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Enhancing Biodegradation of Methyl-Parathion By *Aspergilli Sp.* from Indian Agricultural Soil

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Articalinfo

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Abstract

The fungal strain *Aspergilli* isolated from an Indian agricultural soil was evaluated for the biodegradation of methyl parathion. The fungal sp. *Aspergilli* showed a better methyl parathion degradation at 36 h than the *Kosakonia* strains. By HPLC-UV analysis, it was observed in the presence of both strains that all methyl parathion was biotransformed and biodegraded in 24 h of incubation. HPLC-ToF and GC-MS analysis were employed for the identification of metabolites from the methyl parathion reactions. The first reaction of the biodegradation pathway was the direct hydrolysis of the pesticide to yield p-nitrophenol by a phase I reaction. The biotransformation of methyl parathion occurs via the nitro group reduction with the formation of an amine group in the phenolic moiety, followed by the amine acetylation to yield an acetamide derivative by phase II reactions. Further biodegradation proceeded with the hydrolysis of the acetamide product, forming N-(4-hydroxyphenyl) acetamide. The fungal strain *Aspergilli* can promote the reduction of p-nitrophenol levels in 12 days, showing potential for future bioremediation studies.

Introduction

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During the last century, advances in synthetic chemistry have enabled chemists to create numerous novel compounds, including xenobiotics (Xu et al., 1989). These are manmade compounds with a structure that microorganisms have never been exposed to. Many of these are recalcitrant i.e, remaining unchanged in the environment. These include some detergents, pesticides, halogenated aliphatic, aromatics, nitrosamines and polycyclic aromatic hydrocarbons. Modern agricultural practices emphasize on extensive use of chemicals, specially designed to target and destroy insect and pests. Accumulation of pesticide residues into the soil and direct entry into the environment make it a potentially dangerous pollutant. Their recalcitrant properties are supposed to be due to polymerization, branched polymer structure, presence of stable bond not subjected to hydrolysis, heterocyclic, aromatic & polycyclic component with presence of chlorine, nitro and sulphonate groups.

Pesticides are broadly classified into *organochlorines, organophosphorus & carbamates.* An important group among pesticides are organophosphorus pesticides. Also known as '**organophosphates**' (OP). An organophosphate is general name for esters of phosphoric acid. These are known for their high specificity & long term impact. The best known of these are 'Metaphos' (synonym ; Methyl parathion). These are among the highly effective organophosphorus insecticides used extensively worldwide compared to other organophosphates.

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Applications of fungi in *Bioremediation.* Excessive use, accidental spillage and the production of large volumes of waste result in pesticide pollution of the environment. Bioremediation provides a cheap and environmentally friendly way to remove these toxic elements from the environment. There has been some success in the use of bacteria and bacterial enzymes for the bioremediation of OP compounds. The use of live cells in bioremediation has inherent practical difficulties (for example, nutritional requirements, availability of fresh inocula and oxygen demand), and as a result the use of purified enzymes has increased. A company in Australia (Landguard; see further information) now sells carrier-based OPH enzyme for removal of OP from sheep-dip waste before it can be applied to soil. Gene and protein engineering have been used to increase the catalytic activity and efficiency of microbial PTEs, using site-directed mutagenesis to eliminate preferential selection of particular enantiomers for PTEs. Other approaches, such as DNA shuffling, have been successfully used to increase the efficiency of degradation of a poor substrate.

Other biotechnological applications of fungi, OPH(MPH) have been successfully used to develop and evaluate biosensors for OP contamination. Two different approaches have been employed for OPH biosensors: a potentiometric approach to measure local pH change and an amperometric measurement of electroactive enzyme products.

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Both of these methods have been combined to produce an improved biosensor tool.

Medical applications of fungi, Various biotechnological efforts have been made to formulate therapies for OP poisoning. OP poisoning, caused either unintentionally or in suicide attempts,has resulted in hundreds of thousands of deaths, and exposure to several millions of individuals, every year. furthermore, the ease of synthesis and availability of OP compounds makes them suitable for terrorist purposes. It is therefore imperative to develop an efficient therapy for patients with severe OP exposure. A limited number of PTEs (mainly enzymes of bacterial and human origins) could be used to treat OP poisoning. The dermal and intravenous administration of bacterial PTEs (obtained from *B. diminuta* and *A. radiobacter*) to experimental animals has been shown to confer prophylactic and therapeutic protection against OP poisonings. Human butyrylcholinesterase and paraoxonase have also been used with limited success. against OP poisoning *in vitro.* The objectives of the study:- To isolate & screen Methyl parathion Hydrolase producing *Aspergilli*.To compare the potential of seven different *Aspergillus niger* towards Methyl Parathion degradation and Methyl parathion Hydrolase production.To quantify the production of methyl parathion hydrolase, by determing enzyme activity.

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Materials and Methods

Collection of samples

Eight soil samples were collected for isolation of fungi , were from eight different sites. Samples from each site were collected randomly just 1 cm below the soil surface with the help of a spatula in sterile polybags. Each polybag was well labelled with the site of collection. Agricultural soil from Makroniya, Sagar(M.P.), Agricultural soil from Pathariya, Sagar (M.P.), Garden soil from Botany Department (Dr. H.S.G.V.V,Sagar), Garden soil from Microbiology Department (Dr. H.S.G.V.V, Sagar), Road side soil, Compost soil and Soil from Chemistry Department (near lab. discharge)

Preparation of samples and Media for Fungal Isolation

The soil samples were processed by removing root pieces & stones. All samples were dried at room temperature. The samples now powdered by grinding & finally filtered through sieve. For isolation of fungi, Sabouraud Dextrose Agar (SDA) media has been used. Dextrose -40.0 gm L^{-1}, Peptone-10.0 gm L^{-} , Agar – agar-20.0gm L^{-1}, Distilled water 1L, pH -7.0. Media were autoclaved at 121˚C , 15 lbs pressure for 20 minutes. After sterilization the medium was poured in sterilized petri plates which after solidification, used for isolation of fungi. Direct plate Method has been used for Isolation of fungi from soil. Fine soil has been sprinkled on SDA plates using sterilized spatula. All plates kept in incubation at 28˚c for 5 days. The fungi thus obtained were point inoculated on fresh plates of

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SDA with the help of inoculation needle. All plates were kept in incubation at 28˚C for 5 days. The isolates of fungi were maintained as stock culture on SDA slants at 4˚C.

Identification of isolated fungi: Fungal isolates were identified on the basis of cultural & morphological characteristics. Cultural characteristics includes front & reverse colour, pigmentation & colony appearance. Morphological characters observed by microscopic examination followed by Lactophenol cotton blue staining under microscope at 10x & 40x.

Screening for Methyl Parathion Hydrolase(MPH) Production: Twelve Aspergilli Isolated have been screened for production of MPH .

Preparation of different concentrations of Methyl Parathion: Concentrations of Methyl Parathion were prepared in ppm.Four concentrations i.e., 15 ppm(0.15 mg in 100 ml Distilled water) ,10 ppm (0.1 mg in 100 ml distilled water),20ppm(0.20mg in 100ml distilled water) & 30 ppm. Composition of Media: $NaNO_3$-2.0 gm L^{-1},KCL -0.5 gm L^{-1},$MgSO_4.H_2O$ -0.5 gm L^{-1},$FeSO_4.7H_2O$-Trace K_2HPO_4-1 gm L^{-1}, Tween 80 -4 ml, Distilled water -1000 ml**,** Vishniac Solution (gm/ltr) **–** Contain EDTA (10), ZnSo4.7H2O (4.40), $CaCl_2.2H_2O$ (1.47).

It is used particularly to enhance the growth of *Aspergilli*. Medium were sterilized at 121˚C , 15 lbs pressure for 15 minutes. After sterilization 1 ml of sterilized Vishniac solution & 1 ml of different concentration of Methyl parathion was added. All test fungi were inoculated in liquid media having Methyl parathion as sole

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carbon source in Modified Czapeks Dox Broth.50 ml of media were taken in well labelled 150 ml Erlenmeyer flask each for 10 ppm & 15 ppm concentration of Methyl parathion. Three disc of each fungal isolate were inoculated in respective flasks. One – one control for each concentration was also prepared having only media but no fungal discs. Flasks now kept in shaker at 28˚C, 120 rpm for 7 days.

Assay of Methyl Parathion Hydrolase : Media after incubation were subjected to filteration & centrifugation (10,000 rpm,10 minutes). The supernatant thus obtained were used for assay of Methyl Parathion Hydrolase activity. Release of para -nitrophenol, an indication of hydrolysis of methyl parathion was taken as a criterion for the estimation of production of methyl parathion hydrolase, which was assayed spectrophotometrically at 405 nm(Absorbance of PNP).

Citrate buffer (0.05 M,pH 5)-1.05 gm of citric acid was dissolved in 100 ml of distilled water. Adjust pH 5 with 0.2 M NaOH.

Substrate solution-50 mg of methyl parathion were dissolved in 50 ml distilled water.

Stopping reagent (1.0 M Na_2CO_3)-Dissolve 10.6 gm of Na_2CO_3 in 100 ml of deionized water.

Standard solution (Stock)-0.0695 pnp (0.01 M) taken in 50 ml of volumetric flask & filled up to mark with citrate buffer.

Table-1

S. No.	Dilutions	Concentration(µmol/ml)	Concentration(nkats/ml)
1	1:20	0.50	0.833

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2	1:50	0.20	0.333
3	1:100	0.10	0.167
4	1:200	0.05	0.083

Enzyme Blank: – Add 1.8 ml of substrate solution , incubate at 50˚C for 60 minutes. Add 1 ml stopping reagent & 0.2 ml of culture filterate.
Blank: – Add 1.8 ml substrate solution; incubate at 50˚C for 60 minutes. Add 1 ml stopping reagent & 0.2 ml buffer.
Standard Solution**: -** Add 1.8 ml of substrate solution; incubate at 50˚C for 60 minutes. Add 1 ml stopping reagent & 0.2 ml of standard solution (Prepare earlier).

Table-2: Take absorbance at 405 nm.

Concentration (µmol/ml)	**Absorbance(405nm)**
0.05	0.057
0.1	0.135
0.2	0.246
0.5	0.631

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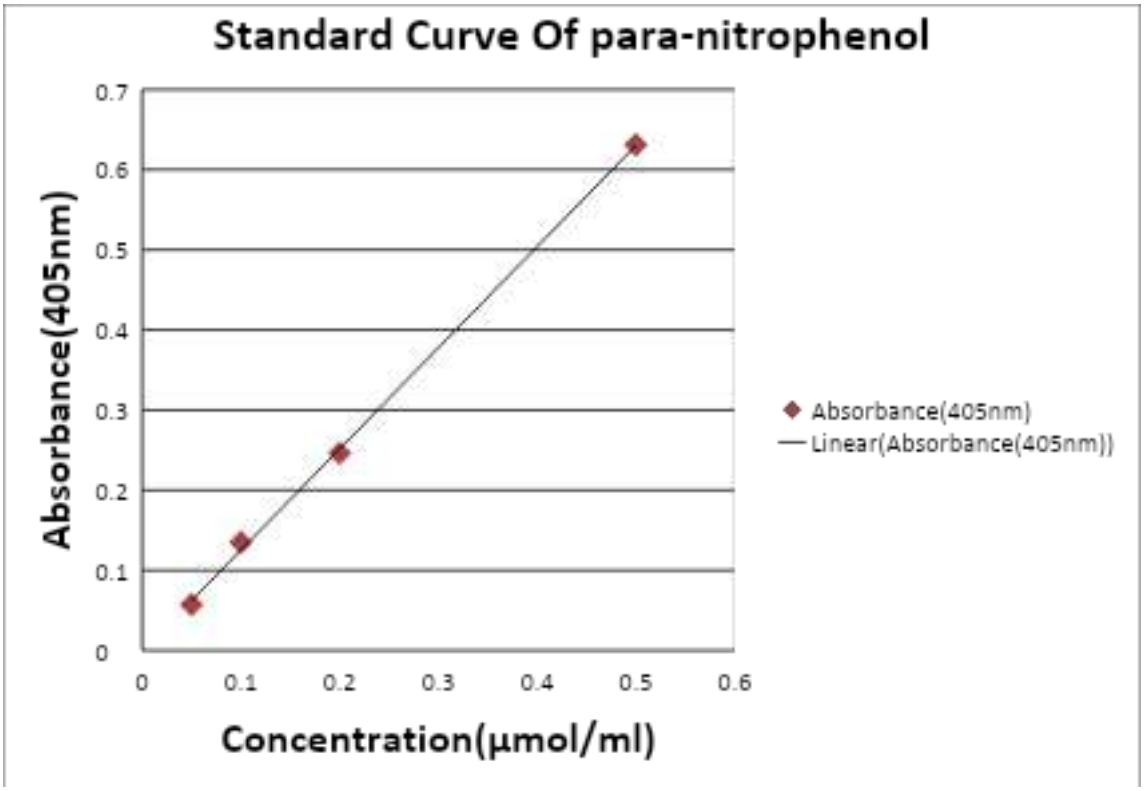

Results and Discussion

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Isolation and Characterization of Fungi Isolated

A total of 12 *Aspergilli* were isolated during the work, from 8 different soil samples. Samples were collected from eight different sites from which twelve different forms of Aspergilli were isolated. All of the isolates were screened under standard conditions of temperature and nutrients for the production of Methyl parathion hydrolase in different concentrations of methyl parathion (ppm). The screening was carried out in modified Czapeks broth in shaker at 120 rpm at 28˚C for 7 days. Quantification of MPH was done by carried out MPH activity based on liberation pnp i.e as much as the MP degraded and MPH produced, more will be the pnp liberated. On screening the different Aspergilli, maximum MPH activity was shown by *A.niger* in both 15ppm &10ppm concentration.While *A.terreus*, *A.versicolor, A.melleus, A.ustus & A. glaucus*, showed less MPH activity in 15 ppm & more activity in 10 ppm. *A.fumigatus* gave less activity in 10 ppm but no activityin 15 ppm. Where as no activity was found in *A.flavus, A.flavipes ,A.penicilloides, A.nidulans & A.tamari.* From all the above results, *Aspergillus niger* was found as potent MPH producer at both 10 & 15 ppm concentration. Thus it was supposed t be valuable to screen different *A. niger* isolated from different sources at more higher concentration of methyl parathion (20 and 30 ppm). The result thus obtained were more promising that many of *A. niger* gave more MPH activity even in higher concentration of methyl parathion.

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Table-3: Number of fungi isolated their source & isolate number.

S. No.	Samples	Isolate number	Fungus Identified
1.	Garden soil(Dept. of microbiology)	GSM1	*A.niger*
		GSM2	*A.fumigatus*
2.	Garden soil(Botany Dept.)	GSB1	*A.niger*
		GSB2	*A.fumigatus*
		GSB3	*A.terreus*
3.	Chemistry Dept.(Near lab. discharge)	CLS1	*A.flavipes*
		CLS2	*A.niger*
		CLS3	*A.fumigatus*
4.	Compost soil	CS2	*A.versicolor*
		CS3	*A.niger*
5.	Agricultural soil(Makroniya),sagar	ASM1	*A.niger*
		ASM2	*A.versicolor*
		ASM3	*A.fumigatus*
6.	Agricultural soil(Kanera),Sagar	ASK1	*A.flavus*
		ASK2	*A.terreus*
		ASK3	*A.fumigatus*
7.	Agricultural soil(Pathriya),sagar	ASP4	*A.niger*
		ASP1	*A.tamari*
		ASP2	*A.glaucus*
		ASP3	*A.melleus*
8.	Road side soil	RS2	*A.ustus*
		RS3	*A.niger*

Primary screening in Broth

All isolated Aspergilli were inoculated in Czapeks Dox Broth, having Methyl Parathion as sole carbon source, in 10 ppm & 15 ppm concentration. The media now kept in shaker incubator at 28˚C for 7 days with control having no fungal discs. After 7 days, media of some fungal flasks became yellow or pale yellow colored as compared to control which was transparent in colour. This observation kept as basis for degradation of Methyl Parathion, which on Hydrolysis by Methyl Parathion Hydrolase librates para-nitrophenol, which gives yellow colour appearance.It was observed that *A.niger* & *A.terreus* gave bright yellow

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colour as compared to other Aspergilli & some of them as transparent as control.

Quantification of Methyl Parathion Hydrolase Production(Secondary screening)

Quantification was done by determining MPH activity in cultural filtrate of different *Aspergilli.* For this Standard curve of para-nitrophenol was used. Absorbance was taken at 405 nm in Spectrophotometer.

Table-4:- Enzyme Activity of Different *Aspergilli***.**

ORGANISM	ENZYME ACTIVITY	
	10PPM	**15 PPM**
A.flavus	NIL	NIL
A.flavipes	NIL	NIL
A.fumigatus	O.145	NIL
A.glaucus	0.736	0.503
A.melleus	1.213	0.937
A.nidulans	NIL	NIL
A.niger	**4.021**	**3.869**
A.penicilloides	NIL	NIL
A.tamari	NIL	NIL
A.terreus	3.546	3.286
A.ustus	3.676	1.866
A.versicolor	0.296	0.198

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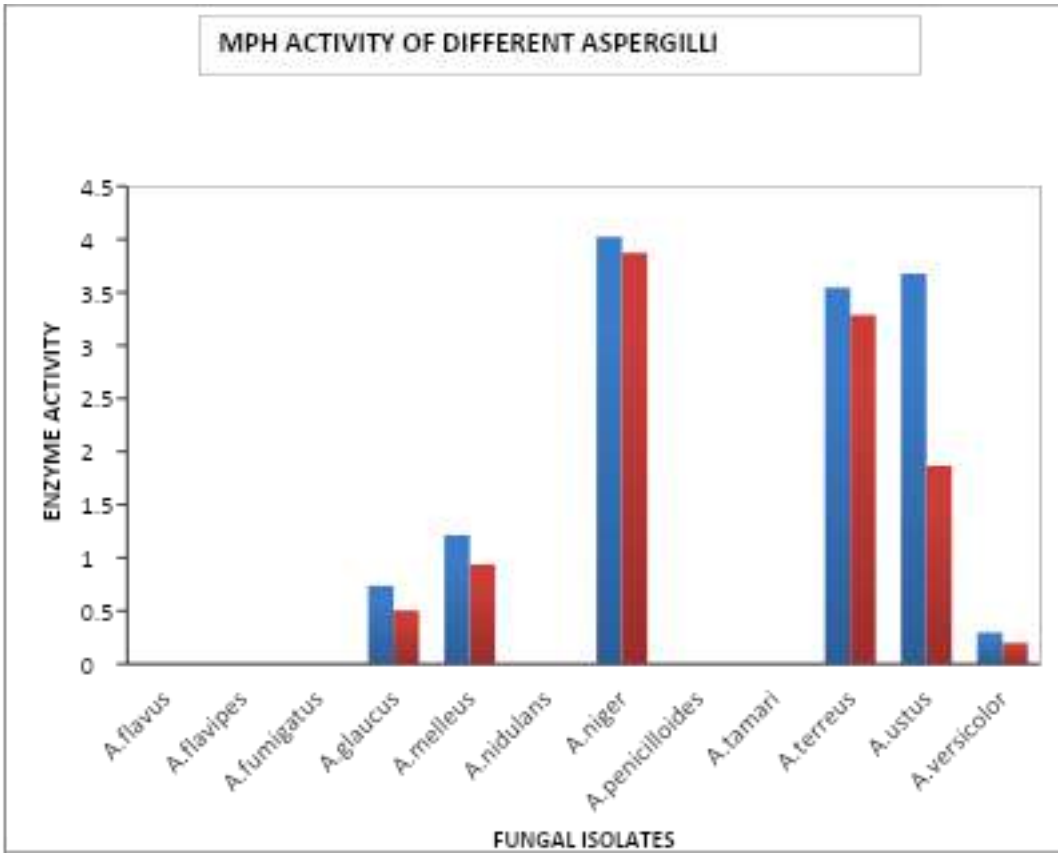

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Fig-1: Enzyme Activity of Different *Aspergilli.*

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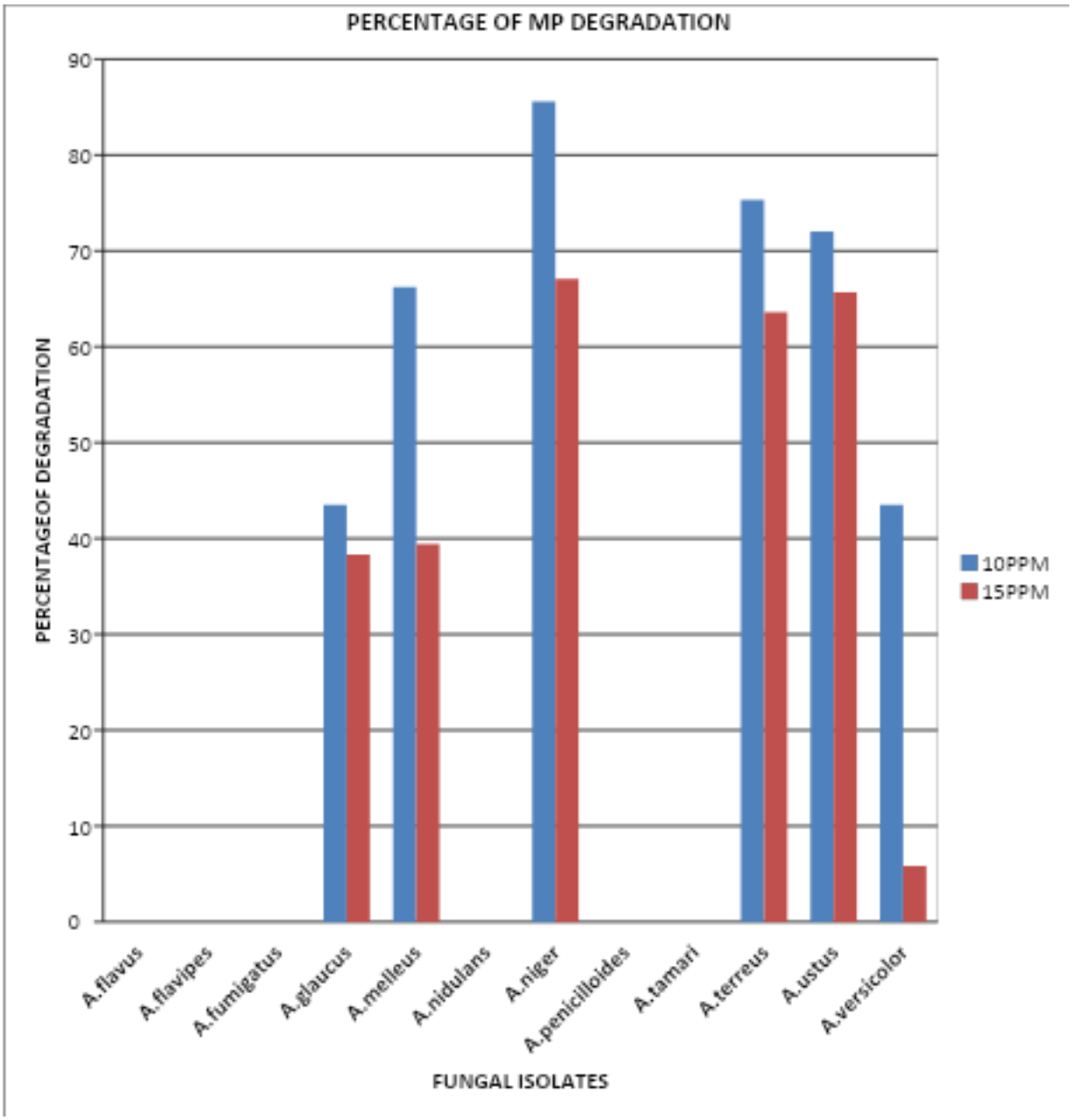

Fig. 3: Percentage of MP degradation by *Aspergilli* in Different concentrations

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Calculation for Methyl Parathion Degradation

After determining Enzyme activity in culture filtrate of Test Aspergilli, percentage of Methyl Parathion was also calculated. The calculation was done by using following formula: *Percentage of Degradation= [1-Absorbance of test sample/Absorbance of control] × 100*

Screening of *A.niger* isolated from different soil samples for MPH activity

After determining the MPH activity & percentage of MP degradation in different Aspergilli, it was noted that only A.niger gave maximum enzyme activity & percentage of MP degradation in both concentrations.Thus it was supposed to be valuable to screen different A.niger isolated from different sources. 3 fungal discs of each isolated A.niger inoculated in Czapeks Dox Broth with Methyl Parathion as sole carbon source. Kept in incubation at 28˚C for 7 days. After incubation broth was filtered & centrifuged(10,000 rpm for 10 min.).Similarv results were indicated . Besides having the highest amount of exoenzyme and endoenzyme, the highest exoenzyme ratio in strain D2 also contributed to the highest atrazine degradation, as exoenzyme facilitated the direct reaction with atrazine in a short time and alleviated the stress of pollutant on strains, while more endoenzyme for D6 and D17 indicated degradation acted only after across the membrane, which needs more time to degradation. Furthermore, trzN in strain D2 can degrade atrazine to hydroxyatrazine. The biodegradation product of atrazine by strain D2 was

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cyanuric acid via dichlorination, hydroxylation, hydrodealkylation, methylation, dealkylation, elimination, and hydrolysis (Yuan et al., 2022). Enzyme Activity now determined as described earlier. Similar results demonstrate that the activity of acetylcholinesterase was unaffected after MP degradation by bacteria(Fernández et al., 2017).

Similarly, the biotransformation of methyl parathion occurs via the nitro group reduction with the formation of an amine group in the phenolic moiety, followed by the amine acetylation to yield an acetamide derivative by phase II reactions. Further biodegradation proceeded with the hydrolysis of the acetamide product forming *N*-(4-hydroxyphenyl) acetamide. *Bacillus* sp.(Natália et al., 2018 and Bhatt et al., 2023.

Table-5:- Enzyme activity of t *A.niger* isolated from different samples.

Source of Isolation	Isolate number	Enzyme Activity(nkats/ml)	
		30 ppm	20ppm
Ground nut seed	A.N-1	2.083	3.670
Compost soil	A.N-2	2.117	4.221
Botanical Garden soil	A.N-3	1.733	3.369
Agricultural soil (Kanera)	A.N-4	**4.912**	**6.542**
Agricultural soil (Makroniya)	A.N-5	2.467	3.503
Chemistry Deptt. Soil	A.N-6	2.413	3.119
Agricultural soil (Pathriya)	A.N-7	**3.72O**	**3.787**

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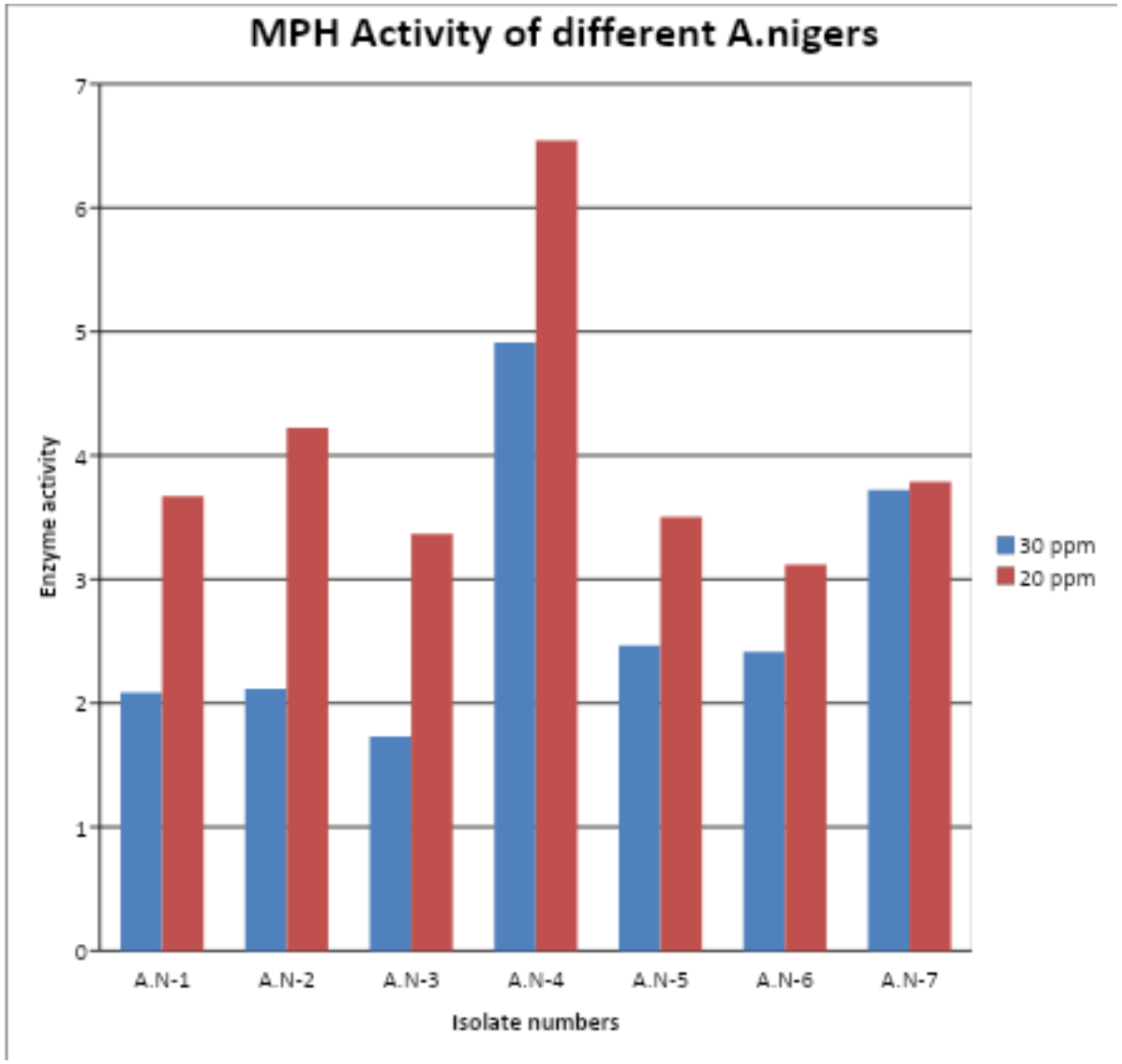

Fig-3:- Enzyme activity of different *A.niger* isolated from different samples

Conclusion

Bacillus sp. CBMAI 1833 and B. cereus P5CNB isolated from a Brazilian peat

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showed two pathways forcommercial MP metabolization. Both strains were capable of not only degrade MP by direct hydrolysisyielding PNP, but also to biotransform the pesticide to a acetamide der ivative, further promoting itshydrolysis in a subsequent step of the degradation process. Bacillus sp. CBMAI 1833 and B. cereus P5CNBwere also capable of biodegrade PNP, showing potential for future bioremediation studies.

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