Journal of Hazardous Materials 344 (2018) 883–892

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Efficiency of the formulated plant-growth promoting *Pseudomonas fluorescens* MC46 inoculant on triclocarban treatment in soil and its effect on *Vigna radiata* growth and soil enzyme activities

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HIGHLIGHTS

- A plant-growth promoting, triclocarban-degrading inoculant for soil treatment.
- A 20-week stable sawdust-based bacterial inoculum of *Pseudomonas fluorescens* MC46.
- Biodegradation and detoxification of triclocarban in soil at up to 31.6 mg kg^{-1}.
- Role of the formulated inoculant MC46 as biofertilizer and bioaugmented agent.
- Successful demonstration of Triclocarban removal and soil fertility improvement.

ARTICLE INFO

Article history:
Received 27 July 2017
Received in revised form
24 November 2017
Accepted 25 November 2017

Keywords:
Triclocarban
Biodegradation
Pseudomonas fluorescens
Bacterial inoculant
Plant-growth promotion

ABSTRACT

For bioaugmentation-based treatment of triclocarban (TCC), an emerging soil pollutant that is recalcitrant to biodegradation and phytotransformation, efficient TCC-degrading bacteria with an effective soil-delivering means are required. This work developed the formulated bacterial inoculant, and successfully demonstrated its TCC removal and detoxification performance in pot soil experiment with *Vigna radiata* plants. The soil bacterium *Pseudomonas fluorescens* MC46 was isolated as TCC-degrading, plant-growth promoting bacterium. The characterizations were conducted in vitro revealing that it could utilize TCC as a sole carbon source, and at a wide and higher concentration range from 1.6–31.6 mg kg^{-1} than those previously reported, while the detoxification was assessed by cytogenotoxicity and phytotoxicity tests. The developed sawdust-based inoculant formula combined with molasses (5% w/w), and either PEG or CMC-starch blend (1% w/w) could maintain a 20-week shelf-life inoculant stability in terms of cell viability, and TCC-degrading activity. Bioaugmentation of the formulated inoculants into TCC-contaminated soil efficiently removed TCC up to 74–76% of the initial concentration, mitigated toxicity, restored plant growth and health, and enhanced soil enzyme activities. This work is the first to demonstrate potential application of the formulated plant-growth promoting bacterial inoculant for the treatment and detoxification of a persistent TCC contaminated in soil.

1. Introduction

Triclocarban (TCC) is a high production volume chemical widely used as an antimicrobial chemical in a variety of personal care and consumer products [1]. Due to its persistence during wastewater and sewage sludge treatment, the levels of TCC at concentration range of μg L^{-1} and mg kg^{-1} levels have been detected in wastewater and treated sewage sludge commonly known as biosolids, respectively [2,3]. The ubiquity of TCC in ecosystem has raised health hazard concerns due to its toxicity to mammals such as human [4,5], aqua-organisms such as freshwater green alga *Pseudokirchneriella subcapitata* [6], and terrestrial organisms such as carrot, tomato, and other plants [7]. The removal of TCC in aque-

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https://doi.org/10.1016/j.jhazmat.2017.11.046
0304-3894/

ous phase was reported by using ozone [1], and chemical catalytic process [8]. On the other hand, TCC removal from soil suffers from adsorption to soil because of its hydrophobicity [9,10], resistance to degradation [11], and potential to be taken up by plants [11–13]. Although some pollutants can be *in vivo* transformed or broken down by plants [14], so far several studies have shown that TCC is not transformed in plants, but rather bioaccumulated especially in plant roots, while translocation from root to shoot or leaf is limited [11–13,15].

With increasing level of TCC in soil from increasing application of biosolids on to land and agricultural fields [16,17], mitigation of TCC in soil is important, and can be enhanced by bioaugmentation of TCC-degrading bacteria to reduce its existence in soil, and to prevent transportation and bioaccumulation of TCC in edible portion of plants. To date, it has been shown that complex microbial community from wastewater [18] or soil [19] is able to degrade TCC. In addition, several TCC-degrading bacteria such as *Sphingomonas* sp. YL-JM2C [20], *Ochrobactrum* sp. TCC-1, TCC-2 [21,22], and *Ochrobactrum* sp. MC22 [23], were isolated from wastewater or soil. While most studies that focused on TCC biodegradation by single bacterial strains were successfully carried out in defined medium, the application of these bacteria as a bioaugmented culture for soil treatment requires further studies. For a successful soil bioaugmentation approach, the most important requirements are that the bioaugmented bacterial cells should survive and maintain degradative activity after soil inoculation [24]. This can be facilitated by developing a bacterial formula that is a bacterial inoculant in a suitable carrier materials to protect cells against harsh chemical and environmental conditions when applied into contaminated soil [25].

Accordingly, to facilitate TCC removal in agricultural soil, this work began with the selection and *in vitro* characterization of TCC-degrading bacterium with plant-growth promoting (PGP) activities, which are defined as bacterial activities to enhance plant growth and protect plant from abiotic stresses including toxic chemical stress in surrounding environment [26].

Then, the formulation of bacterial inoculant was developed and its efficacy on TCC removal in TCC-contaminated soil was investigated using pot soil experiments. In addition, the efficiency of the formulated bioaugmented plant-growth promoting bacteria (PGPB) was further assessed with growth of mung bean (*Vigna radiata*) plants in the presence and absence of TCC, while soil enzyme activities before and after the treatments were also evaluated.

2. Materials and methods

2.1. Chemicals, culture medium, bacterial isolation, and plant growth promoting activity test

TCC (99% purity; Sigma Aldrich, USA) was dissolved in acetone and filtered sterile to prepare a stock solution. Other chemicals were of analytical grade.

Mineral salt medium (MSM) comprised of (g L^{-1}) 5.8 Na_2HPO_4, 3.0 KH_2PO_4, 0.5 NaCl, 0.25 $MgSO_4$, 1.0 NH_4Cl, dissolved in deionized water, and adjusted pH at 6.8–7. When yeast extract was included at 5 g L^{-1}, the medium was referred to as MSMY. Luria-Bertani (LB) medium was used as indicated. The solid media were prepared by adding 20 g L^{-1} agar into the liquid media. When indicated, TCC was included at 30 μM or 50 μM (equivalent to 9.5 or 15.8 mg L^{-1}, respectively).

Bacterial enrichment was carried out using rhizosphere soil samples collected from *Vigna unguiculata* subsp. *sesquipedalis* soil field in the central part of Thailand in 100 mL of MSM medium supplemented with 30 μM TCC under the conditions described in Supplementary materials. The fast growing isolates were tested for PGP activities with and without TCC including phosphate solubilization, and the production of indole acetic acid (IAA), *exo*-polysaccharide (EPS) [27], siderophore [28,29], and ammonia [30] (details in Supplementary materials). The selected isolate was further identified using the partial 16S rRNA sequence with phylogenetic analysis.

2.2. Biodegradation of TCC in liquid medium, kinetic analysis, and toxicity assessment

Biodegradation of TCC was conducted under growth-dependent condition. Initially, TCC stock solution was added to a 250-mL Erlenmeyer flask and air-dried for 15 min allowing acetone to evaporate. Then, 100 mL of MSM and LB-grown cell inoculum (5%, v/v) were added to the flask. The cell cultivation was incubated at 28 ± 2 °C and at 120 rpm in a rotary shaker. At the indicated time interval, the sample was taken to determine cell growth using a spectrophotometer at 600 nm, then centrifuged to remove cells, and the supernatant was analyzed for TCC residuals using high performance liquid chromatography (HPLC) as previously described [23] (details in Supplementary materials). Abiotic control was conducted without cells. Heat-killed cells were also used to represent TCC sorption on to cells.

TCC biodegradation and microbial growth kinetics were studied. The batch experiment was set up in MSM with several of TCC concentrations from 5–100 μM. The growth and TCC biodegradation were monitored. The data were then fitted with appropriate kinetics models [31]. Regression analysis was achieved with the data analysis tool pack of Microsoft Excel®, and the model equations were analyzed using GraphPad Prism 6.07 (CA, USA).

The degradative intermediates were analyzed using Liquid chromatography-Mass spectrometry (LC–MS) using Waters Alliance e2695 coupled to Bruker MicrOTOF Q-II instruments (details in Supplementary materials). Effect of co-substrate on TCC degradation was investigated with various types of carbon source (glucose, succinic acid, and sodium acetate) or nitrogen source (ammonium sulfate, ammonium nitrate, sodium nitrate, and urea), each of which was included into MSM at 1 g L^{-1}. Cell growth determined by the optical density at 600 nm, and TCC degradation were monitored in comparison to that without the addition of co-substrate.

Toxicity of TCC and the degradation metabolites was determined using cytogenotoxicity assessments [32] and phytotoxicity [33] as described in Supplementary materials.

2.3. Growth chamber experiments: effect of TCC on plant growth and root anatomy, and role of TCC-degrading bacterium bioaugmentation

Seeds of *V. radiata* were surface-sterilized [34] and were pregerminated for 3 days. Then, five seeds were aseptically transferred into a 500-mL glass jar containing 300 mL of half-strength semisolid Hoagland's medium containing 30 μM TCC. Then, cell suspension (1 mL of 1.2×10^8 CFU mL^{-1}) was added. The jars were placed in a growth chamber at a constant temperature of 25° C, 80% humidity, and a cycle of 14 h of light and 10 h of darkness. The biomass, height, and root length of plants grown in the presence of TCC and the bacterial cells were determined after 7 days of incubation in comparison to 1) those grown in a normal condition (without TCC and without the bacterial isolate strain MC46), and 2) those grown in the presence of TCC without cells. All experiments were conducted in triplicates.

After the treatments, root tissues of *V. radiata* were prepared by cross sectioning at a distance of 1.2–1.5 cm from the apex to get 2-mm-thick semi-thin sections, stained with Safranin O [35], and observed under a light microscope (Olympus CX31) equipped with