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Biodegradation of Organophosphonates by *Aspergillus* Specie

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ABSTRACT

The utilization of organophosphonates as sole source of phosphorus by *Aspergillusniger* was studied. *Aspergillusniger* was found to utilize the phosphorus in diethylethylphosphonate, 2-aminoethylphosphonate and potassium hydrogen phosphate for its growth. *Aspergillusniger*was isolated from rotten pap and identified using standard manuals.The growth of the fungus was monitored by weighing while the phosphate ion released was monitored by UV-Visible spectrophotometer (Genesys IOS VI) at 660nm. The release of phosphate ion was an indication that the first step in organophosphonates degradation by fungus was cleavage of the Carbon-Phosphorus (C-P)bond. There was an inverse relationship between the fungus growth and the amount of phosphate ion in the broth culture medium. The products of degradation were determined by Thin Layer Chromatographic method. The results showed that the phosphate ion in potassium hydrogen phosphate was released into the culture medium faster than that of diethylethylphosphonate and 2-aminoethylphosphonate.

Key word: Organophosphonates, biodegradation,*Aspergillusniger*.

INTRODUCTION

Phosphonates are a class of organophosphorus compounds characterized by Carbon-Phosphorus (C–P) bond[1]. Phosphonatesoccur widely among xenobiotics. Pollution of the environment by phosphonatesaroused interest in their pathways and mechanisms of biodegradation[2]. As a result of their structural similarity to some phosphate esters, phosphonatescould act as inhibitors of enzymes[3]. Bacteria and fungi play a major role in phosphonate biodegradation in soil[4,5,6]. The first phosphonate to be identified to occur naturally was 2-aminoethylphosphonic acid, found in membranes of plants and many animals[3].Organophosphonates are used in large quantities as pesticides, flame retardants, corrosion inhibitors and plasticizers [7]. The Carbon-Phosphorus bond is resistant to chemical degradation[8]. However, there exists a good number of micro-organisms possessing the ability to biodegrade Carbon-Phosphorus bond of

phosphonates through enzymatic hydrolysis [9]. These are widespread among bacteria [10, 5] and many strains of fungi[11,12]. *Bacillus* and Arthrobacterand yeasts were able to grow on phosphonates as the sole source of phosphorus, nitrogen or carbon[1].

Some organophosphorus insecticides, such as diazinon, chlorpyrifos, ethion, parathion, fonofos, malathion and gusathion, are susceptible to microbial hydrolysis and serve as phosphorus source for growth of pure and mixed cultures *Flavobacterium sp.*, *Pseudomonas sp.* and *Arthrobacter sp.*[13-14] reported that *Streptomyces pilosus* is capable of growing on several insecticides such as carbofuran, cloethocarb, trimethacarb, isofenphos, fonofos and phorate.

Several methods such as titrametry, spectrophotometry, gas-liquid chromatography (GLC), High Performance Liquid Chromatography (HPLC), and photometry, have been used for the determination of organophosphorus compounds [15, 16, 17]. The analysis of orthophosphate ions (PO_4^{3-})in fermentation broth of microorganisms has been carried out with the use of Fiske-Subbarow method [18]or through the oxidation of organophosphorus pesticides with slight excess of N- Bromosuccinimide(NBS) and the unconsumed NBS determined with rhodamine-B through spectrophotometry [19]. Various analytical methods have been used to monitor the growth rate of microorganisms in biodegradation studies. The growth kinetics of *Pseudomonas sp.* Strain PG2982 was monitored by measuring the turbidity of culture broth medium using spectrophotometric and colorimetric methods[20, 21]. The growth of *Penicilliumcitrinium* was monitored by weighing bacteria dry mass[12].

The complete mineralization of 2-aminoethylphosphonate, a biogenic organophosphonate of wide distribution occured via its conversion to phosphonoaceltaldehyde and subsequent cleavage of the C-P bond by phosphonoaceltaldehydephosphonohydrolase (phosphonatase). This pathway has been described in cells of *Bacillus cereus* and *Pseudomonas aeruginosa*, which utilized 2-aminoethylphosphonate as a sole phosphorus source[1, 23]. The microbial mineralization of Diethylethylphosphonate to diethylhydroxyl phosphonate has also been previously reported[24]. In this study, we report the capability of fungal strain, *Aspergillus niger* to utilize 2-aminoethylphosphonate anddiethylethyl phosphonate as phosphorus source for its growth.

MATERIALS AND METHODS

Chemicals

The chemicals used were of analytical grade (products of Sigma-Aldrich, USA). They included 2-aminoethylphosphonate, phosphon oacetaldehyde, diethylhydroxylphosphonate, potassium hydrogen phosphate(K_2HPO_4), acetic acid, acetone, potato dextrose agar, iodine, ethanol, calcium chloride, sodium chloride, sodium nitrate, magnesium sulphate, ferrous sulphate, sucrose,tris-buffer, trichloroacetic acid(TCA), 1,2,4-aminonaphthosulphonic acid, sodium sulphite, sodium hydrogen sulphite, and ammonium molybdate.Diethylethylphosphonate was synthesized in our laboratory according to the procedure described previously[25].

Culture Media

The culture media (Czapekdox broth) contained(g/l): sucrose, (30.0); magnesium sulphate, (0.5); calcium chloride, (0.5); ferrous sulphate, (0.1); sodium nitrate, (3.0);tris-buffer, (7.88);phosphorus source(0.0005 mol). 100cm^3 of deionized water was added to the flask to dissolve the salts on a water bath (30^{0}C) with occasional swirling. The pH of the medium was adjusted to 7.2 with 1cm^3 of 1.0 mol/L NaOH . The flask was finally made to mark with deionized water. 2-aminoethylphosphonate (0.0005mol, 0.11g/l) served as phosphorus source in culture medium A, diethylethylphosphonate (0.0005mol, 1.0 ml/l) served as phosphorus source in culture medium B while potassium hydrogen phosphate (0.0005mol, 0.087g/l) served as phosphorus source in culture medium C. Culture medium D contained all the nutrients except phosphorus source.

Isolation of *Aspergillus niger*

Microorganisms were collected from a rotten pap. Individual colonies of the microorganisms were picked up with sterilized

inoculation loop and isolated. The isolated microorganisms were sub-cultured on potato dextrose agar slants. *Aspergillusniger*fungus strain was isolated, purified and identified according to colony characters as described in standard manuals[26-28].

Biodegradation experiments and fungus growth kinetics

The biodegradation experiments were carried out with pure isolated *Aspergillusniger* fungus colony for a period of twenty days. Glass bottles with covers were sterilized for the biodegradation experiments. Into each of the ten sterilized glass bottles were dispensed 47.5cm^3 of culture medium A. This was followed by addition of 2.5cm^3 of the innoculum. The mixture was incubated on a rotary shaker (Uniscope SM 101) at 120rpm at 30^{0}C and assayed for fungal growth by filtering the broth culture in each sample bottle at 48 hours intervals and the dry mass of the mycelia determined. The experiment was carried out in duplicates. Filtrates from the broth culture were used for the determination of the rate of utilization of the phosphorus source by the isolate through spectrophotometric analysis and the detection of the metabolites of organophosphonatesthrough Thin-Layer Chromatographic analysis. The biodegradation experiments were also carried out on culture media B, C, D and E. Culture medium E (Control) is culture medium A without the isolate.
Determination of rate of utilization of organophosphonates by *Aspergillus niger* through spectrophotometric analysis of the filtrates.

The spectrophotometric experiment was carried out on the filtrates of the ten sample bottles containing broth culture medium A.

The following procedure was used for the preparation of the Test and Blank solutions:

Filtrates of Broth culture medium A were used. The analysis of phosphorus in form of inorganic phosphate (PO_4^{3-}) was carried out on UV-Visible Spectrophotometer (Genesys IOS VI) at 690nm, using Fiske-Subbarow method [29, 30]. Trichloroacetic acid[5%(wt/vol) and 9.5cm^3] was placed in a centrifuge tube, followed by addition of 1.0cm^3 of filtrate of broth culture medium A from sample bottle 1. The mixture was stirred by stirring rod and allowed to stand for 5 minutes. The clear supernatant (5.0cm^3) was transferred into a test tube, followed by addition of 1.0cm^3 of ammonium molybdate and 0.5cm^3 mixture of 1,2,4-aminonaphtholsulphonic acid, Na_2SO_3, and $NaHSO_3$. The mixture was thoroughly mixed and allowed to stand for 10 minutes. The procedure was repeated for filtrates of broth culture A in sample bottles 2 to 10. The absorbance for each solution in a cuvet was determined at 690nm against distilled water. Analyses were carried out in duplicates. The experiment was repeated for the filtrates of broth culture of other phosphorus source (i.e. filtrates of broth culture media B and C).

Detection of organophosphonates and metabolites through Thin Layer Chromatography (TLC)

Filtrates from broth culture medium A (sample bottles 3, 6, and 9) were used for the Thin-layer chromatographic analysis. Broth culture filtrates were examined by TLC on hp-TLC (aluminium plastic sheets pre-coated with silica-gel (20 × 20 cm) (Merck, Darmstady, Germany). Chromatograms were developed using n-butanol-acetic acid-water (12:3:5) as a solvent system. Spots were visualized in an iodine chamber. Sample spots were identified by comparing with their corresponding standards. The R_f value of each spot was determined. The TLC of filtrates of culture medium B was also carried out.

RESULTS AND DISCUSSION

The enrichment culture media were able to utilize organophosphonates as sole source of phosphorus. The growth rate of *Aspergillusniger* was observed to increase in all the culture media except the Control as shown in the Table 1 below: *Aspergilllus niger* was able to grow more in broth culture media containing phosphorus sources through enrichment procedure(Table 2). The results of the study showed gradual increase in dry mass of fungus, attaining the highest dry mass in few days and followed by gradual decrease in mass (Table 1 and Figure 1). These observations had been reported by other workers[17,31].The decrease in dry mass of fungus was an indication that phosphorus source was being utilized by fungus

Table 1: Dry mass of *Aspergillus niger* in different broth culture media

Time	Dry mass of *Aspergillus niger* in broth culture media(47.5cm^3)				
(Hours)	A	B	C	D	E
0	0.080	0.030	0.100	0.059	0
48	0.145	0.064	0.148	0.053	0
96	0.189	0.081	0.285	0.060	0
144	0.226	0.085	0.500	0.072	0
192	0.234	0.091	0.700	0.083	0
240	0.268	0.100	0.852	0.096	0
288	0.232	0.160	0.927	0.118	0
336	0.220	0.175	0.921	0.110	0
384	0.185	0.134	0.842	0.090	0
432	0.165	0.062	0.825	0.036	0

and little or nothing was left to support the growth of the fungus. These observations were confirmed by the determination of phosphate ions from phosphorus source.

The fungal growth of *Aspergillus niger* was more in culture media A, B and C than that of culture medium D (Table 2). This showed that phosphorus played a vital role in the growth of the fungus.

*Aspergillus niger w*ould act on phosphorus source by enzymatic hydrolysis of Carbon-Phosphorus (C-P) bond thereby forming phosphate ions.The determination of rate of utilization of the released phosphate ions by*Aspergillusniger* was carried out through spectrophotometric analysis using the method of Fiske –Subbarow. The

Table 2: Effect of phosphorus source on the growth rate of *Aspergillus niger*

Broth Culture Media	Phosphorus source	Fungal growth (dry mass g/l) [using the highest dry mass]
A	2-aminoethylphosphonate	5.64
B	Diethylethylphosphonate	3.68
C	Potassium hydrogen phosphate	19.52
D	No phosphorus source	2.48
E	No fungus	0.00

Table 3: Determination of rate of utilization of phosphorus sources by *Aspergillus niger* through spectrophotometric analysis

Time (hours)	2-aminoethylphosphonate Absorbance (Broth Culture filtrate A)	Diethylethylphosphonate (Broth Culture filtrate B)	Potassium hydrogen phosphate (Broth Culture filtrate C)
48	2.000	2.253	1.500
96	1.500	1.880	1.000
144	0.670	0.890	0.500
192	0.200	0.400	0.110
240	0.050	0.300	0.025
288	0.048	0.298	0.020
336	0.046	0.123	0.010
384	0.044	0.072	0.008
432	0.043	0.040	0.006
480	0.032	0.037	0.001

concentration of available phosphate ions in the filtrates broth culture media A, B and C decreased with time as shown in Table 3.

The disappearance of phosphate ions with the corresponding increase in the growth rate of fungus was as a result of their utilization by the fungus for its growth (Figures 1 and 2).

The fungus was able to utilize potassium hydrogen phosphate, 2-aminoethylphosphonate and diethylethylphosphonate as sole phosphorus source. However, *Aspergillus niger* grew more significantly when potassium hydrogen phosphate served as sole phosphorus source. Potassium hydrogen phosphate being an inorganic compound, would release its phosphate ions more easily than the two organic compounds (2-aminoethyl phosphonate and diethyethylphosphonate).

Therate of growth of *Aspergillusniger*was more in 2-aminoethylphosphonate than diethylethyl phosphonate. This observation could be as a result of the presence of nitrogen in 2-aminoethyl phosphonate which would complement the effect of phosphorus(Table 1).

Certain essential ions are important for

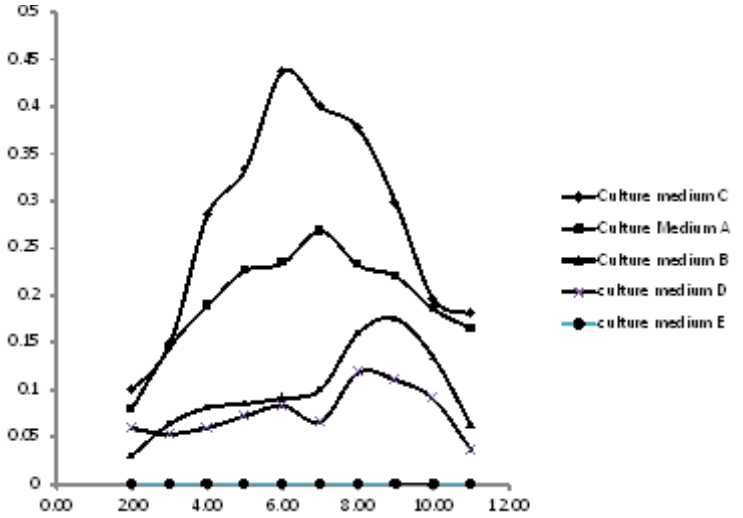

Fig. 1: Growth rate of *Aspergillus niger* in broth culture media

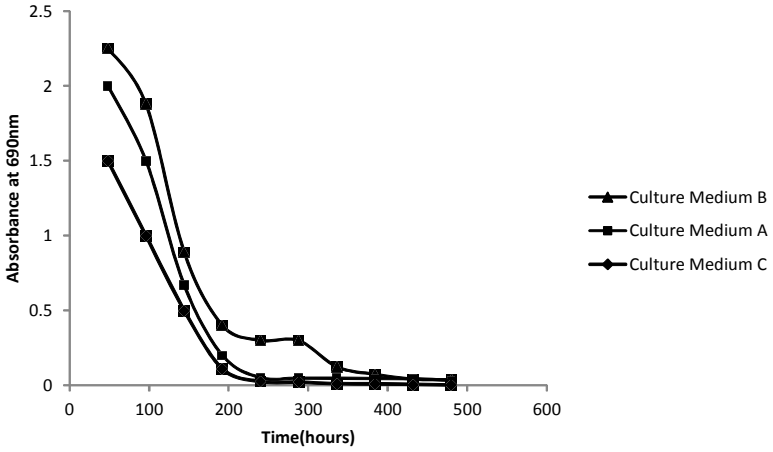

Fig. 2 : Determination of rate of utilization of phosphorus sources by *Aspergillus niger* through spectrophotometric analysis

fungi growth and phosphate is one of them. With the absence of phosphate ions in culture medium D, there was a decrease in the growth rate of *Aspergillusniger*when compared with the broth culture media A, B and C.The microbial activity and organophosphonates degradation followed an inverse relationship(Figures 1 and 2).

The biodegradation products of the organophosphonate compounds were determined by Thin Layer Chromatographic analysis. The organophosphonates and their degradation products were identified by comparing their Retention factor (R_f) with their corresponding standards. 2-aminoethylphosphonate and its metabolite(phosphonoacetaldehyde) were not detected after the 12th day (sample bottle 6) of the biodegradation experiment. This could be as a result of the quantitative release of their phosphate ions. On the other hand there was detection of Diethylethylphosphonate and its metabolite (diethylhydroxylphosphonate) even after the 18th day of the biodegradation experiment. This showed that *Aspergillus niger* degraded 2-aminoethyl phosphonate faster than Diethylethylphosphonate. The present study demonstrates that the isolated *Aspergillus niger* possessed the ability to degrade organophosphonates. This findings could be applied in the bioremediation of soil contaminated with organophosphonate pesticides.

CONCLUSION

The enrichment culture study over a period of time gave indication that organophosphonates could serve as sole phosphorus source for microbial growth. Application of microorganisms could be used to remediate environments that have been contaminated with organophosphonate pesticides. The ability of this isolate to utilize organophosphonate pesticides effectively provides a means of removing them from the environment. The most promising approach for reducing the presence of pollutants in the environment is bioremediation by microbes since they exist in abundance in the environment. This approach is preferred to other methods since it is safer, cost effective and has recorded high degradation rate.

REFERENCES

1. McGrath, J.W.;Ternan, N.G. and Quinn, J.P. Utilization of organophosphonate by environmental microorganism.*Letters in Applied Microbiology* **1997**, *24, 69-73.*
2. Kononova, S.V. and Nesmeyanova, M.A. Phosphonates and their degradation by microorganisms. *Biochemistry (Moscow)* **2002**, *67, 184-195.*
3. Kim, A.D.; Baker, A.S.; Dunaway-Mariano, D.; Metcalf, W.W.;Wanner, B.L. and Martin, B.M. The 2-aminoethylphosphonate-specific transaminase of the 2-aminoethylphosphonate degradation pathway.*Journals of Bacteriology* **2002,** *4134-4140.*
4. Nawaz, K.; Hussain, K.; Choudary, N.; Majeed, A.; Umbrin, I.; Ghani, A.; Lin, F.; Ali, K.; Afghan, S.;Raza, G. and Lashari, M.I., Eco-friendly role of biodegradation against agricultural pesticides hazards.*African Journal of Microbiology Research* **2011**, *5(3), 177-183.*
5. Ortiz-Hernandez, M.L. and Sanchez-Salinas, E., Biodegradation of the organophosphate pesticide tetrachlorvinphos by bacteria isolated from agricultural soils in Mexico. *Rev. Int. Contam.Ambient* **2010**, *26(1), 27-38.*
6. Slaoui, M.;Ouhssine,M.; Berny, E. and Elyachioui, M., Biodegradation of the carbofuran by a fungus isolated from treated soil.*African Journal of Biotechnology* **2007**, *6(4), 419-423.*
7. Hilerbrand, R.L. and Henderson, T.G, Phosphonic acids in nature.In the Role of Phosphonates in Living Systems ed. Hildebrand, R.L. pp5-30. Boca Raton, F.L: CRC Press Inc, **1983**.
8. Kanekar, P.P.;Bhadbhade, B.J.; Deshpande, N.M. and Sarnaik, S.S., Biodegradation of organophosphorus pesticides. *Proc. Indian NatnSci Acad.* **2004**, *57-70.*
9. Singh, B.K. and Walker, A., Microbial degradation of organophosphorus compounds. *F.E.M.S. Microbiol. Rev.* **2006**, *30, 428-471.*

10. Massiha, A.; Pahlaviani, M.R.M.K. and Issazadeh, K., Microbial degradation of pesticides in surface soil using native strain in Iran.*IPCBEE* **2011**, *18, 76-81.*
11. Romero, M.C.; Reinoso, E.H.; Kiernan, A,M. and Cordoba, S., Biodegradation of glyphosate by wild yeasts.Revista Mexicana De Micologia **2004**, *19*, 45-50.
12. Zboinska, E.; Maliszewska, I.;Lejczak, B. and Kafarski, P., Degradation of organophosphonates by *Penicilliumcitrinum*. *Letters in Applied Microbiology* **1992**, *15, 269-272.*
13. Digrak, M. and Kazanici, F., Effect of some organophosphorus insecticides on soil microorganisms.*Turk J Biol* **2001**, *25, 51-58.*
14. Gauger, W.K.; MacDonald, J.M.; Adiran, N.R.; Matthees, D.P. and Walgerbach, D.D., Characterization of a *Streptomycete* growing on organophosphate and carbamate insecticides.*Arch. Environ. Contam. Toxicol.* **1986**, *15, 137-141.*
15. De Pasquale, C.; Fodale, R.; LoPiccolo, L.; Palazzolo, E.; Alonzo, G. and Quatrini, P., Biodegradation of organophosphorus pesticides by soil bacteria.*World Appl. Sci. J.* **2009**, *6(7), 987-998.*
16. Ghosh, P.G.; Sawant, N.A.; Patil, S.N. and Aglave, B.A., Microbial biodegradation of organophosphate pesticides. *International Journal of Biotechnology and Biochemistry* **2010**, *6(6), 871-876.*
17. Sailaja, K.K. and Satyaprasad, K., Degradation of glyphosate in soil and its effect on fungal population.*Journal of Environ. Science and Engg.* **2006**, *48,189-190.*
18. Sudol, T. and Krzysko-Lupicka, T., Direct indicators of determination of glyphosate decomposition by filamentous fungi. *Physicochemical Problems of Mineral Processing* **2005**, *39, 257-261.*
19. Matthew, S.B.; Pillai, A.K. and Gupta, V.K., A rapid spectrophotometric assay of some organophosphorus pesticides in vegetable samples.*Electron J. Environ. Agric. Food Chem* 2006, *5(6), 1604-1609.*
20. Moneke, A.N.; Okpala, G.N. and Anyanwu, C.U., Biodegradation of glyphosate herbicide *in vitro* using bacteria isolates from four rice fields. *African Journal of Biotechnology 9(26), 4067-4074.*
21. Shinabarger, D.L and Braymer, H.D., Glyphosate catabolism by Pseudomonas sp. strain PG 2982. *Journal of Bacteriology* **1986**, *168(2),* 702-707.
22. Dumora, C.; Lacoste, A.M. and Cassaigne, A., Phosphonoacetaldehyde hydrolase from *Pseudomonas aeruginosa* : purification properties and comparison with *Bacillus cereus* enzyme. *Biochimica et Biophysica Acta* **1989**, *997*, 193-198.
23. Cook, A.M.; Daughton, C.G. and Alexander, M., Phosphonate utilization by bacteria. *Journal of Bacteriology* **1978**, *133*, 85-90.
24. Fawole , M.O. and Oso, B.A., Laboratory Manual of Microbiology.Spectrum Books Ltd. **2001**, 127-128.
25. Adelowo, F.E., Synthesis, characterization and fungicidal activity of some dialkylalkylphosphonates and dialkylphenylphosphonates.*International Journal of Research and Reviews in Applied Science* **2012**, *12(1),* 107-114 .
26. Holt, J.G.; Krieg, N.R; Sneath, P.H.A.; Staley, J.T. and Williams, S.T., Bergye's Manual of Determinative Bacteriology, 9th Ed. Williams and Wilkins, Baltimore, Maryland, U.S.A, 1994.
27. Olutiola, P.O.; Famurewa, O. and Sonntag, H.G., An introduction to General Microbiology.HeideibergerVerlagsanstalt Und Druckerel, Heidelberg, Germany, 1991, 267.
28. Christian, G.D., Analytical Chemistry 3rd Edition. John Wiley and Sons Ltd. U.S.A., **1980**, 410-411.
29. Fiske, C.H. and Subbarow, Y., The colorimetric determination of phosphorus. *The Journal of Biological Chemistry* **1925**, *66(2), 375-400.*
30. Olawale, A.K. and Akintobi, O.A., Biodegradation of glyphosate pesticide by bacteria isolated from agricultural soil. *Report and Opinion* **2011**, *3(1),* 124-128