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Research Article

Isolation and Characterization of Efficient Lambdacyhalothrin Degrading Actinobacteria from Paddy Field Soil

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ABSTRACT
Actinobacteria isolated from the Lambdacyhalothrin exposed paddy field soil by enrichment method designated as JMCTTKA8. In characterization and identification studies, mineral medium spiked with 0.05% Lambdacyhalothrin showed good growth which indirectly proved that the capacity to degrade it. Morphological characters like silvery ash color pigmented, smooth, waxy, flat matt, glistening colonies, pale brown pigmented were appeared on ISP4 and ISP6 media, contrastingly milk ash pigmented colonies in ISP7 medium. In BA and CM media, good levels of growth with milky whitish to pale whitish pigmented, smooth to rough colonies were appeared. The strain strongly utilizes carbon and nitrogen sources. JMCTTKA8 SEM images exhibited thin and very long filamentous without any elementary branching in early phase of growth, rod shaped cells were observed as length of 1.062µm and 0.429µm diameter. Rod to coccoid-shaped elements were appeared, which are the characteristic property of the genus *Rhodococcus*. 16S rRNA phylogenetic analysis revealed that strain JMCTTKA8 should be assigned to the genus *Rhodococcus*. The strain JMCTTKA8 is capable of degrading Lambdacyhalothrin pesticide and used its metabolites as a sole carbon and nitrogen source for their growth. Therefore, the strain *Rhodococcus* is suitable for the efficient and rapid bioremediation of Lambdacyhalothrin pesticide contaminated environment.

KEYWORDS: Paddy field soil sample; Lambdacyhalothrin; 16S rDNA; *Rhodococcus* sp.

INTRODUCTION

Agricultural practices are one of the largest and most important economic activities in the world. Since 1950s the usage of chemicals to control crop pests has continuously grown. Pesticides usage becomes an essential activity of agriculture to protect crops and livestock from various pests for many decades and their application considered as the most effective mechanism against pest [1]. Natural insecticide pyrethrin obtained from *Chrysanthemum cinerariaefolium* and *Chrysanthemum coccineum* flowers were used to produce pyrethroid pesticides [2], the third most employed class of insecticide used worldwide, more than 320 million hectares [3]. Very less percentage (<1%) of an applied pesticide only reaches the target pest and the remaining were ends up in soil, water and air [4]. Through this abiotic factors the pesticides entered into the food chain and affects various non-target species like aquatic invertebrates, fishes, mammals like humans [5],

flora and fauna [6, 7] and also disturbs the soil enzyme activities [8].

Lambdacyhalothrin[(RS)-a-Cyano-3-phenoxybenzyl-(Z)-(1RS,3RS)-(2-chloro-3,3,3-trifluoro propenyl)-2,2-dimethylcyclopropanecarboxylate] is one of the main pyrethroids, their usage been gradually increasing globally for agriculture, especially to reduce the usage of organophosphates compounds in residential home and some agricultural applications [9]. It has higher light stability and longer effect duration than natural pyrethroids and their half-life in water varied from 17 to 110 days [10] and highly toxic to aquatic invertebrates and fishes [4]. Pesticide exposure increased the incidence of cancer, hormone disruption and problems with reproduction and fetal development and also develops hyperexcitation, aggressiveness, skin allergy and seizures [11, 12].

In India, Rice is cultivated in about 44.3 million hectares producing 141 million metric tons of grains annually [13]. Lambdacyhalothrin, broad spectrum of pyrethroid insecticide applied on a large scale in rice fields of Tamil Nadu (India) to control foliar insects, while reaches the soil surface it accumulates nearly up to 15 cm of top soil layer [14] which is the vital site of the highest microbial activities for maintaining soil fertility [15] while arbitrary usage of pesticides causes great damage to the microflora in the paddy field [16].

Bioremediation has received increasing attention as an efficient and cheap biotechnology approach to retrieve the polluted environments. Microbial degradation process have been successfully removed various chemicals from soil and aquatic environments [17]. Actinobacteria is a kind of soil bacterium present in high percentage in soils which has great potential to biodegrade various organic and inorganic toxic compounds [18]. Actinomycetes belongs to the genera *Arthrobacter, Brevibacterium, Clavibacter, Corynebacterium, Micromonospora, Mycobacterium, Nocardia, Nocardioides, Rhodococcus* and *Streptomyces* were actively involved in pesticide-degradation [19]. Recently pyrethroid-degrading strains and their mechanisms have been studied by various researchers such as *Serratia* sp. JCN13 [20], *Streptomyces parvulus* HU-S-01 [21] and *Brevibacterium aureum* DG-12 [22] and the three genes (Estp, pytH and PytZ) encoded for pyrethroid-hydrolyzing carboxylesterases from *Klebsiella* sp. ZD112 [24], *Sphingobium* sp. JZ-1 [25] and *Ochrobactrum anthropi* YZ-1 [26], one new monooxygenase CMO from a *Streptomyces* sp. [23] respectively.

The biodegradation process plays an alternative approach to control synthetic pesticide and its residues hazards due to its cost-effective and ecofriendly properties. Only a few reports were available about the Lambdacyhalothrin degradation by actinobacterial isolates. Therefore, there is an immediate need for effective strategies to remove Lambdacyhalothrin from environment. So, the present work mainly focused on the Lambdacyhalothrin degradation with the following objectives. (1) Isolation of actinobacterial strains from pesticide exposed paddy field soil samples by enrichment method (2) Morphological, biochemical and molecular characterization of the isolated actinobacterial strain.

MATERIALS AND METHODS

Soil sample collection

The Lambdacyhalothrin pesticide exposed (more than 17 years) paddy field soil samples were collected in an aseptic manner at a depth of 5-10cm according to the 'V' shaped method at different sites at Paithur, Attur, Salem district, Tamil Nadu (India). Within six hours soil samples were brought to the laboratory and spread in clean aluminum trays, dried at room temperature. After sieving to a maximum particle size of <2mm mesh and these soil samples were stored at 4°C until further use [27].

Chemicals

The synthetic pyrethroid pesticide used in the present study was commercial grade pesticide, Lambdacyhalothrin 5% EC w/v named KARATE® (Syngenta Agrochemicals, India Private Limited) procured from the local market Attur, Salem district, Tamil Nadu (India). All other chemicals and reagents used were of analytical grade and purchased from Hi-Media Pvt Ltd Mumbai, India.

Isolation and enumeration of Actinobacteria by enrichment method

Lambdacyhalothrin exposed paddy field soil sample 10g was collected and the fine soil

particle has added to 100ml of sterilized Mineral medium (MM pH 6.8–7.0) containing (gL-1) Na_2HPO_4-5.8, KH_2PO_4-3.0, NaCl-0.5, NH_4Cl-1; $MgSO_4$-0.25 spiked with 0.05% pesticide (Lambdacyhalothrin) in 250ml Erlenmeyer flask for the isolation of Actinobacteria [28]. The flasks were placed on a rotary shaker and incubated in 28±2°C, 121rpm for 25 days. After twenty five days, 1ml aliquots of treated samples was taken and mixed in 9ml of sterile deionized water, serially diluted upto 10^{-8} dilutions. The 0.1ml of the serially diluted sample from 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} was taken and spread over the petridishes containing Starch-Casein Nitrate media [29] supplemented with cycloheximide (50µgmL^{-1}) for the enumeration of Actinobacteria and uninoculated plate was used as control. All the plates were incubated at 28°C for up to 15 days and observed daily from the third day onwards for the growth and characteristics.

Morphologically different actinobacteria colonies were appeared as tough leathery or chalky texture, smooth and colloidal nature, dry and branching filamentous with different colour of spores were picked from the isolated plates [28] and streaked on ISP4 medium supplemented with Lambdacyhalothrin (0.05%) and incubated at 28°C for 7 days.

Morphological and Cultural Characteristics

The colour of spore mass of the matured aerial mycelium like grey, white, yellow and pigmentation of substrate mycelium (yellow) and diffusible pigments (yellow) of the selected isolates JMCTTKA8 were determined by naked eye examinations of 15 days old cultures grown on various International Streptomycetes Project (ISP) media [28] ISP1 to ISP7, Bennet's agar and Calcium malate agar. The intermediated colour of the substrate mycelium was also recorded.

Utilization of Carbon sources

Different types of carbon sources (1.0%) such as arabinose, xylose, meso-inositol, D-mannitol, fructose, rhamnose, raffinose, galactose, maltose, glycerol, sucrose, lactose, D-mannose, D-sorbitol, dextrin and dulcitol were sterilized with diethyl ether overnight and added separately on the molten basal medium. The isolates streaked on various slant tubes and incubated for 15 days at 28°C. The basal medium with 1.0% of glucose was considered as positive control. The basal medium alone represented as negative control. The selective JMCTTKA8 isolate was streaked on various sugars and incubated for 15 days at 28°C along with its positive control as well in negative control [28].

The utilization of carbon is expressed as strongly positive (++), when the growth on tested carbon in basal medium is equal to or greater than growth on basal medium plus glucose positive (+), when growth on tested carbon is significantly better than on the basal medium without carbon and significantly less than with glucose. Negative (-) showed the growth is similar to or less than growth on basal medium without carbon.

Utilization of Nitrogen sources

The ability of selected JMCTTKA8 isolate in utilizing various nitrogen compounds as a source of energy was studied following the method recommended by ISP [28]. Chemically pure nitrogen sources, certified to be free from the add mixture with other contaminating materials were used. Nitrogen sources of the test were L-Proline + basal medium (positive control), Basal medium alone (negative control) basal medium with L-Glutamine, L-Methionine, L-Valine and L-Alanine, 0.1% each. The sterilized media was prepared and inoculated glass tubes were observed from the 5^{th} day onwards. The growth on a given nitrogen sources was compared with controls, growth on basal medium alone and growth on basal medium with L-proline. For each of the nitrogen sources, utilization is expressed as positive (+), negative (-), on doubtful (+ or -). In the doubtful strains, there was only a trace of growth which was slightly higher than that of the controls.

Scanning Electron Microscope

For electron microscopic observations, the culture strain JMCTTKA8 grown on ISP4 medium and 2 to 3 sterilized small discs were inserted at an angle of 45°C and incubated for 5 days at 28°C. After 5 days, a small disc of cultures were doubly washed with PBS (0.1M) and fixed with 2% glutaraldehyde in PBS at 4°C for 2hr [21]. After fixation, the samples were dehydrated with 30%, 40%, 50%, 60%, 70%, 80%, 90% ethanol series at one time each and then dehydrated with 100% ethanol by three times for 10min. The dehydrated samples were kept

overnight in desiccators and thereafter gold coated by sputtering. The samples were then analyzed by HR-FE-SEM (JEOL, JSM-670IF) at 30kV.

Molecular Characterization: DNA Extraction

Genomic DNA was prepared according to standard methods [30]. PCR amplification was performed for the respective samples of purified DNA using Actino-F (5'-CGCGGCCTATCAGCTTGTTTG-3') and Actino-R (5'-CCGTACTCCCAGGCGGGG-3') primers [31]. The polymerase chain reactions conditions include initial denaturation of template DNA was achieved at 94°C for 6min. Further denaturation was carried out 94°C for 1min; annealing at 52°C for 1min, extension at 72°C for 30sec and a final extension at 72°C for 10min for 35 cycles. Amplified products were isolated by electrophoresis on 1.2% agarose gel using 1×TAE buffer at a constant supply of 60V for 30min. The resulting sequences were compared with the genes available in the GenBank nucleotide library by a BLAST search through the National Center for Biotechnology Information (NCBI) and phylogeny was analyzed using MEGA 7.0 [4]. An unrooted tree was built using the neighbor-joining method. Based on the expressed polyphasic characters, the potential of actinobacteria strain was tentatively identified with the help of the keys of Nonomura [32].

RESULTS & DISCUSSION

Isolation and enumeration of Actinobacteria by enrichment method

In the present study, the screening of pyrethroid-degrading strains by enrichment procedure Lambdacyhalothrin pesticide exposed paddy field soil samples used to select some potential isolates with a high survivability in the environment and maximal degrading activity towards pyrethroids. Finally, Actinobacteria isolated from Lambdacyhalothrin pesticide exposed agricultural soil samples from Paithur village, Attur Taluk, Salem district, Tamil Nadu (India).

0.05% concentration of commercial lambdacyhalothrin pesticide incubated in 28±2°C, 121rpm for 25days. After that, 1ml of treated sample was taken and mixed in 9ml of sterilized deionized water and serially diluted upto 10^{-8} dilutions. From the dilution factor 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were used to isolate the actinobacteria by spread plate method on SCN media. In most cases reported, pesticide degraders were isolated from corresponding contaminated sources, including pesticide-containing soil, water, sediment, as well as industrial wastes [33, 20, 34].

Characterization of JMCTTKA8

Morphological and cultural characteristics of JMCTTKA8 actinobacterial strain was studied by using various ISP media (Table 1). Moderate as well as poor levels of growth were observed with cream and whitish color in ISP1 and ISP3 media respectively. Pinkish milky white pigmented, wavary edges in the outer layer of the culture with brownish riverside pigmented was also noted in ISP2 medium.

Silvery ash color pigmented, smooth, waxy, flat matt, glistening colonies were appeared on ISP4 medium, where the mass of young colonies were emerged from the mature colonies. Pale brown pigmented, smooth, waxy, glistening colonies were observed in the ISP6 medium. Contrastingly milk ash pigmented, smooth, waxy, glistening and wavary natures of the colonies were recorded in ISP7 medium. Good levels of growth was achieved with milky whitish to pale whitish pigmented, smooth to rough types of colonies were noticed in BA and CM media.

Utilization of Carbon Sources

The isolates strongly utilized the positive control (Basal medium + 1.0% glucose) and other sugars such as D-mannitol, fructose, glycerol, sucrose and dextrin and moderately utilized raffinose, galactose, maltose, lactose, D-mannose and dulcitol as a sole carbon and energy source. But arabinose, xylose, meso-inositol, rhamnose and D-sorbitol was not utilized as a sole carbon and energy source. Growth was absent in negative control (Table 2).

Table 1: Morphological characteristics of JMCTTKA8 in various media

Medium	Growth	Colour of the aerial mycelium	Colour of the substrate mycelium	Soluble pigment	Melanin pigment
ISP1	++	Creamy	Pale white	0	0
ISP2	++++	Pinkish Milky white	Brownish	0	0
ISP3	++	Whitish	Whitish	0	0
ISP4	++++	Silver ash	Pale yellow	0	0
ISP5	+	Pale yellow	Pale yellow	0	0
ISP6	++++	Pale brown	Pale yellowish	0	0
ISP7	++++	Milky ash	Pale whitish ash	0	0
BA	+++	Milky whitish	Pale whitish ash	0	0
CM	+++	Pale whitish	Pale whitish ash	0	0

-: No growth; +: Poor growth; ++: Moderate growth; +++: Good growth; ++++: Very good growth; x: Not determined; 1: Produced; 0: Not produced

Table 2. Utilization of Carbon source by JMCTTKA8 actinobacterial strain

Sl.No.	Carbon Source	Growth	Result
A	Basal medium (Positive control)	+ +	Strongly positive
B	Basal medium with glucose (negative control)	-	Negative
1	Arabinose	-	Negative
2	Xylose	-	Negative
3	Meso-inositol	-	Negative
4	D–mannitol	+ +	Strongly positive
5	Fructose	+ +	Strongly positive
6	Rhamnose	-	Negative
7	Raffinose	+	Positive
8	Galactose	+	Positive
9	Maltose	+	Positive
10	Glycerol	+ +	Strongly positive
11	Sucrose	+ +	Strongly positive
12	Lactose	+	Positive
13	D–mannose	+	Positive
14	D–sorbitol	-	Negative
15	Dextrin	+ +	Strongly positive
16	Dulcitol	+	Positive

++: Strongly Positive; +: Positive; -: Negative

Utilization of Nitrogen sources

The isolate strongly utilized the 0.1% of the L-glutamine, L-valine and L-alanine along with the positive control L-proline. Moderate level of growth was achieved in L-methionine (Table 3). These nitrogen sources were strongly used as a sole nitrogen and energy source. Obviously the growth was absent in negative control.

Table 3. Utilization of Nitrogen source by JMCTTKA8 actinobacterial strain

S.No.	Nitrogen Source	Growth	Result
1	Basal medium + L-proline (Positive control)	+ +	Strongly positive
2	Basal medium (Negative control)	-	Negative
3	L – Glutamine	+ +	Strongly positive
4	L – Methionine	+	Positive
5	L –Valine	+ +	Strongly positive
6	L- Alanine	+ +	Strongly positive

++: Strongly Positive; +: Positive; -: Negative

Scanning Electron Microscope

In scanning electron microscopic observations, JMCTTKA8 colonies exhibited as a thin and very long filamentous without any elementary branching in the early phase of growth. Later, the thin long filamentous cells divided and produced a number of small fragmented cells. In the final phase, rod shaped cells were observed with the length of 1.062µm and 0.429µm diameter. A beautiful connection thread was observed in between the two rod cells. The rod to small rod cell cycle was only observed instead of rod to coccus type of cell cycle. Rod- to coccoid-shaped elements are the characteristic property of the members of the genus *Rhodococcus* (Fig. 1).

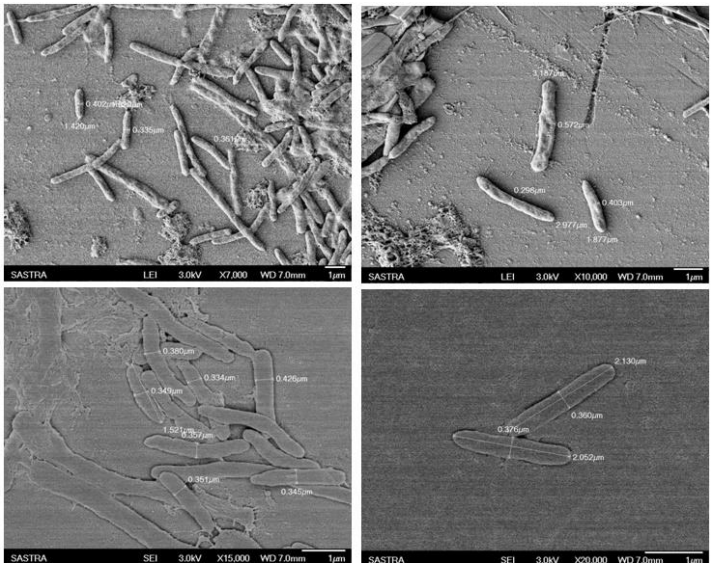

Fig. 1. Scanning Electron Microscope images showing the rod to coccoid shaped cells of JMCTTKA8

Molecular characterization

According to BLAST analysis, the resulting sequence had high similarity to the 16S rDNA gene sequence of genus belonging to *Rhodococcus* group. The phylogenetic tree (Fig. 2) indicated that the isolate was closely clustered with *Rhodococcus baikonurensis* (GenBank accession No. NR024784.1) and *Rhodococus erythropolis* (GenBank accession No. NR119125.1), with sequence identities of 100% respectively. The 16S rDNA gene sequence was deposited in the GenBank database with the accession number MG208089. Based on the morphological, biochemical characteristics and 16S rDNA sequence analysis, the potential strain JMCTTKA8 was identified as *Rhodococcus* sp.

Members of Actinomycetes, *Streptomyces* genus were widely present in the soil and their bioactive secondary metabolites isolates showed various inhibitory effect toward microorganisms [35, 36, 37]. However, only a few literatures were available about Actinomycetes ability to degrade pyrethroids which belongs to *S. aureus.* The genus *Rhodococcus* was classified into the family *Nocardiaceae* of the suborder

Corynebacterineae [38]. The genus *Rhodococcus* have been assigned to four 16S rRNA subclades, represented by *R. equi*, *R. rhodnii*, *R. rhodochrous* and *R. erythropolis* [39] and their discovery were of novel ones which has been greatly facilitated [40].

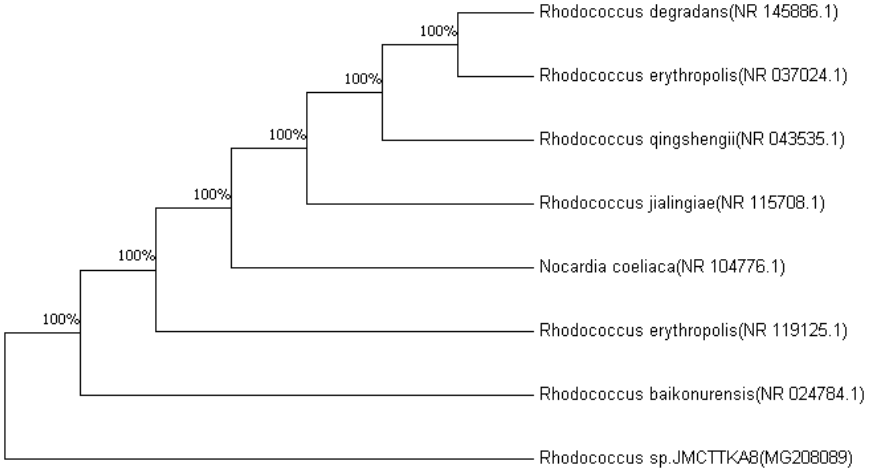

Fig. 2. The phylogenetic tree based on the 16S rDNA sequence of strain JMCTTKA8 and related strains

Previous studies indicated that the potential pyrethroid degrading microbes were mostly from genera *Bacillus* and *Pseudomonas* [41, 42, 43], while *Streptomyces* sp. appeared to be a new genus that was found highly effective in degrading pyrethroids. Gram-positive, aerobic, non-motile, non-spore forming strain, designated DSD51WT, was isolated using a resuscitative technique from a soil sample collected from Kyoto park (Japan) and characterized by using a polyphasic approach. The morphological and chemotaxonomic properties of the isolate were typical of those of members of the genus *Rhodococcus*. Strain DSD51WT was found to form a coherent cluster with *Rhodococcus hoagii* ATCC 7005T, *Rhodococcus equi* NBRC 101255T, *Rhodococcus defluvii* CallT and *Rhodococcus kunmingensis* YIM 45607T as its closest phylogenetic neighbours in 16S rRNA gene sequence analysis.

Syed *et al.* [44] a novel actinobacterial strain (NIO-1009T) isolated from a marine sediment sample. 16S rRNA phylogenetic analysis comparisons revealed that strain NIO-1009T had the closest sequence similarity to *Rhodococcus kroppenstedtii* DSM 44908T and *Rhodococcus corynebacterioides* DSM 20151T with 99.2 and 99.1% respectively. Silva *et al.* [45] isolated a novel actinobacterium (CMAA 1533T) from the rhizosphere of *Deschampsia antarctica* collected at King George Island (Antarctic Peninsula). Strain CMAA 1533T was found to grow over a wide range of temperature (4–28°C) and pH (4–10). Macroscopically, the colonies were observed to be circular shaped, smooth, brittle and opaque-cream on most of the culture media tested. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain CMAA 1533T belongs to the family *Nocardiaceae* and forms a distinct phyletic line within the genus *Rhodococcus*. Sequence similarity calculations indicated that the novel strain is closely related to *Rhodococcus degradans* CCM 4446T, *Rhodococcus erythropolis* NBRC 15567T and *Rhodococcus triatomae* DSM 44892T (≤96.9%).

Yong-Xia *et al.* [46] a Gram-positive, aerobic, non-motile actinobacterium strain, designated YIM 45607T, was isolated from a rhizosphere soil sample in Kunming, south-west China. Chemotaxonomically, the isolate contained chemical markers that supported its assignment to the genus *Rhodococcus*. On the basis of 16S rRNA gene sequence similarity analysis, strain YIM 45607T formed a new subline within the genus *Rhodococcus*, with *Rhodococcus equi* as its closest phylogenetic neighbour (98.2% 16S rRNA gene sequence similarity to the type strain).

CONCLUSION

This paper evidenced the *in vitro* analysis of biodegradation efficiency of actinobacterial strain JMCTTKA8 in 0.05% Lambdacyhalothrin in mineral medium. This strain isolated from

Lambdacyhalothrin pesticide exposed paddy field soil from Paithur Village, Attur Taluk, Salem district (Tamil Nadu, India). Their characters were observed in ISP recommended media along with BA and CM media. D-mannitol, fructose, glycerol, sucrose, dextrin and L-glutamine, L-valine, L-alanine utilized as carbon and nitrogen sources. In ISP4 media, this strain exhibited thin, very long filamentous rod shaped cells. The 16S rRNA gene sequence analysis of JMCTTKA8 showed peculiar cell cycle pattern as rod to rod cell cycle whereas other *Rhodococcus* genus showed rod to coccus cell cycle pattern. By these preliminary investigations, it is clear that these actinobacterial isolates might be used for biodegradation of Lambdacyhalothrin pesticides as well as for the biotransformation studies.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in this research article.

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