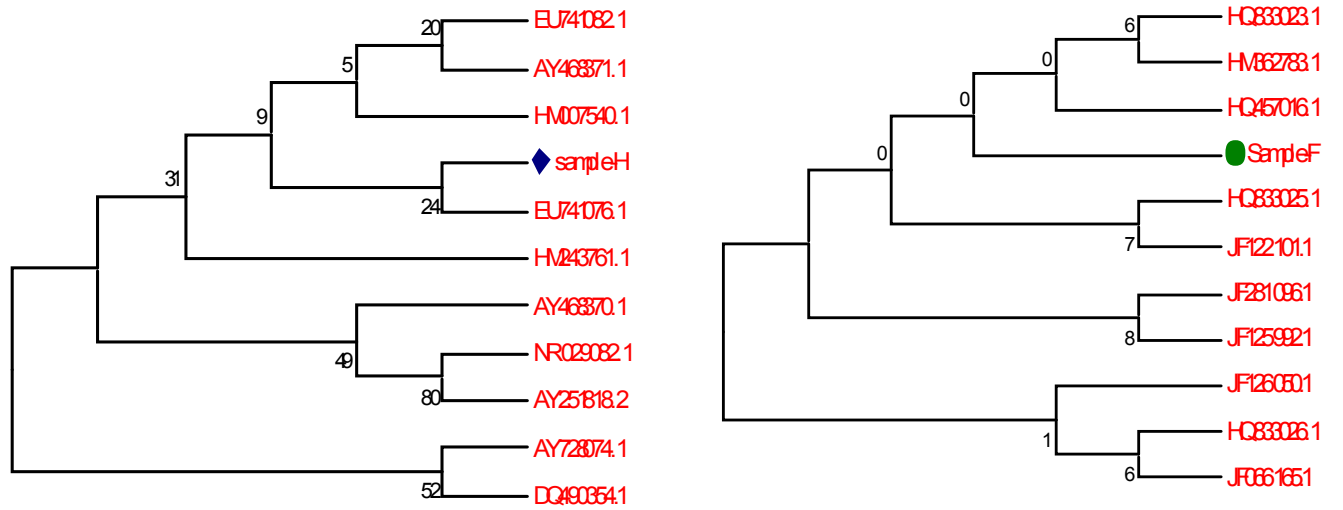

Figure 4. Evolutionary relationships of 11 taxa

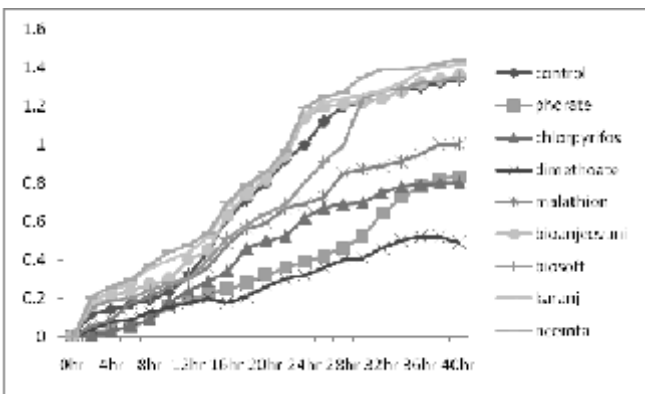

Figure 5. Growth curve of *Bacillus cereus* JY9 in presence of pesticides.

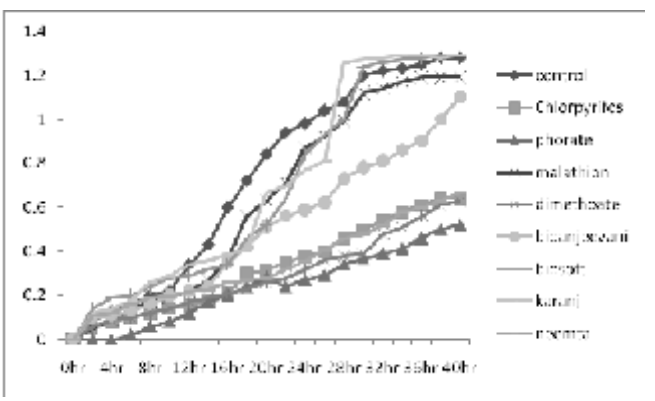

Figure 6. Growth curve *Methylobacterium* sp. HJM27 in presence of pesticides.

favours the normal growth of *Bacillus cereus JY9* as that of control.

Methylobacterium under the influence of Dimethoate (0.2 O.D, 16 hour) and Chlorpyriphos (0.2, 16 hour) showed an increased generation time .Under the influence of Phorate, *Methylobacterium* showed different pattern of growth, at 40 hours O.D. was recorded to be 0.5 which was quiet different from that of control *i.e.* O.D. 1.3 at 40 hours as shown in (Fig.6). This is contradictory with the findings of Aken *et al.* (2004); Suenaga *et al.* (2001) who reported that *Methylobacterium* sp. are known to degrade Phorate Similar to *Bacillus cereus*, the growth curve of *Methylobacterium* was not affected by biopesticides and were similar to the control.

By comparing the mean of all organophosphate treatments with control, it was found that F value is not statistically significant for *Methylobacterium* sp. HJM27 growth, which indicated that organophosphates does not favour the normal growth of *Methylobacterium* , where as the F value in case of biopesticides is found to be statistically significant indicating that biopesticides does not interfere much with the normal growth of *Methylobacterium*.

ACKNOWLEDGEMENT

I would like to thank Dr. M.P.Sinha, Reader, P.G. Dept. of Zoology, Ranchi University Ranchi, for his criticism and valuable suggestions on this manuscript, and Dr. Bharti Singh Raipat, H.O.D., Dept. of Zoology, St. Xaviers College, Ranchi, for her innovative thoughts and conversations concerning this research.

REFERENCE:

Abdullah, A. R., Bajet, C. M., Matin, M. A., Nhan, D. C. and Sulaiman, A. H. 1997. Ecotoxicology of pesticides in the tropical paddy field ecosystem. *Environmental Toxicology and Chemistry,* **16**: 59–70.

Aditya, C. N., Banerjee, H. and Kole, R. K. 1997. An appraisal of pesticide use in Indian agriculture with special reference to their consumption in West Bengal. *Science and Culture,* **63**: 223-228.

Ahmed, S. and Ahmed, M. S. 2006. Effect of Insecticides on the total number of soil bacteria under laboratory and field conditions. *Pakistan Journal of Entamology,* **28**(2):63-67.

Ajaz, M., Jabeen, N., Akhtar, S. and Rasool, S. A. 2005. Chlorpyrifos resistant bacteria from Pakistani soils: Isolation, identification, resistance profile and growth kinetics. *Pakistan Journal of Botany*, **37**(2): 381-388.

Aken, B.V., Yoon, J.M. and Schnoor, J.L. 2004. Biodegradation of nitro-substituted explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-trinitro-1,3,5-triazine and octahydro-1,3,5,7-tetranitro-1,3,5-tetraxocine by a phytosymbiotic methylobacterium sp. associated with poplar tissues (Populus deltodes x nigra DN 34). *Applied and Environmental Microbiology*, **70**: 508-517.

Barr, C. J. 1993. Countryside survey 1990: main report. Department of Environment, Eastcote, UK.

Benton, T. G., Vickery, J. A. and Wilson, J. D. 2003. Farmland biodiversity – Is habitat heterogeneity the key?. *Trends Ecology and Evolution,* **18**: 182–188.

Bianchi, F. J. J. A., Booij, C. J. H. and Tscharntke, T. 2006. Sustainable pest regulation in agricultural landscapes: A review on landscape composition, biodiversity and natural pest control. *Proceedings of Royal Society,* **273**: 1715-1727.

Chamberlain, D. E., Fuller, R. J., Bunce, R. G. M., Duckworth, J. C. and Shrubb, M. 2000. Changes in abundance of farmland birds in relation to the timing of agricultural intensification in England and Wales. *Journal of Applied Ecology,* **37**: 771–788.

Chatterjee, S., Ghosh, T.S. and Das,S. 2010.Virulence of *Bacillus cereus* as natural facultativepathogen of *Anopheles subpictus* Grassi (Diptera:Culicidae) larvae in submerged rice-fields and shallow Ponds. *African Journal of Biotechnology*, **9(41)**: 6983-6987.

Dutta, M., Sardar, D. Pal, R. and Kole, R. K. 2010. Effect of chlorpyrifos on microbial biomass and activitie in tropical clay loam soil. *Environmental Monitoring and Assessment*, **160**: 385-391.

FAO, 2005. Proceedings of the Asia regional workshop. Regional office for Asia and the Pacific, Bangkok, Thailand.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution,***39**:783-791.

Floristean, V., Carmen CreÑu and Carp-Cărare, M. 2007. Bacteriological characteristics of *Bacillus cereus* isolates from poultry. *Bulletin,* USAMV-CN, **64**: 1-2.

Green, P. N. and Bousfield, I. J. 1982. A taxonomic study of some gram-negative facultatively methylotrophic bacteria. *Journal of General Microbiology*, **12**: 623-638.

Guo, J.H., Qi, H.Y., Guo, Y.H., Ge, H.L., Gong, L.Y., Zhang, L.X. and Sun, P.H. 2004. Biocontrol of tomato wilt by plant growth promoting rhizobacteria. *Biological Control*, **29**: 66-72.

Gurtner, C. S., Maca, S., Rolleke, S., Nig, K., Lukas, J., Hirsch, A., Lubitz, W. and Asenbauer, T. B. 2001. 16S rDNA Based Identification of bacteria from conjunctival swabs by PCR and DGGE fingerprinting. *Investigative Opthalmology and Visual Science*,**42**(6): 1164-1171.

Huang, C.J., Wang, T.K., Chung, S.C. and Chen, C.Y. 2005. Identification of an antifungal chitinase from a potential biocontrol agent, *Bacillus cereus* 28-9. *Journal of Biochemistry and Molecular Biology*, **38**: 82-88.

Kang, Y. S., Kim J., Shin, H. D. and Park, W. 2007. *Methylobacterium platani* sp. nov., isolated from a leaf of the tree *Platanus orientalis. International Journal of Systematic and Evolutionary Microbiology,***57**: 2849-2853.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**:111-120.

Knief, C., Frances, L., Blanco, C. A. and Vorholt, J. A. 2010. Site and plant species are important determinants of the Methylobacterium community composition in the plant phyllosphere. *The ISME Journal*,**4**: 719-728.

Li, Y., Kawamura, Y., Fujiwara, N., Naka, T., Liu, H., Huang, X., Kobayashi, K. and Ezaki, T. 2004. *Sphingomonas Yabuuchiae* sp. nov. and *Brevundimonas nasgae* sp. nov., isolated from the Russian space laboratory Mir: *International Journal of Systematics and Evolutionary Microbiology,* **54**: 819-825.

Madigan, M. T., Martinko, J. M. and Parker, J.1997. Brock Biology Microorganism, 8th ed., Prentice Hall International Inc., New Jersey: 1-986 **PP**.

Orengo, M. J., Marrero, E. N. R. and Velazquez, C. R. 2010. Isolation and characterization of bioluminescent bacteria from marine environments of Puerto Rico.

Peixoto, R. S., Coutinho, H. L. C., Rumjanek, N. G., Macrae, A. and Rosado, A. S. 2002. Use of rpoB and 16S rDNA genes to analyse bacterial diversity of a tropical soil using PCR and DGGE: *Letters in Applied Microbiology,* **35**: 316-320.

Pimental, D. 1971. Ecological effects of pesticides on non-target species. Executive office of science and technology, Supt. Of Documents U.S. Govt. Printing office,Stock No.4 106-0029, Washington, D.C.

Pimentel, D., Stachow, U., Takacs, D. A., Brubaker, H.W., Dumas, A. R., Meaney, J. J., O'Neil, J. A. S., Onsi, D. E. and Corzilius, D. B. 1992. Conserving biological diversity in agricultural / forestry systems. *BioScience*, **42**: 354–362.

Pozo, C., Martinez, T. M. V., Salmeron, V., Rodelas, B. and Gonzalez, L. J. 1995. Effect of chlorpyrifos on soil microbial activity. *Environmental Toxicology and Chemistry*, **14**:187-192.

Reddy D M., Paul D., Reddy, H.K. and Reddy, G. 2009. Characterization and identification of *Bacillus cereus* GMHS: An efficient 2-picoline degrading bacterium. *International Journal of Integrative Biology*, **5**(3) : 187-191.

Reinecke, S.A. and Reinecke, A. J. 2007. The impact of organophosphate pesticides in orchards on earthworms in the Western Cape, South Africa. *Ecotoxicology and Environment* Safety, **66**: 244-251.

Robinson, R. A. and Sutherland, W. J. 2002. Post war changes in arable farming and biodiversity in Great Britain. *Journal of Applied Ecology,* **39**: 157-176.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution,* **4**: 406-425.

Shan, X., Junxin, L., Lin, L. and Chuanling, Q. 2009. Biodegradation of malathion by Acinetobacter johnsonii MA19 and optimization of cometabolism substrates. *Journal of Environmental Science* : 76-82

Shishir, A., Akter, A., Hassan, M.H., Kibria, G., Ilias, M., Khan, S.N. and Md. Hoq, M 2012. Characterization of locally isolated *Bacillus thuringiensis* for the development of ecofriendly biopesticides in Bangladesh. *Journal of Biopesticides,* **5**:216-222

Singh,B.,Kaur,R. and Singh, K. 2009.Degradation of organophosphate pesticide,Malathion by *Bacillus* spp. isolated from soil. In: *Proceedings of the 11th International conference on environmental Science and Technology Chania*, Crete, Greece, 3-5 september 2009.

Suenaga, H., Mitsuoka, M., Ura, T., Y. Watanabe,T. and Furukawa, K. 2001. Directed evolution of biphenyl dioxygenase: Emergence of enhanced degradation ..... *Journal* ....., **183**: 5441-5444.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, **24**: 1596-1599.

Van Elsas, J.D., Mantynen, V. and Wolters, A.C. 1997. Soil DNA extraction and assessment of the fate of Mycobacterium chlorophenolicum strain PCP-1 in different soils by 16S ribosomal RNA gene sequence based most-probable-number PCR and immunofluorescence. *Biology and Fertility of Soils*, **24**: 188– 195.

Vetrivelkalai, P., Sivakumar, M. and Jonathan, E.I. 2010. Biocontrol potential of endophytic bacteria on *Meloidogyne incognita* and its effect on plant growth in bhendi. *Journal of Biopesticides*, **3(2)** : 452 - 457

Weller, D., Raaijmakers, J., Mcspadden Gardener, B.B. and Thomashow, L.S. 2002. Microbial population responsible for specific soil suppressiveness to plant pathogens. *Annual Review of Phytopathology*, **40**: 309-348.

Weon, H.Y., Kim, B.Y., Joa, J.H., Son, J.A., Song, M.H., Kwon, S.W., Go, S.J. and Yoon,S.H. 2008.*Methylobacterium iners* sp. nov. and *Methylobacterium aerolatum* sp. nov., isolated from air samples in Korea. *International Journal of Systematic and Evolutionary Microbiology*, **58**: 93–96.

Nazia. S. Sultan[1*], Bharti. S. Raipat[2] and M. P. Sinha[3]

[1]Centre for Biotechnology, Marwari College, Ranchi – 834 001. * Communication author e-mail:naz.cherry@gmail.com

[2]Department of Zoology, St. Xavier's College, Ranchi - 834 001

[3]P.G. Department of Zoology, Ranchi University, Ranchi - 834 008.

Received: 02.08.2012 Revised: 17.12.2012 Accepted: 03.04.2013