J. Environ. Treat. Tech.
ISSN: 2309-1185

Journal web link: https://dormaj.org/index.php/jett
https://doi.org/10.47277/JETT/10(3)194

Bioremediation of Synthetic Pyrethroid by Hydrolases of Bacillus aryabhattai and Bacillus circulans Derived from Indigenous Soil

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Received: 25/05/2022 Accepted: 04/07/2022 Published: 20/09/2022

Abstract

Synthetic pyrethroids are widely used for the improvement of crop production but are also regarded as potentially harmful pollutants. The present study aimed to evaluate the efficiency of different indigenous soil bacteria to degrade Lambda Cyhalothrin (LC), a known synthetic pyrethroid. The sampling area was selected as Rawal Lake and soil samples were collected from alongside upper streams that flow into Lake. LC degrading bacterial strains were isolated and identified as *Bacillus aryabhattai* and *Bacillus circulans*. COD was used as a parameter for measuring % the removal of LC at different intervals. *Bacillus aryabhattai* showed % removal of 55% whereas, *Bacillus circulans* showed a % removal of 83% in Minimal Salt Media after 72 hours. This study revealed that *Bacillus circulans* may tolerate LC more effectively at higher concentrations and therefore may be used as potential hydrolyzing enzymes that may disrupt chemical bonds of pyrethroid and result in the reduction of toxicity. This work exhibited a promising approach for the bioremediation of LC and may hence be used as environmental bioremediations of other pyrethroids as well.

Keywords: Bioremediation, Lambda-cyhalothrin, Minimal salt medium, Optical density, Pyrethroids, Pesticide

1 Introduction

Pyrethroids are a type of synthetic pesticide derived from the natural insecticide pyrethrins. The majority of these compounds are chiral molecules that exist as a mixture of isomers and are used to control pests in agriculture and indoor environments (Saillenfait et al., 2015) due to their potent toxic activity against various insect pests (Liu J et al., 2015). Upon entering the soil habitat, pyrethroids go through a number of processes, such as conversion or decomposition, sorption-desorption, volatilization, absorption by florae, overflow to shallow waterways, and transit to underground water (Galadima et al., 2021). However, excessive use has a detrimental effect on non-target organisms and contaminates terrestrial and aquatic ecosystems leading to environmental pollution and health risks (Cycon and Piotrowska-Seget, 2016; Gonçalves and Delabona, 2022). Furthermore, with 320 million hectares treated, pyrethroids are the third most sold chemical class of insecticides (Housset and Dickman, 2009). Lambda- cyhalothrin (LC) is relatively stable in the environment, being stable to hydrolysis in water at pH 5 and having half-lives of 453 days at pH 7, and 7.3 days at pH 9, resulting in (3-(2-chloro-3,3,3,-tri-fluoroprop-1-en-1-yl)-2,2-dimethylcyclopropanecarboxylic acid and an unstable cyanohydrin by-product that ensue 3-phenoxybenzoic acid) (PBAc) (Collis and Leahey, 1984; He et al.,2008). Furthermore, LC biodegradability in the soil is affected by pH, temperature, light exposure, and microbial activity. For instance, a reported half-life in sterile soil was 161 days, whilst the half-life in non-sterile soil was 120 days, and longer periods were achieved with reduced light exposure (Birolli et al.,2018).

LC, a synthetic pyrethroid sold in Pakistan with brand names such as Karate, Hunter, etc., is extensively applied in cotton cultivation along with its uses in vegetable production and dengue vector control. As a second-generation composite pyrethroid; it is one of the 10 most common pesticides used worldwide. The residues of LC have been detected in runoff water, irrigation water, sediments, and surplus water from domestic and agricultural sources. As reported in a study, LC residues have been found in soil and sediment samples collected from Faisalabad i.e. 0.2 µg/L, and Bahawalnagar i.e. 2.9 µg/L (Khan et al., 2018). Traces of LC have been detected in Rawal Lake, where it may produce direct toxic effects on aquatic organisms due to its rapid bioaccumulation potential. LC in higher concentrations is found to be toxic to humans and non-target species. Large dose exposures may cause significant toxicity in humans including neurotoxicity, genotoxicity, cytotoxicity, reproductive toxicity, and mutagenicity (Dehghani et al., 2022). Whereas it is highly toxic to invertebrates and fish. In fish, the compound is toxic at a concentration greater than 0.36 µg/L and for invertebrates, the toxic concentration is 50 µg/ liter (Chen et al., 2015). In order to prevent these pesticides from further exacerbating the situation, many treatment methods have been established. These methods include physical, chemical, or biological processes which either detoxify or degrade the pesticides. Many conventional strategies have been implemented such as photodecomposition, ozonation, adsorption, fenton

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degradation, and incineration. However, these physicochemical technologies are quite expensive and many times, they aggravate the problem, rather than eliminate it (Kumari et al., 2011). Moreover, these technologies are not eco-friendly as they release toxic substances as by-products. The process of Bioremediation has proved to be more attractive than other conventional methods because it is more cost-effective and far less disruptive (Sharma, 2020). Several bacterial strains have been utilized for the degradation of a number of pesticides as they employ metabolic pathways to mineralize these pesticides, whose presence is causing disruption in the environment (Tran et al., 2021). The discovery of new enzymes and pathways could be aided by the identification of novel bacterial species (Bodor et al., 2020).There application in microbial remediation demands an in-depth knowledge of the physiological, biochemical and molecular biological properties towards employed strains (Zhao et al., 2021). According to various research studies, potential bacterial strains such as *Psychrobacter alimentarius, Rhizobium gallicum, Pseudomonas putida, Pseudomonas aeruginosa, Stenotrophomonas acidaminiphilia, Bacillus cereus, Pseudomonas fluorescens, Serratia plymuthica,* and *Achromobacter spp.* were found to be able to degrade chlorpyrifos, cypermethrin, bifenthrin, permethrin, deltamethrin, fastac, fenvalerate and fluvalinate (Thatheyus & Selvam, 2013; Khalid, S. & Hashmi, I., 2016; Rayu et al., 2017; Cycoń et al., 2017).

In the current study, bacterial strains were isolated from Rawal Lake tributaries' soil for the rapid and efficient degradation of LC. The isolation of indigenous bacterial strains capable of metabolizing LC through biodegradation was favorable given that they are well adapted to the local conditions. Indigenous bacterial strains were examined in liquid media under varying concentrations of LC and nutrients, for their potential to degrade LC. The hydrolytic enzyme of bacterial strain disrupts major chemical bonds in the toxic molecules and leads to the reduction of their toxicity (Zhan et al., 2020). In addition to this, the biodegradative capabilities of different microbial isolates in order to identify more efficient strains for their future use in the bioremediation of other toxic compounds were evaluated.

2 Materials and Methods

2.1 Chemicals and reagents

Commercial grade Lambda-cyhalothrin was purchased from a local Pharmacy. The common name for Lambda-cyhalothrin in the market is Nokout. Methanol for standard stock preparation was acquired from Merck (Germany). All COD chemicals used were purchased from sigma Aldrich. Mineral salt medium (MSM) component salts (Diammonium Phosphate $(NH_4)_2HPO_4$, Magnesium Sulfate Heptahydrate ($MgSO_4.7H_2O$), Dipotassium Phosphate (K_2HPO_4), Calcium Nitrate ($Ca\ (NO_3)_2$), Ferrous Sulfate Heptahydrate ($FeSO_4.7H_2O$) were also purchased from Sigma Aldrich.

2.2 Sampling sites and soil sample collection

Rawal Dam, situated in Islamabad, is constructed on the Korang River. The river generates 84,000 acre-feet of water per year with average rainfall, having a catchment area of 106 square miles. Rawal Lake and its catchment area are key sources of drinking water for Rawalpindi city and cantonment. There are four major streams, 43 small streams, and the Korang River contributing to its storage (Ayaz et al., 2016). These four major streams and Korang River were selected as sampling sites, which are shown in Figure 1 along with their GPS coordinates. Soil samples were collected alongside the streams and rivers. The samples from random points selected alongside streams were collected in sterile polythene bags from the depth of 10cm, as per standard APHA protocols (2017). The samples were then transported to the lab and stored at 4°C before further processing.

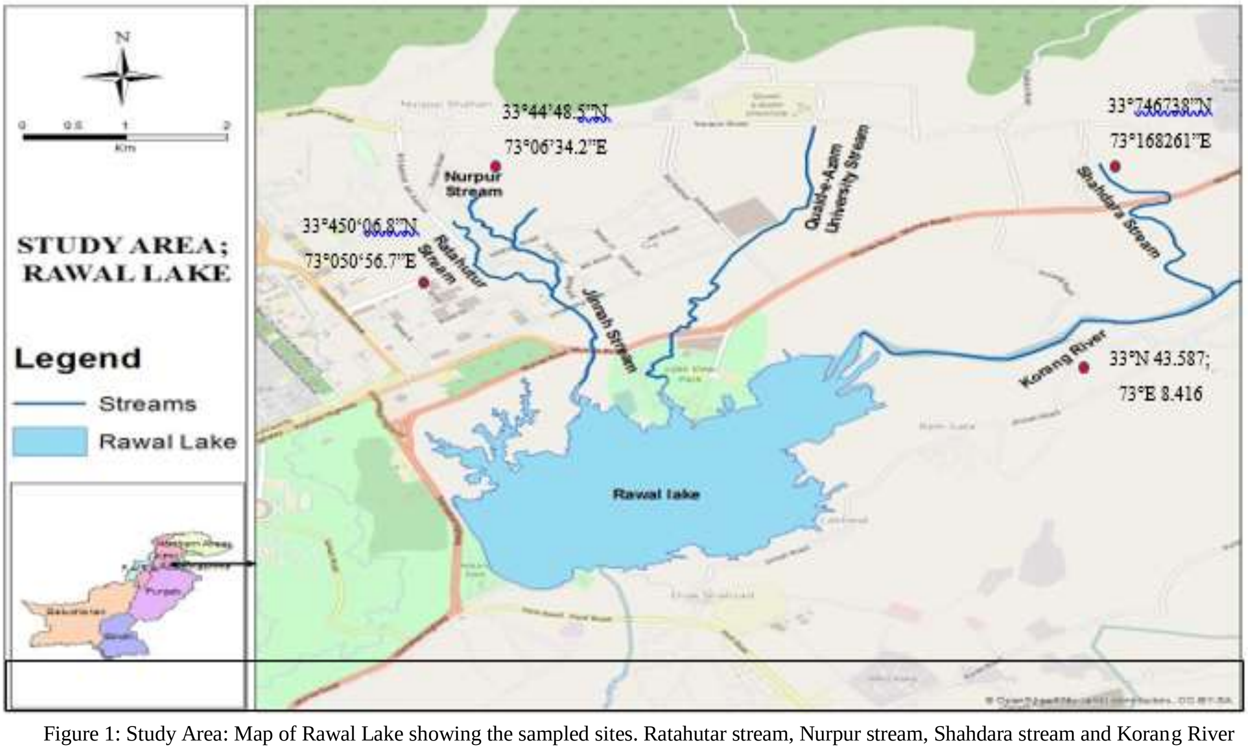

Figure 1: Study Area: Map of Rawal Lake showing the sampled sites. Ratahutar stream, Nurpur stream, Shahdara stream and Korang River

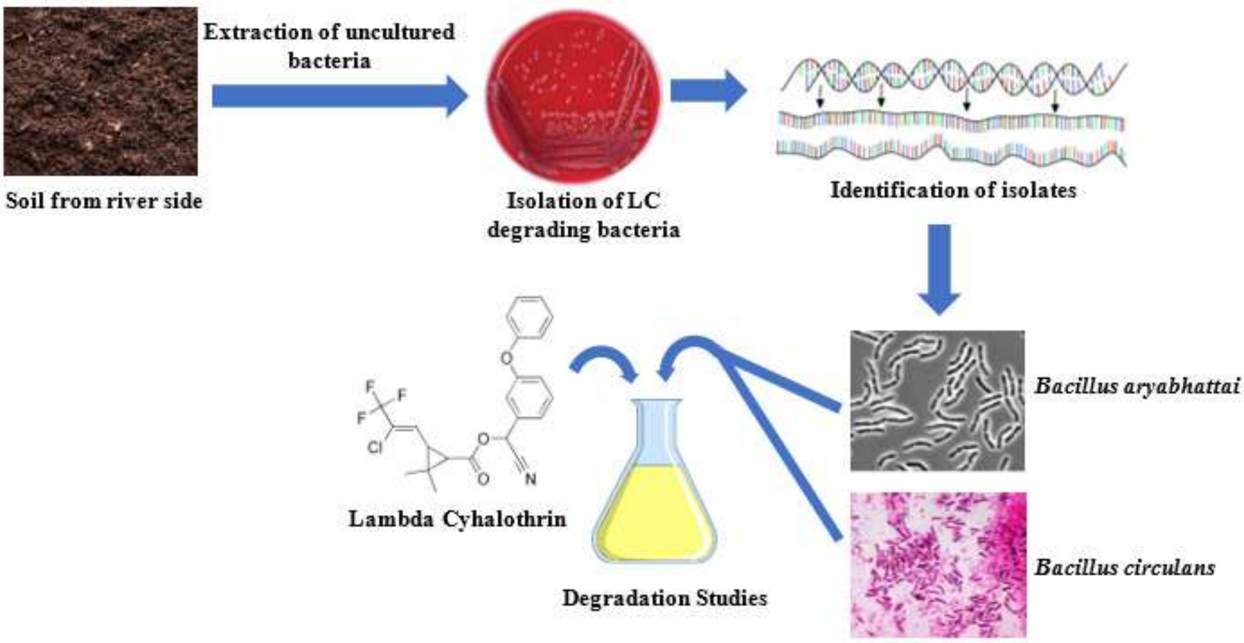

Figure 1a: Flow diagram of the Experimental Work

2.3 Isolation of Lambda-cyhalothrin degrading bacteria

The soil samples were collected from random sites where pesticides were heavily used for improving crop quality and were mixed to form a composite. In order to remove impurities i.e. gravel and stones from soil samples, these were sieved with the help of a 2 mm sieve. Following that, 10g of soil sample was added to 100ml of the minimal salt medium in a 250ml conical flask and incubated for 2 days at 30°C (Abdullah et al., 2016). In order to obtain pure bacterial cultures, aliquots of this suspension were spread on MSM lambda-cyhalothrin agar plates, streaked with the help of the streak plate method, and incubated for 24 hours at 37°C. After 24 hours, distinct bacterial colonies were chosen and further purified using the same procedure mentioned above. Strong resulting colonies in growth were subjected to 4-5 cycles of repeatedly streaking and pure cultures were obtained.

2.4 Screening of potential isolates

The process of screening was performed in order to adapt soil microbes to LC and to evaluate potential isolates capable of utilizing the pesticide more competently. For this purpose, pure cultures were inoculated in MSM medium in 250 ml conical flasks having an LC concentration of 100mg/L. The pesticide in the media served as the sole source of carbon and energy for the growth of strains(Zhang et al., 2017) The conical flasks containing the pesticide and inoculum were kept in an incubator at 37°C. Growth was monitored at regular intervals using a single beam UV-visible spectrophotometer at 600 nm. Strains with appreciable growth curves in the presence of LC were selected for further LC degradation studies.

2.5 Identification of isolates

Isolated strains were identified with various characterization parameters including colony morphology, biochemical test, and gram-staining (Collins & Lyne, 1985). A polymerase chain reaction was performed for species-level identification.

2.6 DNA sequencing and Phylogenetic analysis

DNA from bacterial isolates was extracted using a DNA extraction kit. The extracted DNA was then amplified using PCR. For this purpose, the reaction mixture was prepared with a total volume of 50 µl. The reaction mixture was processed in Thermocycler (Extragene 9600). For the 16SrRNA gene detection, the PCR program includes 5 min at 95°C for template denaturation, and 40 cycles for template amplification consisting of three steps: 95°C for 1 min for DNA denaturation into a single strand, 61°C for 1 min for the primer to anneal to their complementary sequences on either side of the target sequence, 72°C for 1 min for extension of complementary DNA strand from each primer and final elongation at 72°C for 10 min for Taq DNA polymerase to synthesize any un-extended strand left. These isolates were then transferred to the Genome analysis department Macrogen, Seoul, South Korea for 16S rRNA sequencing. The partial genome sequences of the bacterial strains obtained were used to construct a phylogenetic tree using MEGA 7 software. The nucleotide sequence of the 16S rRNA gene of each isolated strain was then submitted to NCBI GenBank for obtaining the accession numbers against each sequence (Ghumro et al., 2017).

2.7 Degradation studies

The capability of selected strains to degrade LC, as the sole carbon and energy source was determined. The MSM medium was supplemented with varying concentrations of LC (100 to 500mg/L) and 24 hours of fresh bacterial cultures in 250 ml conical flasks. The flasks were incubated at 37°C and the samples were monitored for 72 hours. The aliquots were drawn at regular intervals, diluted, and analyzed by the COD titration closed reflux method as per standard methods described by APHA, 2017. Triplicate sets of each concentration with inoculum were maintained in all experiments along with controls. Optical density was also measured for each concentration at 600 nm using a UV-visible spectrophotometer.

3 Results and Discussion

3.1 Morphological and biochemical analysis of LC degrading strains

Environmental factors were always considered crucial factors when isolating potential biodegrading strains (Zhai et al., 2012). As a commonly used pyrethroid, Lambda-cyhalothrin was extensively used in agriculture to increase crop yield and to control pests (Bhatt et al., 2021a). In the current study, two distinct colonies were selected among the 10 isolated strains because of their highest growth rates. These capably growing bacterial strains were designated as S-4 and S-6 and were purified after 7-8 rounds of streaking. Different morphological and biochemical characteristics of these two strains were studied. Both strains were gram-positive and appeared rod-shaped. Morphological analysis of the colonies exhibited that S-4 appeared to be large, circular, undulated, and flat while S-6 was irregular, undulated, large, and convex. The biochemical characteristics of both strains were similar while analyzing for catalase and motility. However, strain S-4 was positive for the citrate and oxidase test while S-6 has shown negative results.

3.2 Growth studies using Lambda-cyhalothrin

The growth of two potential strains S-4 and S-6 with LC (100 mg/L) as the sole source of carbon and energy in the MSM medium was studied. The number of bacterial cells and LC utilization rate exhibited a rapid increase trend in the initial cultivation phase (0-24h). At 48 h, the number of bacterial cells increased to its maximum level, then the number gradually decreased. The growth increased gradually on increasing the incubation time but a decreasing trend on prolonged incubation correlates to depletion of energy sources. The strain S-6 exhibited strong growth with maximum OD >1 as compared to strain S-4. Abdullah and co-workers presented similar findings by supplementing media with the initial concentration of lambda-cyhalothrin as 100 mg/L (Abdullah et al., 2016). Pyrethroids contain ester-bond organic compounds which could be degraded by cleaving, hydrolyzing, or inhibiting the ester bond (Bhatt et al., 2021a). Literature reported that biological methods are proven to be more efficient in the hydrolysis of ester bonds and could effectively bioremediate the pesticide as compared to physical and chemical methods (Bhatt et al., 2019, 2021a; Zhan et al., 2020). In literature the hydrolyzing of 85% of 30 mg/L of LC is reported by the whole cell catalyst (Ding J et al., 2022). Hence the most effective bacterial strains or bacterial consortia for boosting sustainable pesticide bioremediation can be scrutinised using important mechanistic methodologies combined with microbial genetic development (Sarker et al., 2021).

3.3 DNA sequencing and phylogenetic analysis

The sequences obtained from Macrogen were analyzed through the BLAST tool and further processed by MEGA 7 software in order to construct the phylogenetic tree which showed linkages between the isolated strains and those at GENEBANK of NCBI. The Phylogenetic tree is presented in Figure 2. According to various studies on lambda-cyhalothrin, bacteria from the genera *Bacillus* and *Pseudomonas* are recognized as highly proficient microorganisms, playing a vital role in the degradation of a variety of pesticides (Chen et al., 2015; Abdullah et al., 2016). Through molecular characterization, it was found that isolates S-4 and S-6 were *Bacillus aryabhattai* and *Bacillus circulans*, respectively. Earlier biodegradation of pyrethroid was reported through *B. thuringiensis*
ZS-19 (Chen et al., 2015), *S. trueperi* CW3 (Bhatt et al., 2020a), *P. putida* (Gong et al., 2018), *Sphingobium sp.* JZ-2 (Guo et al., 2009), and *O. anthropi* YZ-1 (Zhai et al., 2012), and few studies reported the pyrethroid biodegradation by potential enzymes such as esterases or carboxylesterases (Bhatt et al., 2020b, 2021a).

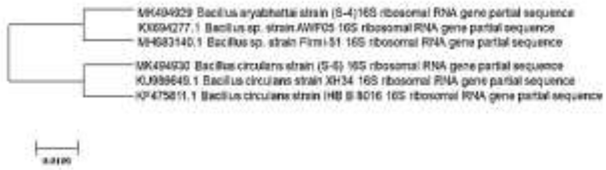

Figure 2: Phylogenetic tree demonstrating relatedness and linkage of S-4 and S-6 to different bacterial strains

3.4 Biodegradation studies of LC

Degradation was observed by analyzing samples at regular intervals (0-72 hrs) through Chemical Oxygen Demand (COD). *Bacillus aryabhattai* (S-4) has shown significant removal in COD when inoculated in MSM containing LC, as illustrated in Figure 3. The strain was capable to metabolize lambda-cyhalothrin with maximum COD removal of 55% at the concentration of 100 mg/L of pesticide in MSM, with maximum growth possible. *Bacillus aryabhattai* exhibited to be a slow degrader when the pesticide concentration increases. Zheng et al.,2012 have also reported that bacterial degradation rate faces declines with increasing substrate concentration. This is probably due to the reason that higher concentrations of Lambda-cyhalothrin exhibited an inhibitory effect on the growth of isolate.

Figure 4 is showing COD removal rates of LC at different concentrations ranging from 100 to 500 mg/L when *Bacillus circulans* were inoculated in MSM. The degradation of LC was recorded to be 55, 64, 83, 79 and 74% at 100, 200, 300, 400, and 500 mg/L concentrations, respectively. *Bacillus circulans* can tolerate higher concentrations of LC by showing maximum percentage removal at a concentration of 300 mg/L concentration after 72 hrs. These findings are correlated to those reported by Zhang et al. (2016), who concluded that higher degradation of LC was observed at higher concentrations in the culture within two days. Results exhibited that these isolated microbial strains have the pyrethroid-degrading ability through hydrolases therefore these strains deserved to be further studied.

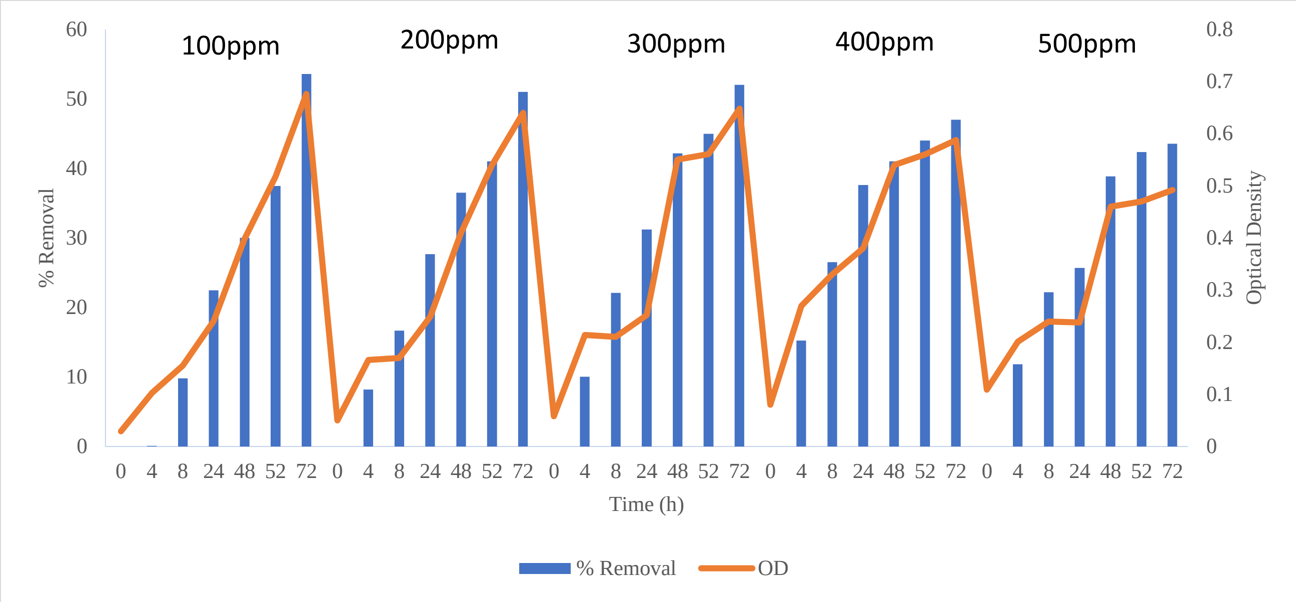

Figure 3: Removal efficiency of *Bacillus aryabhattai* (S-4)

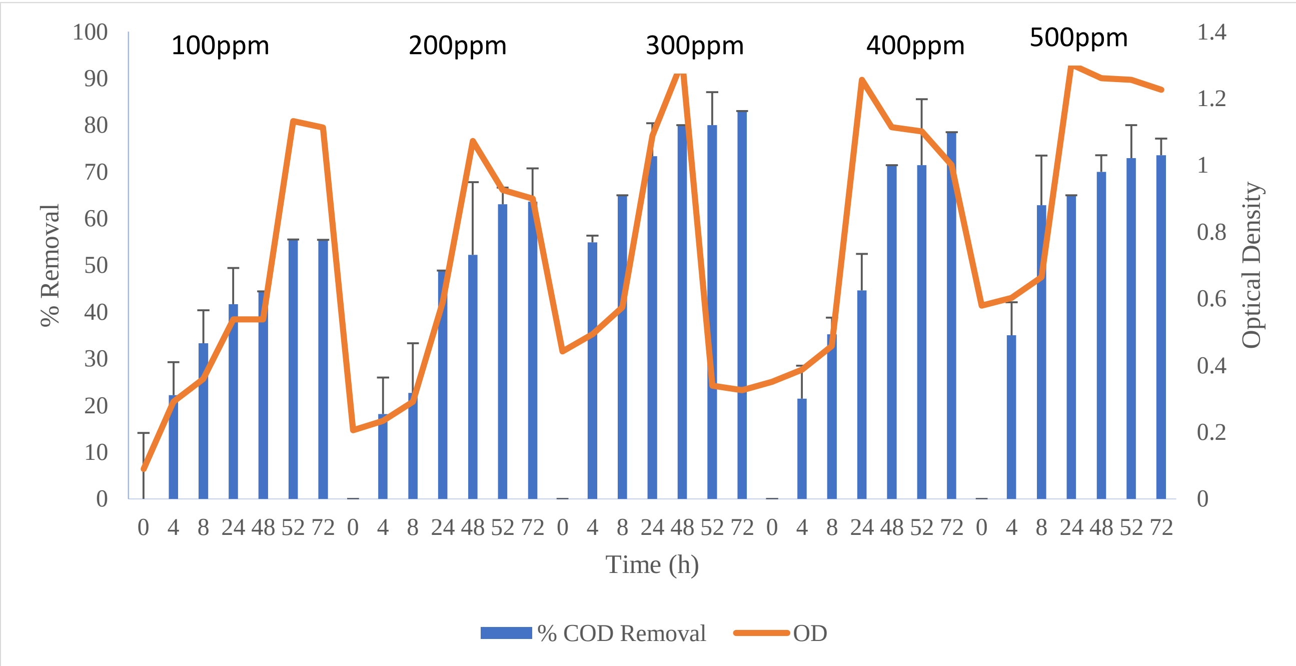

Figure 4: Removal efficiency of *Bacillus circulans* (S-6)

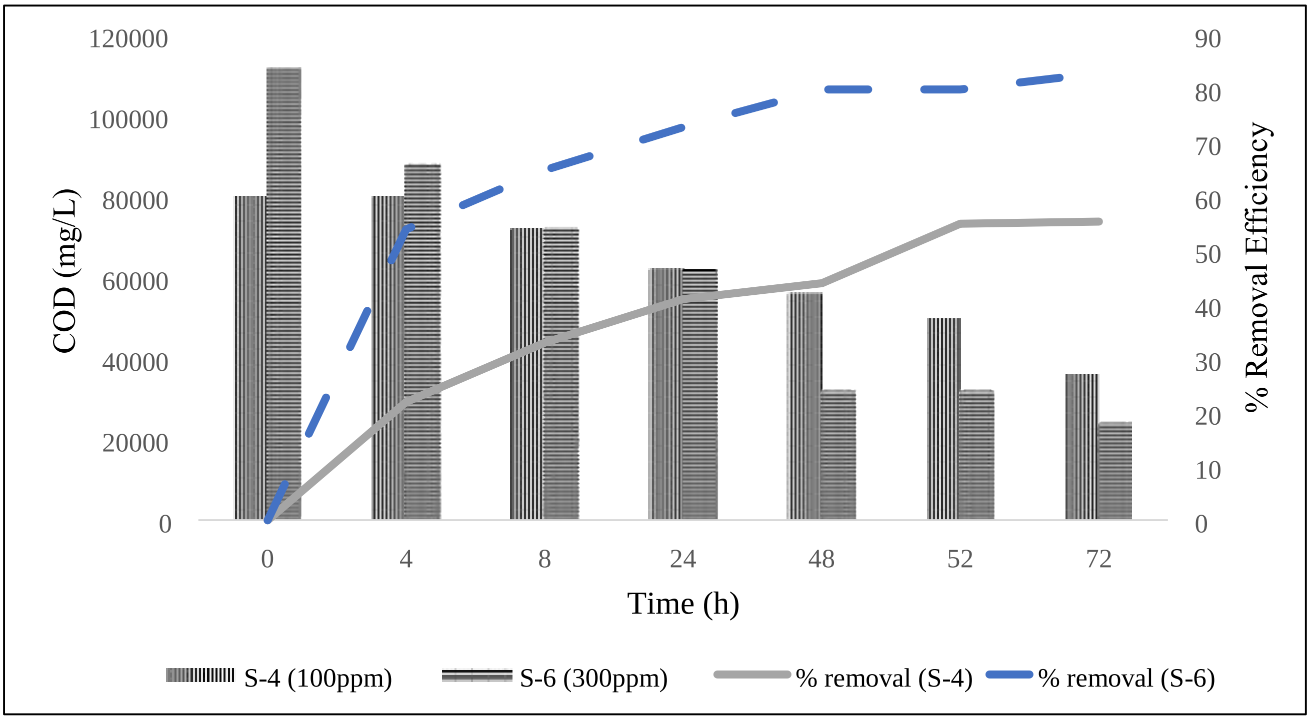

Figure 5: Comparison of COD reduction in MSM media with LC by S-4 and S-6

3.5 Comparison of removal efficiencies by S-4 and S-6

It was observed in the current study that two different species *Bacillus aryabhattai* and *Bacillus circulans* revealed different maximum removal efficiencies at different concentrations of LC. To test the biodegradability of LC COD test was conducted. Results showed that *Bacillus aryabhattai* exhibited maximum percentage removal at a concentration of 400mg/L of LC while *Bacillus circulans* showed maximum removal at a concentration of 300 mg/L of LC. (Figure-5) Moreover, the percentage removal efficiency was observed to be higher in the case of *Bacillus circulans* i.e. 83%, as compared to *Bacillus aryabhattai* i.e. 55%. Thus, the strain *Bacillus circulans* may tolerate lambda-cyhalothrin more efficiently at higher concentrations as compared to other strains *Bacillus aryabhattai* and may play a vital role in the bioremediation of environmental contamination of pyrethroids. Bacillus sp. are involved in the degradation of several organic compounds including pesticides, herbicides, and dyes and proved to be proficient strains in the elimination of these compounds from the environment. Two different species *Bacillus simplex* and *Bacillus muralis*, utilized in a study conducted in 2016, exhibited different COD reduction efficiencies of 94 and 78% in the media containing chlorsulfuron (Erguven and Yildirim, 2016).

4 Conclusions

The ultimate goal of the bioremediation process is to convert the toxic compounds into less toxic and more environmentally friendly substances. The process ensures a healthy environment and healthy products for human consumption. In this study, it was observed that the bacteria *Bacillus aryabhattai* and *Bacillus circulans* have proved to be efficient strains in LC degradation at varying concentrations (100-500 mg/L) and incubation temperature of 37 °C. The pesticide, Lambda-cyhalothrin was degraded in liquid culture of *Bacillus aryabhattai* and *Bacillus circulans*, where the degradation percentage reached 55 and 83% after 72 hours, respectively for the first time. This indicates that these bacterial strains may be utilized as the source of potential hydrolyzing enzymes and are responsible for the bioremediation of Lambda-cyhalothrin. With reported high degradation efficiency, this strain may provide a novel strategy to degrade lambda-cyhalothrin. Nevertheless, more research should emphasize the metabolic pathways. This approach may consider promising for the biodegradation of LC and many other pesticides from the environment.

Acknowledgments

The authors are highly grateful to the Institute of Environmental Sciences and Engineering of National, the School of Civil and Environmental Engineering, University of Sciences and Technology (NUST) for providing financial assistance. The authors acknowledge the research fund provided by Higher Education Commission (HEC) Pakistan, National Research Program for Universities, Research and Development (5995/Federal/NRPU/R&D/HEC/2016), and NUST postgraduate research program.

Ethical issue

Authors are aware of and comply with, best practices in publication ethics specifically about authorship (avoidance of guest authorship), dual submission, manipulation of figures, competing interests, and compliance with policies on research ethics. Authors adhere to publication requirements that the submitted work is original and has not been published elsewhere in any language. Also, all procedures performed in studies involving human participants were following the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All procedures performed in this study involving animals were following the ethical standards of the institution or practice at which the studies were conducted.

Competing interests

The authors declare that no conflict of interest would prejudice the impartiality of this scientific work.

Authors' contribution

All authors of this study have a complete contribution to data collection, data analyses, and manuscript writing.

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