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Biodegradation of cypermethrin metabolites using terrestrial actinobacterium, *Streptomyces diastaticus* (PA2) and its GC-MS analysis

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Abstract : Pesticide contaminated soil and its remediation is considered as trivial and phenomenly affects of human life expectancy. Cypermethrin is a insecticide, act as a carcinogen and drivenly affect human reproductive system. Hence this study focuses the novel and environmental friendly bioremediatin of cypermethrin contaminated soil by using actinobacteria. 8 potencial stain of actinobacteria were isolated and screened from cypermethrin polluted jasmine garden soil and PA2 showed the good utilization of cypermethrin as the sole carbon and energy sources for growth against 100 mg of cypermethrin with optimizated medium conditions at 28°C, pH 7.2, which was supported by UV-Spec and protein estimation. The growth curve experiment was performed at 20 -100 mg L^{-1} dose of cypermethrin in the medium and found the viable count of *Streptomyces diastaticus* (PA2: UV-spectroscopy at 590 nm) is higher in 7^{th} days. In addition, gas chromatography-mass spectrometry found the hydrolyses products such as benzaldehyde 3-phenoxy, lopropane carboxylic acid and 1-propanone 2, 2-dimethyl on 5^{th} and 7^{th} day respectively under the optimal conditions. The potential PA2 strain was identified as *Streptomyces by* morphological and *Streptomyces diastaticus* by molecular. This study gives the novel and potential strain PA2 for approximately 92-96% of bioremediation of cypermethrin polluted soil within 7days with 0.1ml of inoculum as a source. The PA2 will be a good alternative for biodegradation of toxic compounds and pollutants in our world.
Keywords : Biodegradation; Cypermethrin; GC-MS; *Streptomyces diastaticus.*

1. Introduction

The use of pesticides has increased since the end of the Second World War and the researches have been conducted into the impact of pesticides on the environment. The last few decades, highly toxic organic

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compounds have been synthesized and released into the environment for direct or indirect application over a long period of time[1,2]. Pesticide is any substance or mixture of substances intended for preventing, destroying, repelling any pest, insects, mites, nematodes, weeds, rats, including insecticide, herbicide, fungicide, and various other substances used to control pests[2]. Pesticide scenario in worldwide Around 4.5 million metric tonnes of pesticides is consumed in the world annually to protect crops and meet the demands of public health. Globally, nearly 45 percent of the total pesticides used to consist of herbicides while 30 percent consists of insecticides[3]. A total of 234 pesticides have been registered by The Central Insecticides Board and Registration Committee. Around 20 pesticides have been considered are commonly used and recommended pesticides in India[4].

Cypermethrin is a pyrethroid insecticide. It was first synthesized in 1974. Cypermethrin is a synthetic chemical similar to the pyrethrins in pyrethrum extract from the chrysanthemum plant[5]. Cypermethrin extensively used in cotton, fruit, and vegetable crops as well as in animal health, home and garden pest control worldwide[6]. Pesticide exposure is also linked to cancer, hormone disruption, and problems with reproduction and fetal development. Pyrethroids can cause hyperexcitation, aggressiveness, skin allergy whole-body, and seizures[7,8]. Bioremediation can occur either *in-situ* or *ex-situ* , both are have advantages and also disadvantages[7,8]. However, *in-situ* remediation is more constructive in biological oriented degradations to control the pesticide pollution. Bioremediation is the use of microbes to clean up contaminated soil and groundwater. Microbes are very small organisms, such as bacteria, fungi, protozoa, algae, actinobacterium that live naturally in the environment. The contaminants treated using microorganisms include oil and dye, petroleum products, solvents, and pesticides. The environment factors were the type of soil, temperature, pH, the presence of oxygen and nutrients factor depend on upon the microbial population capable of degrading the pollutants[9].

Especially, the actinobacteria has a great potential for biodegradation of organic and inorganic toxic compounds[10]. Several actinobacterial strains from composts are now being investigated to evaluate their capacity to degrade some petroleum hydrocarbons and to decolorize several synthetic dyes in order to reveal their potential application in bioremediation. But such works from south India is still in infant stage[10-17]. Thus, the aim of this work was to evaluate the ability of actinobacteria isolated from contaminated environments of Namakkal district, to degrade a cypermethrin using *Streptomyces diastaticus*. This study aims to predict the cypermethrin biodegradation ability of the actinobacteria, which is isolated from cypermethrin contaminated soil.

2. Material and Methods

2.1. Sample collection and isolation of actinobacteria

The pesticide-polluted soil samples were collected from jasmine garden located at [Long.11.4700°N and Lat.78.1700°E] Namakkal district, Tamil Nadu. Soil sample was serially diluted from 10^{-2} to 10^{-6} and then 0.1 ml of sample from serial dilution was inoculated in starch casein medium using the L-Rod (spread plate technique) and incubated at 28°C for 4 to 7 days[11]. After the incubation period, morphologically different colonies were isolated and sub-cultured in International Streptomycetes Project -2 medium and the subcultures were stored at 4°C.

2.2. Primary screening of actinobacteria for cypermethrin resistance

The isolated actinobactrial cultures were screened in mineral salt agar medium with 100 mg L^{-1} concentrations of cypermethrin in the each conical flask. After the solidification, the plates were divided and one loopful of 8 actinobacterial cultures was spotted on the mineral salt medium[12,13, 19-22] and incubated at 28°C for 4 to 7 days. After incubation period, the potential strain was selected on the bases of culture growth.

2.3. Secondary screening of actinobacteria for cypermethrin degradation

According to the primary screening result, the potential strain will be chosen for the secondary screening. For that, mineral salt liquid medium was prepared and added 20, 40, 60, 80, and 100 mg concentrations of cypermethrin in the conical flask containing medium separately. The flasks were divided into

5 and one loopful of PA2 actinobacterial cultures was inoculated in each. The mineral salt liquid samples with 1,3,5,7 day intervals were taken and measured the UV- Vis spectrophotometer at 590 nm[24-26].

Total protein estimation by Lowry's method

After the seconday screening, the protein content of the 100 mg cypermethrin treated PA2 culture in mineral salt medium was determined with a stock solution of standard protein bovine serum albumin (BSA), at a concentration of 1000 µg mL^{-1}. The absorbance was measured at 660 nm using UV-spectrophotometer and the protein content determined[26].

2.4. Detection of cypermethrin metabolites by GC-MS method

PA2 actinobacterial cultures inoculated in mineral salt liquid medium with 100 mg concentration of cypermethrin was taken at 1, 3, 5 and 7day intervals and centrifuge at 8,500 rpm for 10 minutes at 4°C[14,24]. Then the supernatant was taken and added with 1ml of dichloromethane and allow it for without disturbing and discarded another layer leave evaporated from the tube. The dichloromethane extract was evaporated and the residue dissolved in acetone[24-26]. The extracts were analyzed by GC-MS. The GC–MS analyses were performed (Agilent, USA) in electron ionization mode 70 eV with an Agilent gas chromatograph, equipped with an MS detector.

2.5. Cultural characteristics and molecular identification of potential strain PA2

A cultural characteristic of actinobacterial strain was studied by morphological and staining. Then, the DNA extraction was carried out and 16S ribosomal RNA gene was amplified by using the PCR method with primers 518f (5'CCA GCA GCC GCG CTA ATA CG 3') and 800r (5' TAC CAG GGT ATC TAA TCC 3'). The PCR product was sequenced by an automated sequencer (AB13730, Eppendorf Master Cycler personal) [26].

3. Results

3.1. Isolation of actinobacterium on ISP2 medium

Fig.1: Sample collection, Isolation and screening of actinobacteria from cypromethrin contaminated soil

The pesticides polluted soil samples from jasmine garden, Ponparappipatti village, Namakkal District, Tamil Nadu shows around 33 actinobacterial colonies with different morphological characters in ISP2 medium (Fig. 1a and b). From the 33 strains, 8 different kinds of actinobacterial colonies were selected. All the 8 actinobacterial strains were streaked on ISP2 agar plates and incubated at 28°C for 7 days. After the incubation period, 8 actinobacterial strains showed good growth with different aerial mass colour, reverse side pigment, and soluble pigment on the ISP2 medium (Table 1). It was necessary to evaluate the ability of the actinobacteria to tolerate the pesticides mixture. Thereby, the primary screening for potential

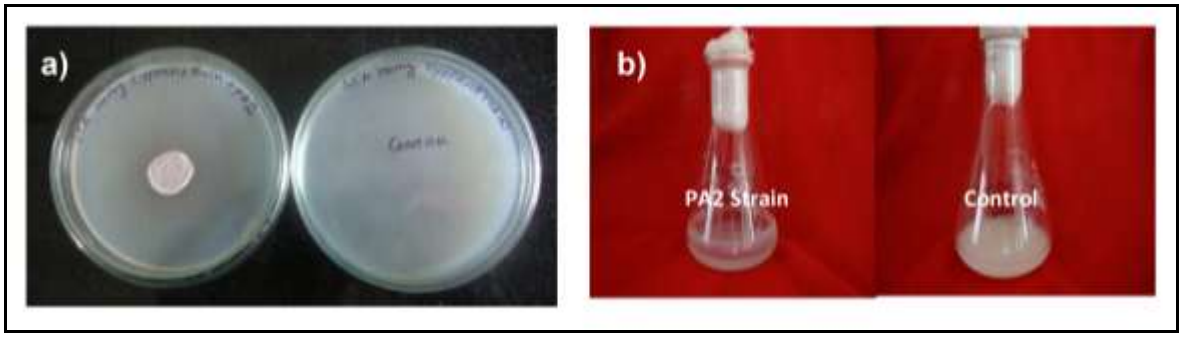


Fig.2: screening of PA2 strain in the presence of starch casein agar medium supplemented with cypermethrin 100 mg (plate method)

Table 1 : Morphology of actinobacteria cultures in soil samples

Strain no.	Growth	Colour	Consistency	Mycelium		Pigment	
				Aerial	Substrate	Reverse	Soluble
PA1	good	Grayish white	powdery	+	+	Brown	_
PA2	good	Grayish white	Smooth	+	+	Black	+
PA3	good	Grayish white	Smooth	+	+	Brown	_
PA4	moderate	Dirty white	Smooth	+	+	Brown	_
PA5	good	Dirty white	Rough	+	+	Brown	_
PA6	Good	Pure white	Smooth	+	+	Brown	_
PA7	Good	gray	Powdery	+	+	Chocolate brown	_
PA8	Good	gray	Powdery	+	+	Brown	_

actinobacteria was tested in 100mg of cypermethrin in the medium. All the 8 strains were showed good growth on starch casein medium supplemented with different concentration of cypermethrin**. C**ypermethrin was utilized as a carbon source by actinobacterial strain (Fig. 1c), however, four strains showed a high degree of tolerance to the toxic mixture, while the other four strains presented moderate tolerance. The previous study represents that the one bacterial strain, named HU-S-01, expressed good growth rate and greater cypermethrin degradation capacity in Starch casein broth. It degraded about 92.1 % substrate within 24 hours and 100 % after 30 hours at the concentration of 50 mg/L cypermethrin in the medium[18.] Strain PA2 utilized cypermethrin (10-100 mg/L) as a source of energy during the process of enrichment in MSM hemostat culture. In shake flask culture the growth of bacterium and utilization of cypermethrin was determined from 1 to7 days. Maximum utilization of cypermethrin was shown by strain PA2 mainly in 100 mg concentration[19] (Fig. 1c). Hence, the strain PA2 was taken as potential actinobacteria for further cypermethirin degradation studies[17] (Fig. 1d).

3.2. Secondary Screening

In the present study mineral salt medium supplemented with 100 mg cypermethrin and also adjusted pH 7.2 the potential strain spot on the plate and incubated at 28°C for 7 days. PA2 strain utilized cypermethrin as its sole carbon and energy source in Mineral salt medium (Fig. 2a). In our report compared with enrichment and utilization of cypermethrin by actinobacteria in mineral salt broth culture S*treptomyces* sp strain PA2 utilized

cypermethrin (100 mg L^{-1}) as a source of energy (**Fig. 2b).** These results narrate our strain PA2 play a crucial role in the degradation of cypermethrin faster in high concentration too. It was done in the previous report.

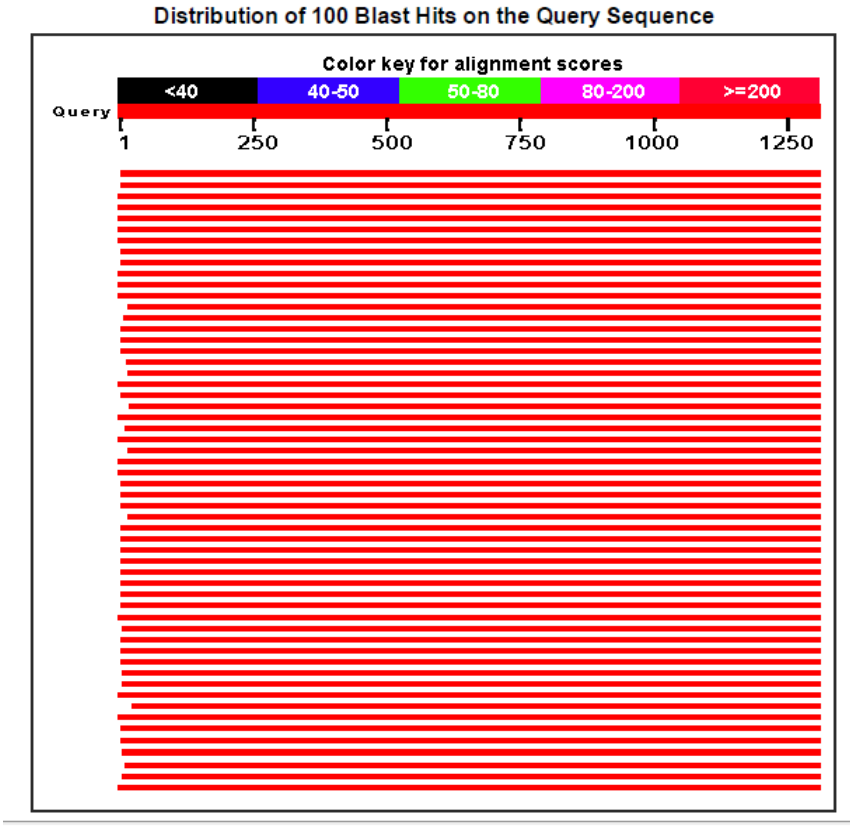
3.3 Cultural characterization and molecular identification of potential strain PA2

Cultural characteristics of actinobacteria strain were studied by using media **(Table 1)** described by Media. In this, the screening methods were carried out and found PA2 strain is the potential one among the isolated culture (**Fig. 3).** Hence, the sequencing analysis of 16S rRNA gene from PA2 processed with primer 518f and 800r. The GenBank accession no. KF537573 was obtained through NCBI direct submission for PA2. The PA2 strain was identified as *Streptomyces diastatiscus* with sequence identities of 99% (**Fig. 3).** Previous reports narrate the antimicrobial activity, enzyme production, azo dye degradation of Streptomycin *diastatiscus*[24]. There was no report on the degradation of pesticide cypermethrin by *Streptomyces diastatiscus.* So the present study is an eye-opening research on the degradation of pesticides by *Streptomyces diastatiscus*. In future, it will be an eco-friendly valuable compound for pesticide degradations.

3.4. Screening of actinobacteria able to grow in the presence of cypermethrin by using mineral salt broth medium and also cypermethrin metabolites analysis by UV-spectrophotometer

Total of 8 actinobacteria, strain PA2 given good result in all the concentrations mainly in a high concentration of 100 mg and 5th and 7th days of broth showed decreasing of OD values when compared to control (Fig. 4). It narrates the degradation of cypermethrin by actinobacteria. In our study, a strain PA2 showed comparatively good growth rate and greater cypermethrin degradation efficiency at a high concentration of 100 mg, it is comparatively higher activity than the previous study[20,21] **(Fig. 4).** In the study 0.1mL of the pure culture was inoculated in the sterile medium supplemented with 100 mg/L of pesticide and incubated under the above-

a) Phylogenetic analysis of Potential actinobacteria PA2



b) Phylogenetic actinobacteria PA2

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptomyces diastaticus subsp. ardesiacus strain NRRL B-1773 16S ribosomal RNA, partial sequence	2338	2338	99%	0.0	99%	NR_043486.1
Streptomyces coelicoflavus strain NBRC 15399 16S ribosomal RNA, partial sequence	2333	2333	99%	0.0	99%	NR_041175.1
Streptomyces fragilis strain NRRL 2424 16S ribosomal RNA, partial sequence	2290	2290	100%	0.0	98%	NR_043381.1
Streptomyces violaceolatus strain DSM 40438 16S ribosomal RNA, partial sequence	2287	2287	100%	0.0	98%	NR_027223.1
Streptomyces coelescens strain AS 4.1594 16S ribosomal RNA, partial sequence	2287	2287	100%	0.0	98%	NR_027222.1
Streptomyces violaceoruber strain DSM 40049 16S ribosomal RNA, partial sequence	2287	2287	100%	0.0	98%	NR_041914.1

c) Phylogenetic actinobacteria PA2

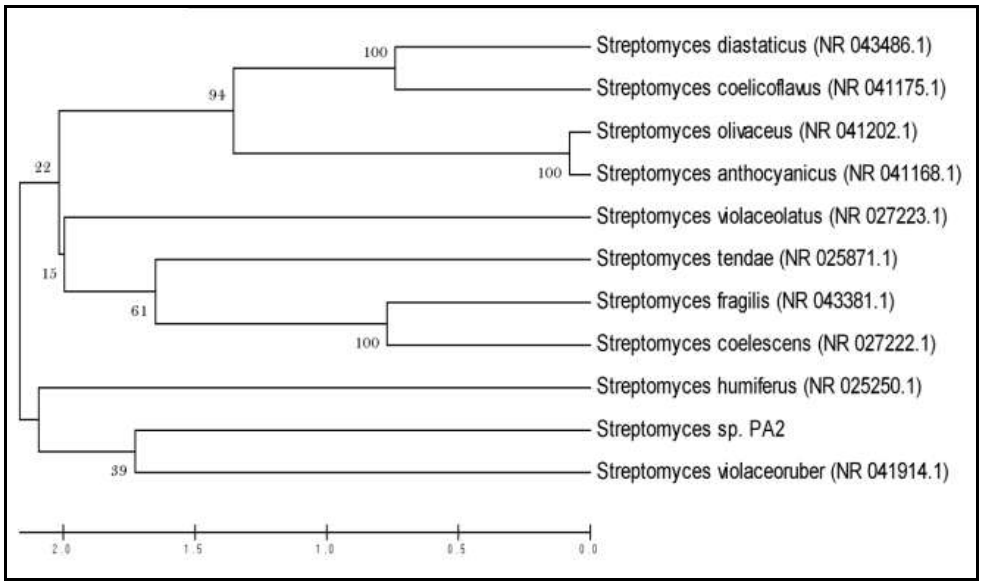


Fig. 3: Molecular identification (Blast) of potential strain PA2 and it graphical summary and phylogenetic tree

mentioned experimental conditions. Samples were collected at 1,3,5,7 day's intervals [20] and the degradation of cypermethrin by *Streptomyces diastaticus* was measured with the help of UV spectrophotometer at 590 nm[21] (**Fig. 4).**

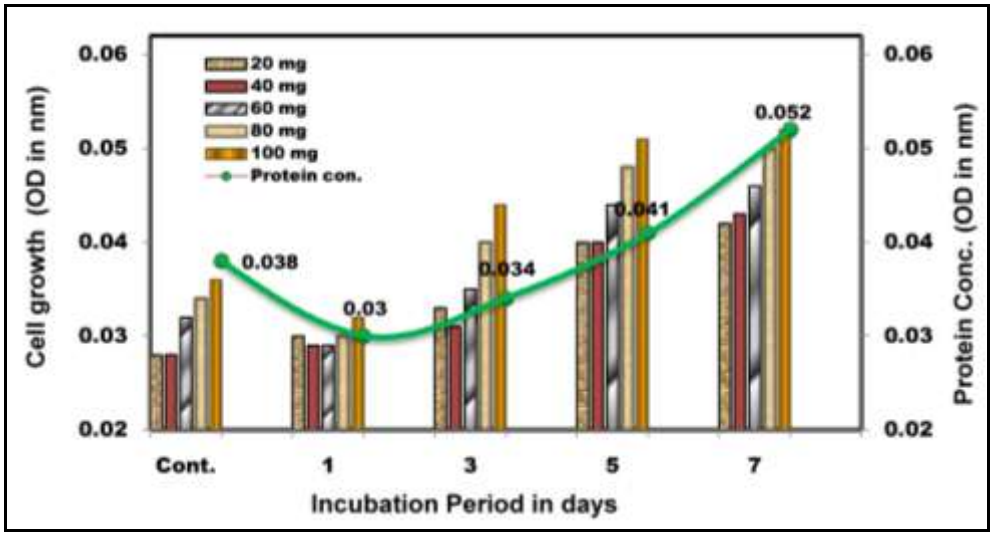


Fig.4: Cell growth and protein concentration of PA2 strain in different conc. of cypermethrin

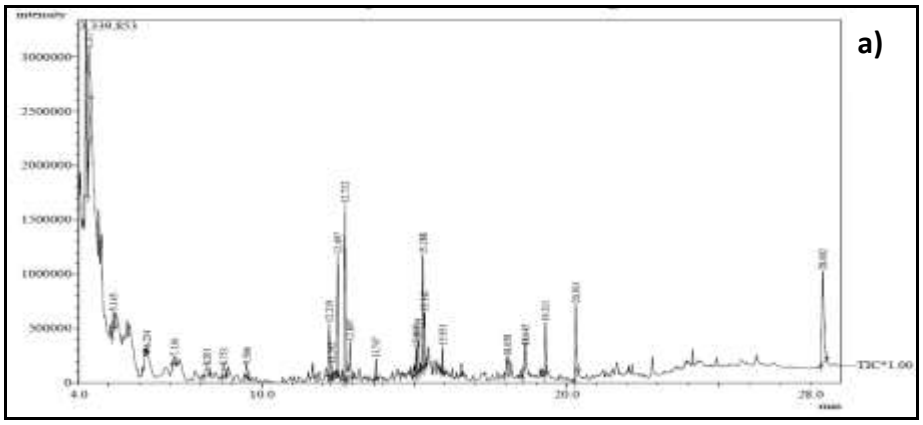
3.5. Total protein estimation by Lowry's method

The protein concentration of the 100mg cypermethrin treated PA2 culture in mineral salt medium was measured 1st to 7th day. It denotes the releasing of the enzyme during the degradation of 100 mg cypermethrin by the potential strain. In the present the study protein content was measured by using Lowry's method at all the day (Fig. 4). The protein content for 5 days and 7 days of mineral salt broth medium with a PA2 strain containing cypermethrin 100 mg showed 720 µg /ml, 810 µg/ml values respectively. The protein concentration was lower during 3rd day and then it's gradually increased than the control; it denotes that the enzyme reaction was held during degradation of cypermethrin. In addition, it indicates PA2 starts to degrade the cypermethrin while it's attaining confluent of growth (after 3 days)**,** which is further supported by GC-MS results.

3.6. Detection of cypermethrin metabolites by GC-MS method

The degradation products of cypermethrin by strain PA2 were extracted and identified by GC-MS **(Fig. 3)**. The metabolite peaks were identified using documented date from National Institute of Standards and Technology (NIST) library database. There are three cypermethrin peaks and three peaks metabolism was observed by GC-MS[22] **(Fig. 3, 3A, 3B, 3C, 3D).** In our present report elaborate that the degradation products of cypermethrin by PA2 strain were extracted and identified by GC-MS **(Table 2 and Fig. 5).** The major peak of 5th and 7th days showed the hydrolysis of cypermethrin compound (RT=15.292, 23.714 and 28.41) benzaldehyde 3- phenoxy, cyclopropane carboxylic acid and 1 propanol 2, 2 dimethyl of cypermethrin by strain PA2.

This was the vital role of PA2 strain on cypermethrin degradation is higher than the previous reports, particularly in high concentrations, which is accordance with UV- spectrophotometer results (**Fig. 5).** According to the previous reports and GC-MS report, the PA2 degraded the 100 mg/L of cypermethrin approximately 70-75 % on 5th days and 92-96% after 7th day in the liquid minimal medium.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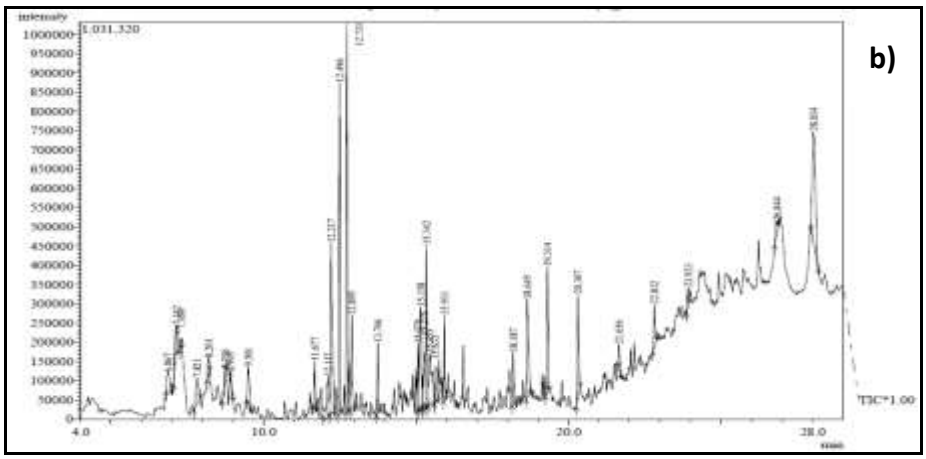
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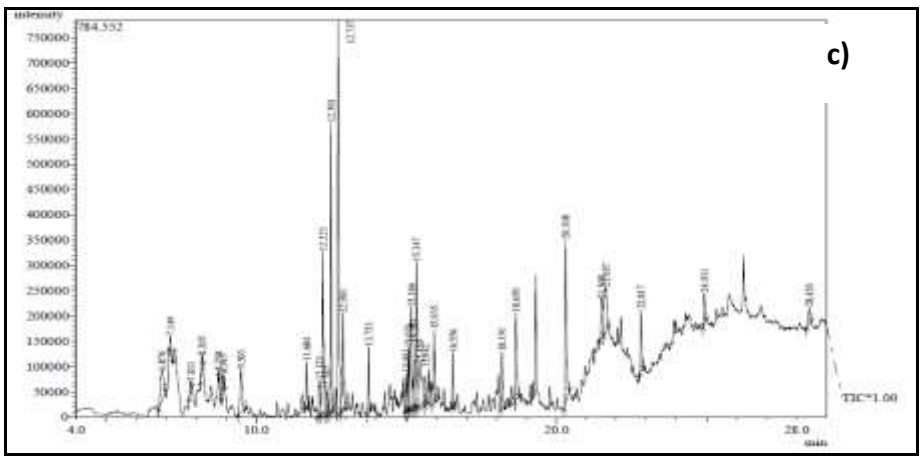
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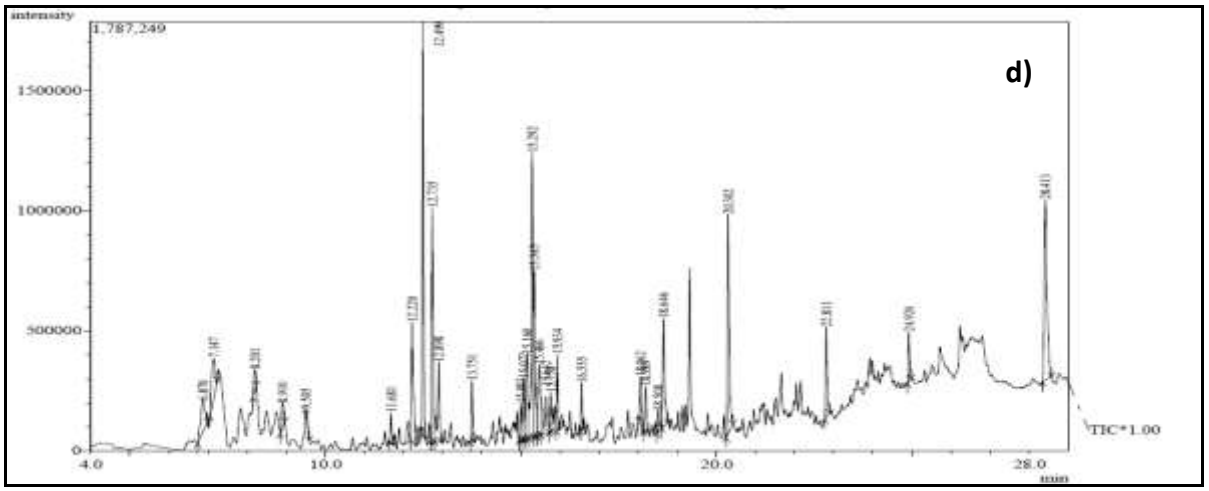
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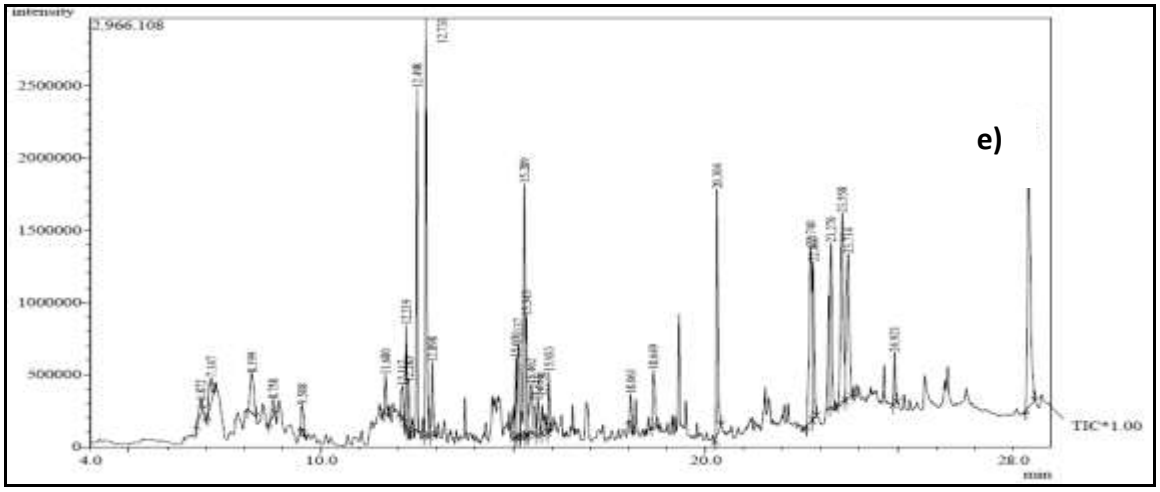

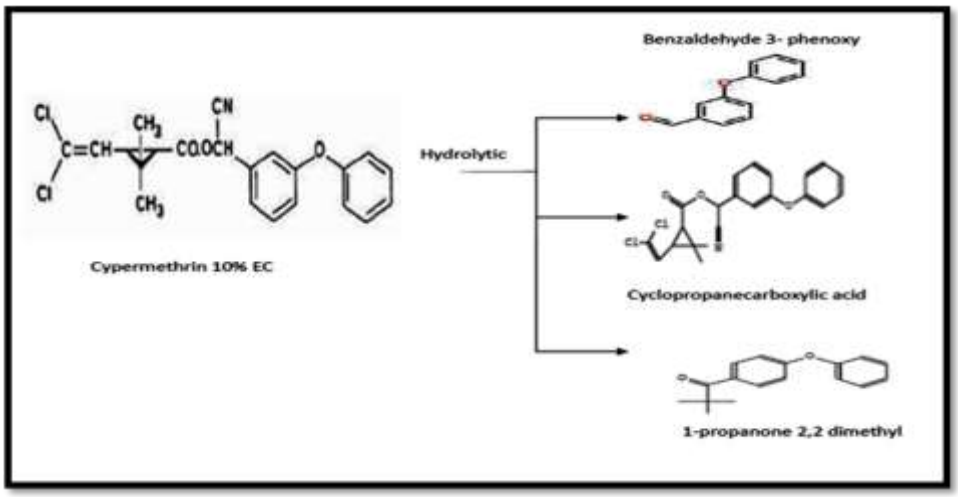

Fig.5 : GC-MS analysis of Cypermethrin degradation (100 mg) in PA2 strain a) Control b)1st day, c) 3rd day, d) 5th day, e) 7th day f) degraded products of cypermethrin

Table: 2 Detection of cypermethrin hydrolysis by PA2 strain

Peak day	R. time	I. time	F. time	Area %	Height %	Chemical name	Chemical Formula
5	15.292	15.192	15.325	10.53	10.45	Benzaldehyde3-phenoxy	$C_{13}H_{10}O_2$
7	23.714	23.633	23.83	6.67	4.31	Cyclopropanecarboxylic acid	$C_{22}H_{19}C_{12}NO_3$
Control	28.41	28.333	28.575	11.61	7.84	1-propanone 2,2-dimethyl	$C_{17}H_{18}O_2$

4. Discussion

In the present study, 33 different types of actinobacterial isolates were obtained from pesticide contaminated soil samples of jasmine garden, Ponparappipatti village, Namakkal district, Tamil Nadu. It was generally considered that the adverse conditions for environmental microorganism's enrichment and screening are crucial in the selection of isolates not only with the desired degrading enzyme systems but having specific regulation of the degradation pathways[16]. In earlier studies, it was described that actinobacteria capable of tolerating and degrading Chlorpyrifos (CP) and pentachlorophenol (PCP) in the contaminated soil[10]. In addition, they described the necessary to evaluate the ability of the actinobacteria to tolerate the pesticides mixture. Fuentes et al 2013 revealed that the 2g L^{-1} of biomass (wet weight) of the single actinobacteria culture has less

tolerance compared than the mixture of four of *Streptomyces* sp. (A2, A11, M7 and AC7) isolated from organochlorine pesticides contaminated Argentinian soils and sediments showed a high degree of tolerance to the toxic mixture (CP and PCP, each at a concentration of 1.66 mg L^{-1}). In our study among the 8 actinobacterial strains PA2 showed faster and efficient growth and tolerance against 100 mg L^{-1} of cypermethrin concentration. So, the strain PA2 is a potential actinobacteria for degradation of single pollutants of soil in very high concentration. In future, along with PA2 multiple xenobiotics which we are screened in may help to bioremediation of mixture pesticide pollutants.

Lin et all 2011 represents that the *Streptomyces sp.* HU-S-01, degraded about 92.1 % substrate within 24 hours and 100 % after 96 hours at the concentration of 50 mg/L (cypermethrin) in the medium[6]. The degradation products of cypermethrin by strain HU-S-01 were extracted and identified by GC-MS. The metabolite peaks were identified by using documented data from National Institute of Standards and Technology (NIST) library database. There are four cypermethrin peaks (B1, B2, B3 and B4) were observed and one peak (A) of metabolism were observed at 24 hours, the peak A (RT=14.793 min) was identified as 3-Phenoxybenzaldehyde, a hydrolyzate of cypermethrin by strain HU-S-01. However, the peak of cypermethrin and 3-Phenoxybenzaldehyde disappear after 96 hours, it suggests that cypermethrin and 3-Phenoxybenzaldehydewere degraded completely by HU-S-01. The first step in the microbial degradation and detoxification of pyrethroid compounds is the hydrolysis of carboxyl ester linkage[23-25]. In our present report elaborate that the degradation products of cypermethrin by PA2 strain were extracted and identified by GC-MS. The major peak on 5^{th} and 7^{th} days showed the hydrolysis of cypermethrin compound (RT=15.292, 23.714 and 28.41) benzaldehyde 3- phenoxy, cyclopropane carboxylic acid and 1 propanol 2, 2 dimethyl of cypermethrin by strain PA2. This was the vital role of PA2 strain on cypermethrin degradation is higher than the previous reports, particularly in high concentrations.

Zhang et al 2011 reports 0.15 g L^{-1} of *Pseudomonas aeruginosa* CH7 biomass inocula, isolated from activated sludge, was able degraded the 25–900 mg L^{-1} concentration of beta-cypermethrin at 90% within 12 days[20]. Similarly, Jilani et al 2006 says 125 mg L^{-1} concentration of beta-cypermethrin was degraded only 17% within 48h of f Pseudomonas (IES-Ps-1) [21]. Yin et al 2013 reports that the degradation experiments showed the isolate *R. sphaeroides* strain S10-1 possessed the relatively higher degradation, capacity of degrading cypermethrin (100 mg L^{-1}) by 90.4% after incubating 7 days at pH 7.0 and Temperature 37°C and S10-1 utilized cypermethrin as its sole carbon and energy source in MSM[19]. In the present study 50, ml mineral salt medium supplemented with 100 mg cypermethrin and also adjusted pH 7.2 the potential strain spot on the plate and incubated at 28°C for 7 days. In our report compared with enrichment and utilization of cypermethrin by actinobacteria in MSB culture S*treptomyces* sp. strain PA2 utilized cypermethrin (100 mg L^{-1}) as a source of energy as like S10-1, HU-S-01, CH7, A2, A11, M7 and AC7. However, the results narrate our strain PA2 play a crucial role in the utmost and faster degradation (7^{th} day) of 100 mg L^{-1} cypermethrin with a low quantity of inoculum. In addition, according to the NIST library database and GC-MS products as like in previous studies, the degradation rate was state that approximately 92-96% on 7^{th} day and 70-75% on 5^{th} day.

Conclusions

The present study concluded that the actinobacterium *Streptomyces diastaticus* (PA2) is a potential source for biodegradation of cypermethrin pesticide in the agricultural soils. Based on the available literature, there is no report on the pesticide degradation by *Streptomyces diastaticus*. So, the present study is open an account for the high concentration of cypermethrin (100 mg) degradation by the actinobacteria, *Streptomyces diastaticus* especially~92-96% degradation rate within sort period and lower concentration of inoculums (0.1mL of pure culture). The future exploitation of this strain will lead to the discovery of many useful eco-friendly pesticide degradation compounds and it increases the soil fertility and prevented health problem in human as well as animals**.**

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