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Bio-degradation of acetamiprid from wetland wastewater using indigenous *Micrococcus luteus* strain SC 1204: Optimization, evaluation of kinetic parameter and toxicity

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ABSTRACT

The fundamental objective of this study is to investigate the feasibility of microbes in removing toxic substances from environment and hopeful consideration that microbial bioremediation can be a cost effective tool for the detoxification of insecticides. Therefore, efforts were made to isolate and characterize indigenous bacteria, capable of degrading the neonicotinoid insecticide acetamiprid (ACE) and to optimize different process parameters in order to effectively treat the Wetland waste water. The enrichment and isolation was done in minimal salts media enhanced with 50 mg L^{-1} of ACE as sole carbon, nitrogen and energy source, incubated at 40 °C on a rotary shaker at 100 rpm for about 7 days. The Face centered central composite design (FCCD) was performed to check the significance of the degradation process in a quadratic model. The mathematical model fitting of growth curve of the isolated bacteria was also studied. The indigenous bacterium capable of degrading ACE was observed to be *Micrococcus luteus* strain SC 1204, having a maximum consumption of 69.84% of ACE in 24 h, quantified using GC–MS. The rate kinetic analysis was investigated using zero-order and three half- order kinetic models. Among the identified metabolites, benzothiazole, 2-(2-hydroxyethylthio) with a prominent peak at RT 6.99 was found to be the end product of ACE bio-degradation. Further, toxicological analysis on *Pseudomonas aeruginosa* exhibited no inhibition zone, suggesting the eco-friendly nature of the degraded metabolite.

1. Introduction

Environment preservation is one of the primary aims of sustainable development. Environmental pollution is growing more and more due to the indiscriminate and frequent deliberate release of hazardous, detrimental substances. Such increasing chemization in all areas of man's activities has deteriorating impact also, besides its benefits [1]. Research efforts have been devoted to develop new, low-cost, low-technology, eco-friendly treatments capable of reducing and eliminating pollution in the ecosystem [2]. In India, alarming levels of pesticides have been reported in air, water, soil as well as in foods and biological materials. Some of these pesticides have also been reported to be toxic [3], mutagenic, carcinogenic and tumorogenic [4,5]. The consumption of technical grade pesticides in India during 2000–2001 was 45,580 MT. Among the insecticides, carbamates, organochlorines, imidacloprids, organophosphates, tops the list of pesticides in Indian market.

The experimental evaluation of chemical degradation in the environment is highly complicated due to multiple reasons. These include the variation of moisture, temperature, chemical and microbiological composition of soil, ability of a chemical to volatilize and photo-degrade. The assessment test for disposability is expensive and usually time-consuming. In some cases, when a chemical is not mineralized or broken down to nontoxic products, there is a possibility of extensive spoilage creating health hazards to fauna and humans [6].

Pesticide biodegradation is a ubiquitous process. It has been documented in a wide range of habitats, including soils, sediments, surface, ground water and sewage sludge, etc. The ubiquity of pesticide degradation suggests that bioremediation strategies can play an important role in the treatment of pesticide wastes. The microbial degradation of hazardous waste offers a promising strategy by which toxic chemicals may safely be detoxified. For that reason there is a need to isolate, identify and distinguish the microorganisms that subsist interact in contaminated environment [7].

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Neonicotinoid insecticides are one of the most important commercial insecticides used worldwide. They are systemic broad-spectrum insecticides which exhibit a novel mode of action because they are agonists of the nicotinic acetylcholine receptor (nAChR), leading to paralysis and death of pest organisms [8–10]. These compounds are active against numerous sucking and biting insect pests, including aphids, whiteflies, thrips, leaf miners, beetles, and a number of coleopteran pests [11,12]. In this chemical class, acetamiprid, a chloropyridinyl neonicotinoid, was considered to be a favorable choice for controlling those pests that are severely resistant to organophosphorus, urethane and synthetic pyrethroid pesticides, so it is regarded by EPA as an important substitute of organophosphorus pesticides [13]. Owing to its broad insecticidal spectrum and relatively low acute and chronic mammalian toxicity, acetamiprid is used widely in crop protection [14,15]. Because of the widespread use of ACE in many areas and its potential toxicity to humans [16,17], the residues present in the environment have received considerable attention, and methods for the biotransformation of neonicotinoids are being actively researched [13].

Understanding the necessity for development of green technology in order to protect environment, it is felt that commercial application of the insecticide resistant bacterial strain in agriculture will greatly help to remove the toxic effects of frequently used chemical insecticide in the Wetland areas. Likewise, in the present study, efforts were made to isolate potent bacterial strain capable of degrading acetamiprid (ACE). Biodegradation of acetamiprid was confirmed using GC–MS, analysis. Estimation of specific growth rate is highlighted using the experimental data obtained for biomass concentration of the exponential growth phase and mathematical growth model is deduced. Emphasis is given on the effect of operating variables and their interactive effect on the overall biodegradation efficiency and optimization of the process conditions to maximize the overall removal percent of ACE insecticide from aqueous solution using Face centered central composite design (FCCD). This study also involves assessment of the related product toxicity.

2. Materials and methods

2.1. Collection of agro–waste water sample

The waste water sample was collected from Mathpukur, East Kolkata Wetlands, West Bengal, (Ramsar site No. 1208, 22°25′40″ North, 88°22′55″ East) India. It was stored at 0° C; the agricultural area has a history of repeated acetamiprid application. The waste water sample was collected from an excess running water flow channel from the agricultural land.

2.2. Chemicals and media

Analytical grade acetamiprid (99% purity) was purchased from Sigma–Aldrich, analytical-grade dichloromethane and ethyl acetate were purchased from Merck, Mumbai, India. Minimal salt media (MM) which is used as a selective media contained 1.5 g L^{-1} K_2HPO_4, 0.5 g L^{-1} KH_2PO_4, 1.0 g L^{-1} NaCl, 0.2 g L^{-1} $MgSO_4$ 7H2O, pH 7.0, acetamiprid (50–300 mg L^{-1}) was added to MM as required. For MM agar preparation 16 g L^{-1} of Agar for bacteriology was added to the MM broth supplemented with acetamiprid. Luria–Bertani (LB) media which is used as a basal media contained 10.0 g L^{-1} tryptone, 5.0 g L^{-1} yeast extract, and 10.0 g L^{-1} NaCl, pH 7.0, acetamiprid (50 mg L^{-1}) was added as an additional component in the LB media. The strain *Pseudomonas aeruginosa* (NCIM No. 2945) was purchased from National Collection of Industrial Microorganisms (NCIM), Pune, India, to assess the toxicological activity of ACE bio-degraded metabolite. All other chemicals were obtained from Merck and Hi-Media.

2.3. Enrichment and isolation of acetamiprid (ACE) degrading bacteria

ACE has been widely used in the control of numerous biting and sucking insect pests in the agricultural fields of the Wetland area. ACE residues were frequently found in water and soil of that area; therefore, it is apparent that several bacteria have adapted to this ACE- contaminated environment. Likewise, for the in vitro isolation of the potential bacterial strains, the agro effluent was serially diluted from 10 $^{-1}$ to 10^{-5}, and aliquots each of 1 mL dilution were inoculated in sterile plates containing of Luria Bertani Agar (LBA) media (composition: 10.0 g L^{-1} tryptone, 5.0 g L^{-1} yeast extract, and 10.0 g L^{-1} NaCl, pH 7.0) supplemented with 10 mg L^{-1} of acetamiprid (ACE) and Minimal salt media (composition: 1.5 g L^{-1} K_2HPO_4, 0.5 g L^{-1} KH_2PO_4, 1.0 g L^{-1} NaCl, 0.2 g L^{-1} $MgSO_4$ 7H2O, pH 7.0,) supplemented with 10 mg L^{-1} of acetamiprid (ACE), using spread plate technique. All plates were incubated at 40 °C in the incubator for 24 h at 100 rpm. Here, acetamiprid (ACE) was used as the sole carbon, nitrogen and energy source for the growth of indigenous ACE utilizing bacteria only [15].

2.4. Screening of the potential strain

Upon noticing very high bacterial counts for the first time on LB plates in 24 h with 10 mg L^{-1} of acetamiprid (ACE); concentration of ACE was increased to 20 mg L^{-1}. Furthermore, with the gradual increase in the concentration of ACE from 20 mg L^{-1} to 40 mg L^{-1}, a considerable reduction in bacterial counts was observed. At concentration of 50 mg L^{-1}, very few distinct colonies formed were picked and purified using the streak plating method. Moreover, the isolated colonies were enriched and stored in LB and MSM slants supplemented with 50 mg L^{-1} ACE using sub culturing technique. After several rounds of sub culturing and purity checking chiefly by simple staining and Gram staining, pure culture of indigenous bacteria capable of degrading ACE were screened for the present research. One strain was investigated to possess the highest ACE-degradation efficiency, was selected for further investigation.

2.5. Identification and characterization of isolated bacterial strain

The isolated bacterial strain was identified on the basis of Gram character, morphological, 16S rRNA sequence & biochemical properties [18] by referring the *Bergey's Manual of Determinative Bacteriology* for the tests. The Gram staining character of the cell and morphological structure was examined by Inverted microscope (Optika XDS-2) and Field Emission Scanning Electron Microscope (FESEM, JOEL JSM 6700 F). The 16S rRNA gene sequencing was performed by BioAxis DNA Research Centre (Pvt.) Ltd., Hyderabad, India.

Furthermore, pair of universal primers was made to amplify the 16S rRNA gene [13] and the sequencing was performed by ABI 3730xl Genetic Analyzer. The 16S rRNA sequence analysis was done using BLAST and the phylogenetic tree was made using Clustal W2- Phylogeny. The sequences were later submitted to GenBank using the online submission tool BankIt. The nucleotide sequence of the isolated strain deposited in the GenBank database was under accession number KJ782459.

2.6. ACE bio-degradation study in batch culture

Initially, 10 mL stock of different dilutions was prepared and mixed with equal volume of Britton Robinson Buffer [mixture of 0.04 M Boric acid (H_3BO_3), 0.04 M Phosphoric acid (H_3PO_4),