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Isolation and Characterization of an Acetamiprid Degrading Bacteria from Cultivated Soils of North Gujarat Region

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ABSTRACT

Microorganisms can survive in polluted environment, which develop potential to degrade substances that are harmful to the environment. Environmental pollution caused by the leaking of chemical fertilizers and pesticides to surface and groundwater, causing serious environmental and social problems throughout the world. Soil samples have been taken from vegetables and cotton plantation that has history of using pesticide for controlling pests. Isolation of these microorganisms has been carried out by using serially diluted sample by streaking on suitable nutrient media. The positive isolates growing at different concentration of pesticides in the nutrient media have been characterized with respect to their morphology and biochemical reactions. The pesticide degradation study is carried out by using potential isolates grown at different pesticide level in the growth media and the degradation has been studied at different concentration during one week of incubation. The degradation products are analyzed by GCMS. The results of pesticide degradation are discussed.

Keywords

Pesticide,
Biodegradation,
Acetamiprid

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Introduction

Pesticide is a substance or mixture of substances which is used for preventing, destroying and also repelling pests. Pesticide may be biological agent or chemical substance used for pest control. Pest include insects, weeds, birds, fishes and plant pathogens that destroy property and vegetation, spread

disease or are vector for disease (1,3,6) Pesticides are most useful in pest control but there are drawbacks of these such as potential toxicity to humans and other animals.

Due to uncontrolled use of pesticide, environment has become contaminated and polluted. (2,9,8,12,13) There are two main reasons for these compounds to persist in

nature. First, the condition necessary for their biodegradation may not be present. Microorganisms that are capable for degrading these toxic compound may be absent at contaminated site.

If the necessary microorganisms are present some limiting factors such as a nutrient storage or some unfavorable environmental condition for biodegradation of contaminant and second is that the compound which is used for pest control may be recalcitrant or resistant to biodegradation. However, there are some microorganisms which can survive in the soil contaminated with pesticide. So identification and characterization of these bacteria is important to study about its potential for degradation of pesticides. Acetamiprid is an organic compound with the chemical formula $C_{10}H_{11}ClN_4$ (see figure 1 for structure). It is an odorless neonicotinoid insecticide. It is intended to control sucking insects on crop such as leafy vegetables and cotton. It is also used for cherry farming to control its fruit fly. Acetamiprid is classified as human carcinogen. The insecticidal activity of neonicotinoid is due to their competitive engagements against the post synaptic acetylcholine (nicotinic) receptor in the central nervous system of insects. It is soluble in most organic solvents and shows increased solubility in water. Neonicotinoid pesticides comprise a fairly new and widely used category of organic compounds which cover about the 17% of global pesticide market and therefore these may be threat to aquatic and terrestrial environment.

Considering the potential of microorganisms to adapt to the environment and capacity to use these as nutrients for growth, their presence is expected at these sites of large use of pesticides for certain crops. Some microorganisms may exist that can degrade the pesticide and also have potential application in bioremediation.

Materials and Methods

Collection of soil sample

Total eight samples collected from vegetable field at different villages in Patan and Mehsana district having history of repeated pesticide application. All the samples are collected in sterile zipper polyethylene bags and stored at 4⁰ C before processing.

Media

Burkes Mineral Medium (BMM) enriched with acetamiprid is used for isolation and characterization of acetamiprid degrading bacteria.(4,5,7,10,11) The BMM has the following composition in (g/l) K_2HPO_4 , 0.2 g ; KH_2PO_4 , 0.8 g ; $MgSO_4$, 0.2 g; $CaCl_6 \cdot H_2O$; $(NH_4)_2SO_4$, 0.2; Yeast extract 0.02%, 0.2g; Glucose 1% (Shivamaiah and kennedy) for the isolation of bacteria. Luria-Bertani(LB) broth is used according to the manufacturer instructions (HI-MEDIA)

Soil Analysis

Soil physical analysis has been determined as per methods described in “Methods of Soil Analysis-Part II, 2nd ed. Am. Soc. Agron., Madison Wisconsin, USA.

Isolation and characterization of bacteria

Soil bacteria capable of degrading acetamiprid have been isolated from cultivated soil treated with pesticide

From 10% soil suspension serially diluted up to 10^{-3} , 0.1 ml is inoculated in BMM agar containing 20, 30, 50, 100 of acetamiprid by spread plate technique. A total of eight different soil samples collected from various sites in Patan and Mehsana districts. Where cotton and in some vegetable like chili is grown. Inoculated plates are incubated at 37⁰C

for several days till isolated colonies developed. Pure cultures are prepared and studied for their colony and cell morphology. These are characterized biochemically.

The potential strains of bacteria which showed good growth on BMM agar with 50 ppm of pesticide were selected for identification. The identification has been carried out using Biolog[®] (MicroLog3 4.20.05) on the basis of sole carbon source utilization and biochemical analysis results are listed in table 3.

Degradation study

Inoculum is prepared in Luria broth by inoculation of bacteria and incubated on rotary shaker at 180rpm, 28⁰C temperature for 4-7days. 10 ml of inoculum is added in 90 ml of BMM which contain 50 ppm acetamiprid pesticide. This is incubated at 28⁰ C & 120 rpm and after 48 hours 10 ml broth is taken as sample. This is homogenized. To this 3-4 drop of concentrated hydrochloric acid is added and mixed well. Equal volume (10ml) Hexane ethyl acetate mixture (20:80) is added and subjected to vortex for 5 min. Then centrifuged at 2000rpm for 25 minutes. The Organic phase is removed and kept at 4⁰C and immediately subjected to moisture remove. Sample is analyzed through Gas chromatograph and mass spectroscopy.

Gas chromatography with Mass Spectroscopy

Perkin Elmer Auto System XL(courtesy: ISTAR, VV nagar) is used to analyze the degraded pesticide sample after incubation in presence of potential degrading bacteria. The incubated sample is subjected to hexane: ethyl acetate extraction. One microliter of sample has been injected to GCMS under following operative conditions:

Injector temp: 250⁰C

Oven temp: 70⁰C

Time: 5 min

Temperature increase rate: 10⁰C for min up to 280⁰C.

Holding time of sample: 20 min

E1 source temp: 250⁰C

Column identification: PE-5MS, 30mm×250μØ and 0.250μ thickness.

Mass range: 30 to 650 AMU

Split ratio: 6:660

Results and Discussion

In the present study bacterial isolates have been worked out for acetamiprid degradation from the field soils of Patan and Mehsana districts. Soil samples are collected from the cropping soils containing mainly the cotton field and vegetable fields (cultivating chilli) which have history of pesticide use to control pests. Soil physical characteristics are worked out to determine the texture by sieve analysis. The soils are mostly silt loam. The soil textural properties are depicted in table 1. Acetamiprid degrading bacteria have been isolated on Burkes Mineral Medium (BMM) incorporated with different concentrations of acetamiprid. Isolated colony has been studied for their morphological characters (see table-2) and biochemical characteristics (see table-3).

The potential isolates which showed resistance against different concentrations of pesticide are studied for their degradative potential of acetamiprid. The active culture of the isolate has been used to inoculate the BMM broth containing 50 ppm of acetamiprid.

Table.1 Soil texture

Soil sample	Coarse Sand	Fine Sand	Silt + Clay
K1	40.20	43.50	16.30
K2	41.86	36.78	21.10
K3	37.80	32.18	29.72
K4	41.11	28.07	30.46
M1	60.10	35.40	04.09
M2	24.01	44.62	30.46
M3	45.95	33.99	19.73
M3	22.26	55.17	22.07

Table.2 Morphological characteristics

Colony character	Ct
Size	Medium
Shape	Round
Margin	Undulated
Elevation	Raised
Surface	Smooth
Pigment	Yellow
Consistency	Moist
Transparency	Opaque

Table.3 Biochemical characteristics of isolate

Sr. No.	Biochemical test	Result
1	Carbohydrate fermentation	
a	Maltose	Acid and gas
b	Lactose	Negative
c	Mannitol	Only acid
d	Glucose	Negative
e	Sucrose	Negative
2	Methyl red	Positive
3	Indole production	Negative
4	H ₂ S production	Negative
5	Gelatin hydrolysis	Negative
6	Catalase	Positive
7	Dehydrogenase	Positive
8	Casein utilization	Negative
9	Lipid hydrolysis	Positive
10	Starch utilization	Positive
11	Citrate utilization	Positive
12	Urea hydrolysis	Negative

Table.4 Chromatogram result of *Pantoea dispersa*

Area Percent Report

Peak List File : D:\2007-08\200809.PRO\2009-10\ACQUDB\201213PEAKDB\300
 Last Modified : Wednesday, May 30, 2012 2:59:16 PM
 Printed : Wednesday, May 30, 2012 2:59:29 PM

#	Name	RT	Area	Height	BL	Conc	Units	Area/Conc	m/z	Area %
1		15.204	9,500.0	45,387	dd	0.00		0.00	TIC	.14
2		15.552	8,512.1	51,986	dd	0.00		0.00	TIC	.12
3		15.699	9,154.2	54,652	dd	0.00		0.00	TIC	.13
4		15.937	8,235.2	48,946	dd	0.00		0.00	TIC	.12
5		16.102	13,281.1	84,988	dd	0.00		0.00	TIC	.19
6		16.359	46,644.2	183,389	dd	0.00		0.00	TIC	.68
7		16.817	30,141.8	123,978	dd	0.00		0.00	TIC	.44
8		17.056	27,618.9	127,353	dd	0.00		0.00	TIC	.40
9		17.441	79,539.6	305,398	dd	0.00		0.00	TIC	1.16
10		17.881	53,247.1	205,998	dd	0.00		0.00	TIC	.78
11		18.486	212,240.5	398,359	dd	0.00		0.00	TIC	3.09
12		18.889	66,016.3	338,402	dd	0.00		0.00	TIC	.96
13		19.402	260,962.1	519,138	dd	0.00		0.00	TIC	3.80
14		19.971	242,884.9	461,993	dd	0.00		0.00	TIC	3.54
15		20.411	119,382.7	589,721	dd	0.00		0.00	TIC	1.74
16		20.722	155,405.9	519,222	dd	0.00		0.00	TIC	2.27
17		21.309	333,333.2	684,384	dd	0.00		0.00	TIC	4.86
18		22.171	445,752.7	704,365	dd	0.00		0.00	TIC	6.50
19		22.281	222,163.7	693,939	dd	0.00		0.00	TIC	3.24
20		23.014	372,517.1	724,865	dd	0.00		0.00	TIC	5.43
21		23.766	507,948.2	760,797	dd	0.00		0.00	TIC	7.40
22		24.078	196,984.0	868,021	dd	0.00		0.00	TIC	2.87
23		24.554	374,434.2	866,179	dd	0.00		0.00	TIC	5.46
24		25.269	1,090,444.0	939,688	dd	0.00		0.00	TIC	15.89
25		26.113	625,745.2	802,196	dd	0.00		0.00	TIC	9.12
26		27.139	308,841.1	603,434	dd	0.00		0.00	TIC	4.50
27		27.689	226,281.5	507,972	dd	0.00		0.00	TIC	3.30
28		28.276	206,966.3	408,979	dd	0.00		0.00	TIC	3.02
29		28.771	74,710.2	367,046	dd	0.00		0.00	TIC	1.09
30		29.009	84,882.5	341,712	dd	0.00		0.00	TIC	1.24

Area percent report

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Page 1

#	Name	RT	Area	Height	BL	Conc	Units	Area/Conc	m/z	Area %
31		29.284	115,158.1	314,477	dd	0.00		0.00	TIC	1.68
32		29.724	173,147.8	254,953	dd	0.00		0.00	TIC	2.52
33		30.550	59,456.7	215,371	dd	0.00		0.00	TIC	.87
34		30.860	99,301.0	190,551	dd	0.00		0.00	TIC	1.43

Table.5 Possible metabolites after degradation

Hit	REV	for	Compound Name	M.W.	Formula	CAS	Library
1	764	612	S-INDACENE-1,7-DIONE, 2,3,5,6-TETRAHYDRO-3,3,5,5-TETRAM	242	C16H18O2	55591-17-8	Nist
2	719	382	1H-INDENE, 3-ETHYL-1-(1-METHYLETHYL)-	186	C14H18	111400-85-2	Nist
3	708	397	5-METHOXY-2-METHYL-4-OXO-1,2,3,4-TETRAHYDRO-1,10-PHE	242	C14H14O2N2	1000227-06-06	Nist
4	702	337	2H-BENZ[E]INDEN-3-OL, 3,3A,4,5-TETRAHYDRO-3A-METHYL-, (	200	C14H16O	71805-81-9	Nist
5	702	351	BENZENE, (2-ETHYL-4-METHYL-1,3-PENTADIENYL)-, (E)-	186	C14H18	74663-87-5	Nist
6	694	314	1H-INDENE, 1,1,3-TRIMETHYL-	158	C12H14	2177-45-9	Nist
7	679	415	1(2H)-PHENANTHRENONE, 3,4,9,10-TETRAHYDRO-7-METHOX	228	C15H16O2	14427-81-3	Nist
8	666	353	ISOLONGIFOLENE, 4,5,9,10-DEHYDRO-	200	C15H20	1000151-55-0E	Nist
9	661	305	BENZENE, 1,2-BIS(1-BUTEN-3-YL)-	186	C14H18	1000162-73-9 ⁹	Nist
10	655	340	3A,9B-DIMETHYL-1,2,3A,4,5,9B-HEXAHYDROCYCLOPENTA[A]N	214	C15H18O	1000194-11-3O	Nist
11	647	312	1H-INDENE, 3-BUTYL-1-METHYL-	186	C14H18	111400-84-1	Nist
12	617	376	PHENANTHRENE, 9-BUTYL-1,2,3,4,5,6,7,8-OCTAHYDRO-	242	C18H26	16703-29-0	Nist
13	617	507	2(1H)-PHENANTHRENONE, 3,4,4A,9,10,10A-HEXAHYDRO-1,1,4	242	C17H22O	61141-19-3	Nist
14	607	477	1,4-NAPHTHALENEDIONE, 2-HYDROXY-3-(3-METHYL-1-BUTEN	242	C15H14O3	4042-39-1	Nbs
15	600	304	TRICYCLO[5.4.0.0(2,8)]UNDECA-1(1),7,9-TRIENE, 2-(4-MORPH	228	C15H19ON	24296-52-4	Nist
16	587	432	1,4-NAPHTHALENEDIONE, 2-HYDROXY-3-(3-METHYL-1-BUTEN	242	C15H14O3	4042-39-1	Nbs
17	584	431	1,4-NAPHTHALENEDIONE, 2-HYDROXY-3-(3-METHYL-2-BUTEN	242	C15H14O3	84-79-7	Nbs
18	581	459	2(1H)-PHENANTHRENONE, 3,4,4A,9,10,10A-HEXAHYDRO-1,1,4	242	C17H22O	55902-80-4	Nist
19	569	389	BIS[METHYL(PENTAMETHYLENE)]DISILOXANE	242	C12H26OSi2	1000245-73-7b	Nist
20	568	399	1,4-NAPHTHALENEDIONE, 2-HYDROXY-3-(3-METHYL-2-BUTEN	242	C15H14O3	84-79-7	Nbs

Table.6 Possible metabolites after degradation

Hit	REV	for	Compound Name	M.W.	Formula	CAS	Library
1	798	314	5-METHYL-2-ETHENYL-CYCLOHEXANE-1-CARBOXYLIC ACID	168	C10H16O2	1000144-53-6 ⁶	Nist
2	757	424	BICYCLO[2.2.1]HEPTANE, 7,7-DIMETHYL-2-METHYLENE-	136	C10H16	471-84-1	Nbs
3	750	530	(-)-NEOCLOVENE-(II), DIHYDRO-	206	C15H26	1000152-83-6f	Nist
4	745	572	CYCLOHEXANE, 1,2-DIMETHYL-3,5-BIS(1-METHYLETHENYL)-	192	C14H24	62337-99-9	Nbs
5	738	510	3-CYCLOHEXENE-1-ETHANOL, BETA, 4-DIMETHYL-, [R-(R*,R*)]	154	C10H18O	13835-30-8	Nist
6	737	519	CYCLOPROPANECARBOXYLIC ACID, 2,2-DIMETHYL-3-(2-METH	302	C19H26O3	28434-00-6	Nist
7	736	534	2,8,10,14-HEXADECATETRAENOIC ACID, 3,7,11,15-TETRAMET	318	C21H34O2	42207-88-5	Nbs
8	733	537	ACETIC ACID, 1-METHYL-3-(2,6,8-TRIMETHYLCYCLOHEX-1-EN	238	C15H26O2	1000190-96-9f	Nist
9	733	545	ACETIC ACID, 1-METHYL-3-(2,6,8-TRIMETHYLCYCLOHEX-2-EN	238	C15H26O2	1000192-15-8f	Nist
10	730	523	2-AZIDOMETHYL-1,3,3-TRIMETHYL-CYCLOHEXENE	179	C10H17N3	1000185-84-7 ³	Nist
11	730	401	3,7,11-TRIMETHYL-3-HYDROXY-6,10-DODECADIEN-1-YL ACETA	282	C17H30O3	1000144-12-7□□□	Nist
12	729	523	1-CYCLOHEXENE-1-PROPANAL, 2,6,6-TRIMETHYL-	180	C12H20O	35477-44-2	Nist
13	728	513	4,8,13-CYCLOTRIDECA TRIENE-1,3-DIOL, 1,5,9-TRIMETHYL-	306	C20H34O2	7220-78-2	Nbs
14	726	545	2-(1,4,4-TRIMETHYLCYCLOHEX-2-EN-1-YL)ETHYL P-TOLUENES	322	C18H26O3S	1000195-70-2B□□	Nist
15	726	539	CYCLOHEXANE, 1,1,2-TRIMETHYL-3,5-BIS(1-METHYLETHENYL)	206	C15H26	62337-97-7	Nist
16	725	535	DIHYDRO-ALPHA-METHYLIONONE	208	C14H24O	56763-84-5	Nist
17	721	517	CYCLOHEXANE, 1,2-DIMETHYL-3,5-BIS(1-METHYLETHENYL)-, (	192	C14H24	74806-55-6	Nist
18	719	490	CYCLOHEXANOL, 2-METHYL-5-(1-METHYLETHENYL)-	154	C10H18O	619-01-2	Nbs
19	713	446	2-(FENCH-2-YL)FENCHANE	274	C20H34	1000105-83-1□□□	Nist
20	711	421	C(14A)-HOMO-27-NOR-14.BETA.-GAMMACERAN-3-ALPHA.-OL	428	C30H52O	24739-08-0	Nist

Fig.1 Structure of acetamiprid

Fig.2 Chromatogram of standard sample of acetamiprid pesticide

Fig.3 Chromatogram for *Pantoea dispersa*

Fig.4 Mass spectra analysis of degradation products of Acetamiprid for *pantoea dispersa* (A)

Fig.5 Mass spectra analysis of degradation products of Acetamiprid for *pantoea dispersa* (B)

The broth was incubated at 26 °C at 150 rpm for 7 days. After incubation the pesticide was extracted in organic solvent and subjected to GCMS analysis.

The potential culture has also been subjected to Biolog analysis for their identification. On the basis of utilization of Hydroxy –L-Proline as sole carbon, the organism has been identified as *Pantoea dispersa*. This bacteria is

a plant pathogen causing disease in plants. The capacity of this organism to degrade acetamiprid is significant because this would increase its virulence. The results of GCMS analysis of the degradation product of this organism is depicted in the figures showing the GCMS data (Fig. 2–5). The peaks shown by the degradation analysis clearly indicates that the pesticide has been completely degraded in seven days of incubation. The

degradation products are also listed in the figures. The 16S rRNA analysis also confirmed that the isolate is *Pantoea dispersa*.

These isolate completely degraded 50ppm concentration of acetamiprid pesticide. The degradation products are measured by GCMS analysis. Standard sample of acetamiprid gives pick at 23.68 min (retention time). At this retention time the degraded products do not give any pick. This means the pesticide is degraded completely. The possible metabolites and chromatogram of all the isolates are in the listed in table 4 to 6.

Among the isolates which are recorded as potential degraders of acetamiprid have been identified on the basis of sole carbon source utilization by Biolog System (Make and model). These isolates belong to genus *Pantoea* and *Bacillus*. These isolates have been isolated from soil contaminated with the pesticide. This pesticide is used for the protection of crops, vegetables namely cotton and chilly in the districts of Patan and Mehsana. The major degradation products in case of both are S-Indacene-1,7-Dione, 2,3,5,6-Tetrahydro-3,3,5,5-tetram and Decane, 3-cyclohexyl. With possible minor products like Cyclohexane, 1,1'-(1-Ethyl-1,2 - Ethanedyl) Bis, 1,4 - Napthalenedione, 2 - Hydroxy - 3 - (3 - Methyl - 2 - Butane), 1(2H)-Phenanthrenone, 3,4,9,10-Tetrahydro-7-Methoxy, Bis[Methyl(Pentamethylene)]Disiloxane, etc.

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