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BIODEGRADATION OF PESTICIDES BY SOIL ISOLATES OF BENEFICIAL MICRO ORGANISMS

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ABSTRACT

It is well established fact that microorganism plays an important role in cleaning of our environment by degrading several chemicals, pesticides and xenobiotic compounds. Microbial degradation is considered to be an efficient and cost effective method for decontamination of toxic pesticides from the environment. An investigation on biodegradation was undertaken *in vitro* to elucidate the role of bioagents in degradation of three group of pesticides *viz*., Imidacloprid, Pendimethalin and Carbendazim. In this study bacterial and fungal isolates of bioagents were isolated by serial dilution from the pesticide contaminated soil from crop field. The bacterial and fungal isolates were identified as *Pseudomonas fluorescens, Bacillus subtilis, Trichoderma viride* and *Trichoderma harzianum*. *P. fluorescens* was able to degrade 37.25% of Carbendazim, 34.48% of Imidacloprid and 25.66% of Pendimethalin after two weeks of incubation. While *B. subtilis* could degrade 41.17% of Carbendazim, 27.58% of Imidacloprid and 18.58% of Pendimethalin. *T. viride* and *T. harzianum* were able to degrade 31.03% and 28.73% of Imidacloprid and 22.12% and 23.89% of Pendimethalin respectively**.** Fungal species in this study could not degrade Carbendazim due to its fungicidal activity.

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INTRODUCTION

One of the major environmental problems facing the world today is the contamination of soil, water and air by toxic chemicals. Xenobiotic compounds are widely distributed in the environment as a result of their widespread use as pesticides, solvents, fire retardants, pharmaceuticals and lubricants. Several of these chemicals cause considerable environmental pollution and human health problems due to their persistence and toxicity. Eighty billion pounds of hazardous organopollutants are produced annually in agricultural farms and only 10% of these are disposed of safely. India is the largest consumer of pesticides in South Asian countries where maximum (44.5%) consumption of the total pesticides is by cotton crop (Agnihotri, 1999). Recently, it has been estimated that 2.5 million tonnes of pesticides are being applied in each year to the culture of planet and increasing with the passage of time. Only 0.3% of pesticides are being taking in agriculture while 99.7% goes elsewhere (Pimentel, 1995). The use of pesticides in agriculture inevitably leads to the exposure of non-targets organism including human and undesirable side effect can appear on whole species, communities as well as ecosystem; that is why, chlordane and DDT were banned in the United States.

Soil is a dynamic medium for microbial growth serves as a respiratory of biological resources that can exploit in bioremediation of soil pollutants. Among the biological approaches, the use of microbes with degradative ability is considered the most efficient and cost-effective option to clean pesticides-contaminated sites. The hydrolytic enzymes produced during microbial activities degrade the pesticides. Bioremediation method to treat pesticides in soil has gained considerable attention due to their eco- friendliness. They have been used in many countries successfully (Ritmann *et al*., 1988).

A reliable method to detoxify contaminant which is cost-effective for pesticide removal is the biodegradation by bacteria (Serdar and Gibson, 1985). The extent of degradation ranges from formation of metabolites to decomposition to inorganic products. In general, microorganisms demonstrate considerable capacity to metabolize many pesticides (Hindumathy and Gayathri, 2013) they possess the unique ability to completely mineralize many aliphatic, aromatic, and heterocyclic compounds. Many microorganisms can specifically hydrolyze the phosphoester bonds of organophosphates and thus reduce the toxicity of organophosphate pesticides.

Soil microorganism, especially bacteria and fungi (*Pseudomonas, Bacillus, Trichoderma, Penicillium, Aspergillus* spp. etc.) have been reported as the important degraders of agrochemicals. The environmental conditions such as temperature, moisture, aeration etc. which favor microbial development and also favor degradation of chemical compounds in the soil (Beulke *et al*., 2004). The objectives of this study are to isolate pesticide degrading beneficial soil microorganism obtained from a contaminated agricultural soil and test their potential and ability of degradation of pesticides.

MATERIALS AND METHODS

Isolation of bioagents

Soil samples were collected from contaminated major crop field in Akola districts of Maharashtra and brought to the laboratory for microbial isolation. All collected samples mixed thoroughly to make a composite sample. Serial dilution plate count method was used for enumeration of fungi at 10^4 and for bacterial count 10^7 dilutions was used. King's B media (Kamei *et al*., 2014) and Nutrient agar media were used for isolation of *P. fluorescens* and *B. subtilis*. One ml of soil suspension from aliquot dilutions 10^5 to 10^8 was aseptically added to sterile Petri plates containing twenty ml of sterile medium and incubated at 28 ± 2^0C for 48 hrs. Colonies of bioagents were identified by biochemical tests. After 48 hrs individual colonies were picked up with sterile loop and transferred to fresh King's B and Nutrient agar slants, the pure cultures so obtained were stored in a refrigerator at 4^0C for furtheruse. For isolation of *Trichoderma* soil sample was serially diluted and plated on *Trichoderma* selective media and incubated for 7 to 10 days. Sub culturing with actively growing colony of *Trichoderma* sp. was done on selected and plated on PDA medium (Islam *et al*., 2008). Biochemical characterization of bacterial isolates was done as per Bergey's manual of systemic bacteriology.

Biochemical studies

Biochemical test *viz*., oxidase test, starch hydrolysis, gelatine liquefaction, catalase test and citrate production test were carried out for biochemical conformation of *P. fluorescens* and *B. subtilis* (Gade *et al*., 2014)

Oxidase test

An inoculating loop was taken. A well isolated colony was spreaded on an oxidase disk. The reaction was observed within two minutes at 20-30^{0}C. Deep purple blue indicates positive reaction.

Starch hydrolysis

Starch is a complex carbohydrate of the polysaccharide type hydrolysed by bacterium. The positive test indicates the presence of amylase enzyme utilized for the hydrolysis of starch. Inoculate the bacteria on the starch agar plates and incubated for two days. After incubation flooded the plates with Lugol's iodine solution. Presence of starch hydrolysis was indicated by the appearance of clear reddish zone *i.e.* the starch was partially hydrolysed to dextrin.

Gelatin liquefaction

Filter paper discs were dipped in a day old culture suspension and were placed on Petri dishes containing gelatin nutrient agar medium. The Petri dishes were incubated at 30^{o}C for two days and then flooded with 12.5 per cent $HgCl_2$ solution. The development of yellow halo around the growth indicates utilization of gelatin (Stolpe and Gadakari, 1981).

Citrate utilization test

This test was performed by inoculating the microorganism into an organic synthetic medium, Simmon's citrate agar, where sodium citrate is the only source of carbon and energy. Bromothymol blue was used as an indicator. When citrate acid was metabolized, the CO_2 generated and combined with sodium and water to form sodium carbonate, which changed the colour of indicator from green to blue and this constitutes a positive test.

Catalase test

This test was used to indicate the presence of catalase enzyme. Inoculate the petriplate with the bacteria and incubate it for three days. A bit of growth was removed from the plate and placed on a slide, to which 3% H_2O_2 was added. Appearance of bubbles showed the positive test for catalase.

Growth promoting activity

Siderophore production

Evaluation of isolates with universal Chromeazurol assay (CAS) helps in detection the siderophore by *Fluorescens pseudomonas*. This assay mainly depends on the colour zone *i.e.* orange zone against dark blue background, apositive indication for the presence of siderophore. All the isolates were screened by CAS method (Schwyn and Neilands, 1987) fortheir ability to produce siderophore.

IAA production

The IAA test is performed by inoculating a bacterium in to tryptone broth, the indole produced during the reaction is detected by adding Kovac's reagent which produces a cherry red reagent layer (Sharma *et al*., 2014).

Degradation studies

The efficiency of bacterial isolates to degrade pesticides was evaluated *in vitro* based on the degradation of pesticides by both the bacterial and fungal isolates in the presence of pesticide in growth media by UV-visible spectrophotometer. The bacterial and fungal isolates were grown in a flask containing 500 ml of Minimal salt media and Czapek-Dox broth with recommended concentration of pesticides (Imidacloprid, Carbendazim and Pendimethalin) and incubated in rotatory shaker at 150 rpm for 4 days and then 5 ml of culture was drawn in a centrifugal tube and centrifuged at 1000 rpm for 10 minutes. After centrifugation pellets were removed and supernatant was used for further step. The pesticide in the supernatant was extracted with the help of acetone for the spectrophotometric analysis at 660 nm. The broth alone with pesticide treated as control (Sahin and Tamer, 2000).

The present degradation was calculated by the formula:

Initial absorbance – Final absorbance / Initial absorbance × 100%

RESULTS AND DISCUSSION

In present study two species of bacteria and two species of fungus were isolated from pesticide contaminated soil, with the hypothesis of pesticide degradation ability of all four beneficial soil microorganism, further experiments were performed.

The result of biochemical tests revealed that all isolates of *P. fluorescens* were rod shaped, and gram negative and produced yellow colonies on kings' B medium (Shweta Sharma *et al*., 2014), showed a positive reaction for gelatine liquefaction, starch hydrolysis, oxidase test, citrate utilization test and

Table 1: Morphological and biochemical reactions of selected Isolates

Sr. No.	Characters	Reactions of isolates					
		Pseudomonas fluorescens			*Bacillus subtilis*		
		Pf 1	Pf 2	Pf 3	B1	B2	B3
Morphological properties of bacterial isolates							
1	Shape	Rod	Rod	Rod	Rod	Rod	Rod
2	Fluorescence	+ve	+ve	+ve	-ve	-ve	-ve
3	Gram reaction	-ve	-ve	-ve	+ve	+ve	+ve
Biochemical properties of bacterial isolates							
4	Gelatine liquefaction	+ve	+ve	+ve	+ve	+ve	+ve
5	Oxidase test	+ve	+ve	+ve	-ve	-ve	-ve
6	Starch hydrolysis	+ve	+ve	+ve	+ve	+ve	+ve
7	Citrate utilization	+ve	+ve	+ve	-ve	-ve	-ve
8	Catalase test	+ve	+ve	+ve	+ve	+ve	+ve

+ ve - Positive, - ve– Negative

Table 2: Growth promoting character of bacterial isolates

Sr. No.	Bacterial isolates		Siderophore production	IAA production
1	*Pseudomonas fluorescens*	Pf1	+ ve	+ ve
		Pf2	+ ve	+ ve
		Pf3	+ ve	+ ve
2	*Bacillus subtilis*	B1	-ve	+ ve
		B2	-ve	+ ve
		B3	-ve	+ ve

+ ve - Positive, - ve– Negative

Table 3: Analysis of degradation of Carbendazim by beneficial microorganisms

Sr. No.	Treatments	Optical density (600 nm)		Per cent degradation (%)		
		Initial	7thDAI	14thDAI	7thDAI	14thDAI
1)	*Pseudomonas fluorescens*	0.51	0.41	0.32	19.60	37.25
2)	*Bacillus subtilis*	0.51	0.39	0.30	23.52	41.17
3)	*Trichoderma viride*	0.51	0.49	0.48	3.92	5.88
4)	*Trichoderma harzianum*	0.51	0.50	0.48	1.96	5.88
5)	Control	0.51	0.51	0.50	0.00	1.96
	SE(m) ±		0.003	0.007		
	CD (P = 0.01)		0.014	0.031		

Average of three replications

Table 4: Analysis of degradation of Imidacloprid by beneficial microorganisms

Sr. No.	Treatments	Optical density (600 nm)		Per cent degradation (%)		
		0th DAI	7th DAI	14th DAI	7th DAI	14th DAI
1)	*Pseudomonas fluorescens*	0.87	0.66	0.57	24.13	34.48
2)	*Bacillus subtilis*	0.87	0.74	0.63	15.47	27.58
3)	*Trichoderma viride*	0.87	0.68	0.60	21.83	31.03
4)	*Trichoderma harzianum*	0.87	0.70	0.62	19.5	28.73
5)	Control	0.87	0.86	0.85	1.14	2.29
	SE(m) ±		0.003	0.004		
	CD (P = 0.01)		0.014	0.018		

Average of three replications

catalase test (Table1). The biochemical tests *i*.e. gelatin liquefaction, H_2S production, starch hydrolysis, casein hydrolysis and Chrome-azurol assay for siderophore production further confirmed to be *P. fluorescens*. All thirty isolates were positive to catalase test and nineteen isolates were positive for gelatin liquefaction and six were able to hydrolysis starch (Mina Koche *et al*., 2011). Our findings also support the findings of Dibua *et al*. (2014) also reported that *Pseudomonas* sp. was gram negative and motile rod shaped bacteria whereas *Bacillus subtilis* were gram positive, motile and spore forming rod. The results are also corroborates with the finding made by Gade *et al*., 2008. In case of *Bacillus subtilis* all isolates were gram positive, rod shaped, white colour flat colony with serrated margin and showed positive response for gelatine liquefaction, starch hydrolysis and catalase test and showed negative response for citrate utilization test (Table1). Similarly, Ratna Kumari *et al*. (2012) also reported that the *Bacillus subtilis* isolates were gram positive, rod shaped and showed a positive reaction for catalase test, starch hydrolysis, casein hydrolysis and gelatin liquefaction.

Table 5: Analysis of degradation of Pendimethalin by beneficial microorganisms

Sr. No.	Treatments	Optical density (660 nm)		Per cent degradation (%)		
		Initial	7th DAI	14th DAI	7th DAI	14th DAI
1)	*Pseudomonas fluorescens*	1.13	0.97	0.84	14.15	25.66
2)	*Bacillus subtilis*	1.13	1.01	0.92	10.61	18.58
3)	*Trichoderma viride*	1.13	0.99	0.88	12.38	22.12
4)	*Trichoderma harzianum*	1.13	0.97	0.86	14.15	23.89
5)	Control	1.13	1.11	1.10	1.76	2.65
	SE(m) ±		0.004	0.004		
	CD (P=0.01)		0.019	0.016		

Average of three replications

Siderophore production and IAA Production

Appearance of zone around the bacterial colony after 48 hrs of incubation indicated the strains of *P. fluorescens* have the ability to chelate Fe^{3+} from chromoazurol S agar medium. All collected isolates of *P. fluorescence* showed positive reaction for siderophore production test. Formation of a red color layer over the tryptone broth which inoculated with bacterial inoculum confirmed the positive reaction for IAA production test. All the isolates of *Pseudomonas fluorescens* showed a positive reaction for IAA production test (Table 2). These results in present findings collaborates with the results of Gate (2009) assayed ten isolates of *P. fluorescens* and found that all isolates were positive for siderophore production. Twenty isolates were able to produce siderophore and seventeen isolates showed positive test for IAA production (Mina Koche *et al*., 2011). Isolates of *Bacillus subtilis* showed negative reaction to siderophore and positive reaction to IAA production (Table 2). Production of indole substance or well known as auxin synthesis is one of the important factor in plant metabolism. Umamaheshwari *et al*. (2008) stated that among the four bacterial antagonists tested, production of IAA was more in *B. subtillis* (BsW1).

Analysis of pesticide degradation efficiency of selected microorganism

The experiment was carried out to analyze the capacity of beneficial microorganism to degrade pesticides at different time intervals. Carbendazim was degraded by *Psudomonas fluorescens* upto 19.60% within one week of incubation while 37.25% was degraded after two weeks of incubation. Whereas degradation of Carbendazim by *Bacillus subtilis* was of 23.52% in one week of incubation. However,it could degrade 41.17% of Carbendazim after two weeks of incubation by taking the fungicide as a sole source of carbon and nitrogen. But in case of *Trichoderma* species the degradation was very meager up to 5.88% after two weeks of incubation (Table 3). This may be because of the availability of compound to restrict the growth of fungus in Carbendazim. These results in present finding collaborates with the findings of Lin *et al*. (2011) who reported that bacterial strain could degrade the Carbendazim fungicide upto 95.5% at a concentration of 600 mg/l. Similarly, Swati Pandey *et al*. (2012) reported that two soil fungus (*Aspergillus niger* and *Aspergillus fumigates*) were isolated from field contaminated with MCP and recognized as competent for biodegradation of MCP (upto 1% concentration) Similarly, Gianfreda and Rao, (2004) who also reported that fungi degrade pesticides by introducing minor structural changes to the pesticides rendering it non-toxic and are released to soil, where it is susceptible to further biodegradation by bacteria. Sharif *et al*. (2011) also reported the similar result that *Bacillus* sp. could degrade fungicides upto 97.36% within two to eight weeks of incubation. The results of present investigation was confirmative with the result of Geeta *et al*. (2014) who reported that the degradation of Carbendazim up to 77.55% by bacterial consortium. Sing and Dube (2010) found that different fungicides including Carbendazim were compatible to *Pseudomonas fluorescens* at a concentration of 0.3 to 0.6%.

P. fluorescens isolate was capable of degrading Imidacloprid upto 24.13% after an incubation period of one week whereas it could degrade 34.48% of Imidacloprid within two weeks of incubation (Table 4). There was a degradation of 21.83% in the media containing Imidacloprid after one week of incubation when it inoculated with *T. viride* and the degradation was increased upto 31.03% after two weeks of incubation, while *T. harzianum* and *B. subtilis* could degrade pesticide upto 28.73 and 27.58 % respectively, within two weeks of incubation (Table 4). This much degradation may because of the ability of microorganism to utilize the pesticide as sole sources of carbon and nitrogen. Fungal enzymes especially, oxidoreductases, lactase and peroxidases have prominent application in removal of polyaromatic hydrocarbons (PAHs) contaminants either in fresh, marine water or terrestrial (Balaji *et al*., 2013). The herbicide Pendimethalin was degraded by *P. fluorescens* upto 14.15% within one week of incubation while 25.66% was degraded after two weeks of incubation and it also indicated 10.61% degradation of Pendimethalin by *B. subtilis* in one week of incubation whereas *B. subtilis* could degraded 18.58% of Pendimethalin after two weeks of incubation (Table 5). The *T. viride* and *T. harzianum* was able to degrade Pendimethalin upto 22.12 and 23.89% respectively after two weeks of incubation (Table 5). Several authors have reported biodegradation of soil chemicals like insecticides, pesticides, xenobiotics etc. by the help of various microorganisms in the nature. These results were also in agreement with the findings of Veena *et al*. (2010) reported that *Bacillus circulans* capable of degrading herbicide, Pendimethalin. The result from the present finding is confirmative with the result of Sherif and Mounir (2013) who also reported that the *T. viride* and *Psedomonas alcaligens* quickly degraded Butachlor and reached nearly 98 and 75% in a medium containing 50 mg/kg of Butachlor after 15 and 21 days, respectively.

It concluded that the isolated bioagents is able to degrade the

pesticides (Imidacloprid, Pendimethalin and Carbendazim), so the organisms are used as a biological agent for the *in situ* bioremediation of pesticides contaminated soil. In pesticide degradation analysis *B. subtilis* could degrade maximum amount Carbendazim fungicide at 41.17% within two weeks of incubation while in case of Imidacloprid, *P. fluorescens* showed maximum degradation at the rate of 34.48%. *Pseudomonas fluorescens* was also able to degrade a maximum of Pendimethalin at 25.66%. Whereas, *Trichoderma* spp. couldn't degrade Carbendazim more due to its fungicidal effect.

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