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ORIGINAL ARTICLE

Prospecting *Ammoniphilus* sp. JF isolated from agricultural fields for butachlor degradation

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Abstract
Butachlor is a chloroacetamide herbicide used worldwide for controlling weeds in plants of rice, corn, soybean and other crops. In this study, indigenous bacterial species *Ammoniphilus* sp. JF was isolated from the agricultural fields of Punjab and identified using 16S ribosomal RNA analysis. The bacteria utilized butachlor as the sole carbon source and showed complete degradation (100 mg/L) within 24 h of incubation. Two intermediate products, namely 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester and 2,4-bis(1,1-dimethylethyl)-phenol were observed at the end of butachlor degradation. To the best of author's knowledge, biodegradation of butachlor by indigenous *Ammoniphilus* sp. JF from the agricultural fields of Punjab has not been reported so far.

Keywords Butachlor · Herbicide · *Ammoniphilus* sp. JF · Indigenous · 16S rRNA

Introduction

Butachlor (*N*-(butoxymethyl)-2-chloro-*N*-(2,6-diethylphenyl) acetamide), is a herbicide widely used as a pre-emergence or early post-emergence herbicide to control weeds in various crop fields. Annually, around 4.5×10^7 kg of butachlor is utilized across Asia (Yu et al. 2003; Tilak et al. 2007; Zheng et al. 2012; Gao et al. 2015). Extensive application and persistence of butachlor in soil and water has ended up in multiple health and environmental complications (Widmer and Spalding 1995; Fang et al. 2009; Yang et al. 2011). Butachlor is a suspected carcinogen and reported to cause stomach tumours, mitochondrial dysfunction, oxidative DNA damage, chromosomal breakage and disruption of endocrine system (Hsu et al. 2005; Chang et al. 2011; Dwivedi et al. 2012). These health implications necessitate its removal from the environment. Although physical and chemical processes are popular for treating pesticide residues, the production of certain toxic reactants inhibited its widespread application (Burrows et al. 2002). Therefore, microbial degradation was considered an effective alternative as it circumvents high cost and secondary pollution (Beulke et al. 2005). Efficiency, versatility and environment friendliness makes microbial degradation a promising option for the treatment of complex pollutants. Beestman and Deming (1974) in their study reported the difference in half-life of butachlor in sterile (640 ± 8 days) and non-sterile soils (11.4 ± 3 days), indicated the degradative dissipation of butachlor by soil microbes.

Butachlor degradation has been studied with different bacteria isolated from varied sources such as rhizosphere soil (Yu et al. 2003; Dwivedi et al. 2010), agricultural fields and sludge (Liu et al. 2012). Liu et al. (2012) isolated *Rhodococcus* sp. from rice fields of China and reported the efficiency of the strain to degrade 100 mg/L of butachlor within 5 days of incubation. Dwivedi et al. (2010) reported that the butachlor-degrading bacterial strain *Stenotrophomonas acidaminiphila* possessed the potential of degrading butachlor (3.2 mmol/L) completely within 20 days of inoculation in soil. In another study, Zheng et al. (2012) observed 81.2% degradation by *Catellibacterium caeni* sp. nov DCA-1^T after 84 h of incubation in 50 mg/L butachlor amended medium. Another bacterial strain *Bacillus* sp. strain hys-1, which could degrade butachlor more than 90% (100 mg/L) within 7 days, was isolated from active sludge in China (Gao et al. 2015). Though butachlor is widely used in India, reports on its degradation studies are very limited. Keeping in view, the

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present study aims to isolate an indigenous strain from the agricultural fields of Punjab, investigate its biodegradation potential and identify the isolated efficient strain by 16S ribosomal RNA (rRNA) analysis.

Materials and methods

Chemicals and medium

Butachlor herbicide of 97.7% purity was purchased from Sigma-Aldrich. Stock solutions of butachlor were prepared with methanol (chromatographic grade) and filtered with membrane filter (pore size − 0.45 µm) to remove the fine impurities. All solvents and media were purchased from Hi-Media and SRL Pvt. Ltd. The reagents used were of the highest analytical grade.

Collection of soil sample

The soil samples were collected from the agricultural fields located in Gurdaspur district of Punjab, India (N 31°32′, E 75°14′) having prior history of extensive butachlor application for around 20–22 years. The purpose was to obtain a native-resistant bacterial strain capable of showing better degradation. Three fields were selected and divided into grids of size 11.61 × 11.61 m^2. From each grid, 200 g of soil was collected at a depth of 15 cm using a hand hoe. Composite sample was made by mixing and homogenising the sample from each grid (Pal et al. 2006).

Screening of soil for butachlor

Butachlor and its residue in collected soil sample was analysed with gas chromatography–mass spectrometry (GC–MS) by following the method given by Yu et al. (2003). Soil sample was mixed with 30 mL of deionized water and 50 mL of acetone and kept in orbital rotatory shaker at 150 rpm for 1 h. The samples were then filtered using Whatman filter paper no. 42. The soil particles retained on the filter paper was washed twice with a mixture of acetone–water (2:1). The filtrate was evaporated on a rotary evaporator to remove the acetone. The impurities were precipitated by adding 1 g of celite and 5 mL of NH_4Cl–H_3PO_4 to the condensed solution and once again filtered. Further, the filtrate was extracted three times with petroleum ether (30–60 °C), 50 mL for the first and 30 mL for the other two extractions, respectively. After dehydration with anhydrous Na_2SO_4, the extracts were collected in a round-bottom flask, concentrated on a rotary evaporator and analysed in GC–MS.

Enrichment of butachlor-degrading bacterial isolate

A conventional enrichment culture technique was carried out for the isolation of butachlor-degrading bacteria. About 1.0 g of the soil sample was added to 100 mL of conical flasks containing 50 mL of minimal salt medium (M9). The media was supplemented with 100 mg/L of standard butachlor, as a carbon source. The flasks were incubated at 30 °C on a rotary shaker at 130 rpm for 5 days. After incubation of 5 days, 3 mL of the culture was transferred into 50 mL of fresh enrichment medium and incubated for next 5 days. After six transfers, the enriched culture was spread on M9 agar plates containing 100 mg/L of butachlor and incubated at 30 °C for 5 days (Zhang et al. 2011). The colonies grown on the plates were isolated, purified by repeated streaking on Luria–Bertani (LB) and designated as strain JF.

Biodegradation of butachlor

To determine the butachlor degradation efficiency of the isolated JF strain, the cells of JF strain in later exponential phase of growth were harvested by centrifugation at 6000 rpm and 4 °C. Cell pellets were washed twice with M9 media and optical density (OD) was adjusted to 0.02 with M9 medium supplemented with 100 mg/L of butachlor. The cultures were incubated at 30 °C, 130 rpm on a rotary shaker for a period of 120 h. After every 6 h, the sample was collected and the bacterial growth was monitored by measuring the OD. The butachlor in the sample was determined after every 24 h, where 2 mL of culture was centrifuged at 12,000 rpm for 5 min and the supernatant was transferred into test tube. The supernatant was extracted with double the volume of dichloromethane, dried over anhydrous sodium sulphate and evaporated in the water bath. The resultant residue was re-dissolved in methanol, filtered and stored for GC–MS analysis (Liu et al. 2012). A control without the bacterial cells was run under the same conditions.

Identification of isolated strain using 16S rRNA gene-sequence analysis

Isolated pure JF bacterial strain was sequenced at Amnion Biosciences for 16S rRNA gene sequencing. Briefly, gene was amplified using (Taq DNA Polymerase) polymerase chain reaction (PCR) using primers: 27F (5′-AGAGTTTGA TCCTG GCTCAG-3′) and 1492R (5′-TACGGYTACCTT GT TACGACTT-3′). Polymerase chain reaction was carried out in 50 µL of the reaction mixture. Thermal cycler was operated for 35 cycles (95 °C = 5 min, 53 °C = 30 s, and 72 °C = 1.3 min). And lastly, the PCR products were sequenced partially with primers: 518 F (5′-CCAGCAGCC

GCGGTAATACG-3′) and 800R (5′-TACCAGGGT ATC TAATCC-3′). The sequence was aligned with the known sequences in the GenBank database by Basic Local Alignment Search Tool (BLAST) for finding the closest homologous microbes. Phylogenetics was analysed by MEGA version 6 software and distances were calculated using the Kimura two-parameter distance model. An unrooted tree was built by the neighbor-joining method.

Analytical methods

The extracted samples were subjected to GC–MS (Shimadzu QP 2010 Ultra) with RTxi-1 ms capillary column (30 m × 1 mm × 0.1 µm) and splitless injection system. Helium gas at flow rate of 1 mL/min was used as a carrier gas for the analysis. The temperature of oven, injection port, column and detector was maintained at 240, 230, 200 and 270 °C, respectively. Around 2 µL of sample was injected for analysis (Yu et al. 2003).

Results and discussion

Identification of butachlor residues in soil

The presence of butachlor and its residues in the collected soil sample was analysed using GC–MS. Figure 1 shows the GC–MS profile of butachlor residues in soil. The detected compounds were identified by matching the mass of the compounds in the information system of the National Institute of Standards and Technology (NIST 2013) library. Some compounds were identified and compared with literatures. Three residues of butachlor, namely acetamide, 2-chloro-*N*,*N*-diethyl, *N*-hydroxymethyl-2-chloro-*N*-(2, 6-diethyl-phenyl)-acetamide and *N*-(2,6-diethyl-phenyl)-*N*-hydroxymethyl-acetamide were detected in the soil samples. Similar compounds were reported by Zheng et al. (2012) while degrading butachlor (50 mg/L) using *Catellibacterium caeni* sp. nov DCA-1^T. The absence of butachlor in soil indicated its degradation. The pathway of degradation can be abiotic, biotic or combinational depending on the physical and

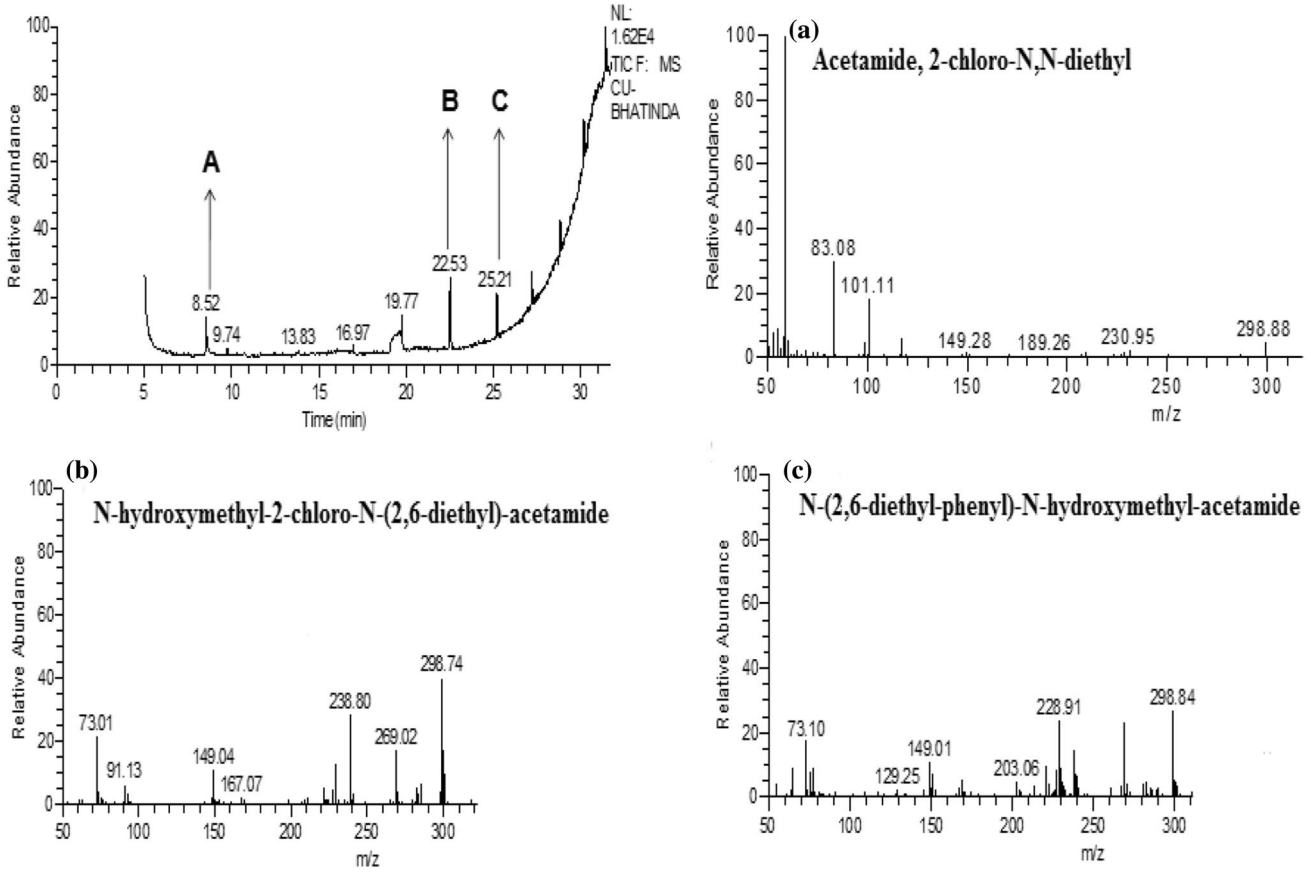

Fig. 1 GC–MS profile of butachlor residues present in soil (**a**, **b** and **c**). The mass spectra of compound A (**a**) and B (**b**) and C (**c**) in GC–MS. Compounds A, B and C were identified as Acetamide, 2-chloro-*N*,*N*-diethyl, *N*-hydroxymethyl-2-chloro-*N*-(2,6-diethyl)-acetamide and *N*-(2,6-diethyl-phenyl)-*N*-hydroxymethyl-acetamide, respectively

chemical properties of butachlor and their interaction with the biotic and abiotic components of soil (Beigel et al. 1999; Sannino et al. 1999; Hafez and Thiemann 2003). However, microbes could have been the major contributing factor for the degradation of butachlor (Pal et al. 2006).

Identification and phylogenetic analysis of isolated strain

Butachlor degrading bacterial strain JF was isolated by enrichment culture technique. The isolated strain was characterized by 16S rRNA-sequencing technique. The 16S rRNA gene sequence was compared with available sequences in GenBank, which showed 100% similarity with the *Ammoniphilus* sp. The isolated strain was designated as *Ammoniphilus* sp. JF and the sequence was submitted to the Genbank database under accession number KP977572. Figure 2 depicts the phylogenetic tree of the isolated *Ammoniphilus* sp. JF strain. Phylogenetic analysis confirmed that isolated strain JF appeared indistinguishable and clustered with members of genus *Ammoniphilus*. Previously, the bacteria has been reported for poly(butylene succinate) (PBS) degradation by Phua et al. (2012). Otto et al. (2013) in his study reported organophorous (OP) hydrolase activity in *Ammoniphilus* sp.

Butachlor degradation studies

The growth curve of *Ammoniphilus* sp. JF grown in 100 mg/L of butachlor as a sole carbon source is depicted in Fig. 3. The maximum growth was observed at 108 h of incubation (OD 0.92). After 108 h, cell growth began to decrease showing an OD of 0.77 at 120th hour of incubation. Efficient growth of bacteria JF in mineral salt medium supplemented with 100 mg/L of butachlor clearly showed its capability of utilizing butachlor as a carbon source and energy. A prolonged lag phase was observed up to 12 h which might be due to the adaptive behaviour of bacterial cells or also, it can be attributed to the cellular processing for signal transduction and consequent induction of metabolic pathway for butachlor degradation. The extended lag phase under such conditions has been reported earlier and supports our results (Chen and Alexander 1989; Jilani and Khan 2006).

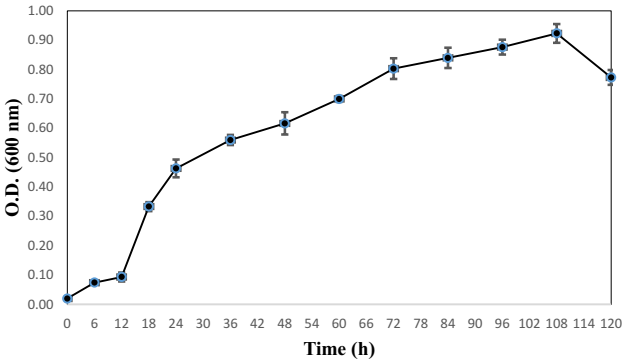

Fig. 3 Growth rate of bacterial strain JF at 100 mg/L of butachlor

Butachlor degradation with *Ammoniphilus* sp. JF strain was investigated at 100 mg/L of butachlor. *Ammoniphilus* sp. JF showed 100% degradation in 24 h of incubation. No significant change was found in the control. A corresponding increase in cell density was observed with increase in time and decreasing butachlor indicating the capability of the bacteria to use butachlor and its metabolites as sole carbon and energy source. The butachlor-degrading bacteria reported in earlier studies showed only 81.2% when incubated in 50 mg/L butachlor for a period of 4 days (Zheng et al. 2012). In another study, Zhang et al. (2011) reported 65.2% degradation when the isolate *Paracoccus* sp. was subjected to 100 mg/L butachlor. The difference in degradation efficiency might be due to the diversity of the butachlor-degrading bacteria and their varied degradation pathways (Zheng et al. 2012). Our findings show that *Ammoniphilus* sp. JF completely metabolized 100 mg/L of butachlor as evidenced from GC–MS analysis. After 24 h of incubation, the presence of 1, 2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester and 2,4-bis(1,1-dimethylethyl) phenol as metabolites was observed which is depicted in Fig. 4. *N*-(Butoxymethyl)-2,6-diethyl-*N*-propylaniline and *N*-(butoxymethyl)-2-ethylaniline are the expected compounds from the degradation of butachlor as depicted in Fig. 5, but GC–MS analysis revealed the presence of 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester and 2,4-bis(1,1-dimethylethyl)-phenol, respectively. These two compounds were obtained due to deacylation at nitrogen group of butachlor and replacement

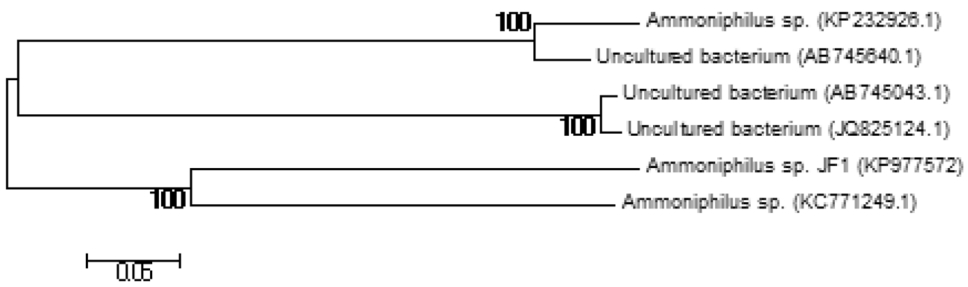

Fig. 2 Neighbour-joining tree showing the phylogenetic relationship between *Ammoniphilus* sp. JF and related species based on 16S rRNA gene sequences. The scale bar represents an evolutionary distance (*k*nuc) of 0.05

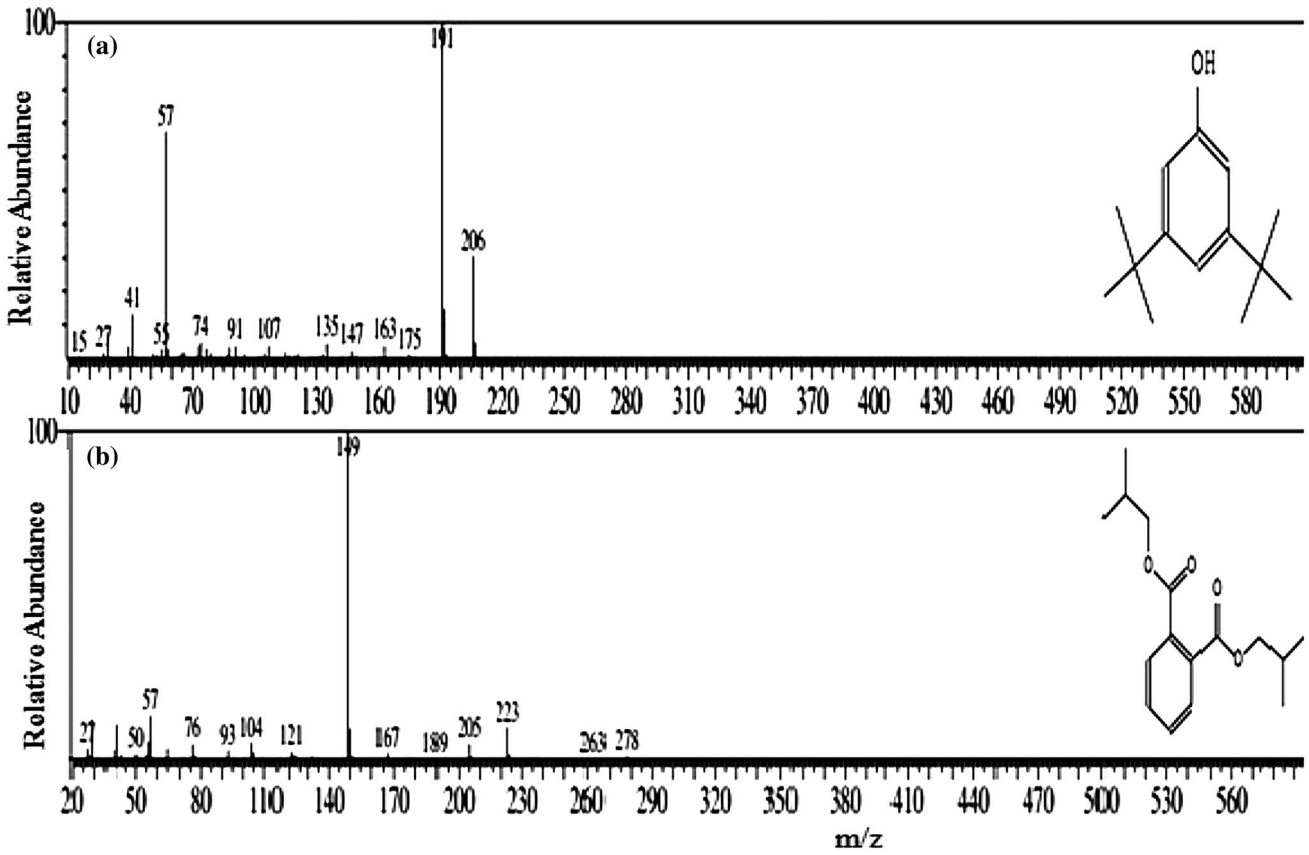

Fig. 4 **a**, **b** Mass spectra for metabolite-2,4-bis(1,1-dimethylethyl)-phenol and 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester, respectively

of acetyl group by alkyl group due to alkylation resulting in the formation of *N*-(butoxymethyl)-2,6-diethyl-*N*-propylaniline. Further, by the action of dealkylation at nitrogen group alkyl chain is removed and *N*-(butoxymethyl)-2-ethylaniline was formed and this metabolite undergoes deamination to release butoxymethylanamine. The butoxymethylanamine is subsequently dealkylated by the action of hydroxylase to phenol. A further oxygenation of phenol by phenol hydroxylase form catechol (Razika et al. 2010) mineralized the compound to carbon dioxide and water (Kim et al. 2013). The mechanism in the proposed pathway is comparable to that of Chakraborty and Bhattacharyya (1991) that reported dechlorination, hydroxylation, dehydrogenation, debutoxymethylation, C-dealkylation, N-dealkylation, O-dealkylation and cyclization as mechanisms of degradation pathways of butachlor involved. The metabolites obtained in the present study are comparable to previous studies. Gushit et al. (2013) reported similar residues, namely 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester and 2,4-bis(1,1-dimethylethyl)-phenol from the extract of herbicide-treated rice plants. Anees et al. (2014) also reported similar degradation products while degrading butachlor using *Nostoc muscorum*. From the previous studies, it is clear that the toxicity (LD_{50}) of the obtained metabolites is comparatively less than the parent compound butachlor. To the best of the author's knowledge, *Ammoniphilus* sp. JF has not yet been reported for butachlor degradation.

Conclusion

The present study demonstrates that it is an efficacious solution to remediate the butachlor using indigenous *Ammoniphilus* sp. JF isolated from the agricultural fields of Punjab having known history of butachlor application since 22–23 years. The isolated strain showed better characteristics than previously reported butachlor-degrading strains, as this strain has a high butachlor-degrading rate and could efficiently degrade 100% of 100 mg/L butachlor within 24 h of incubation. Complete degradation of butachlor in a very short span proves that the isolate possess good potential for cleaning up of butachlor-contaminated sites.

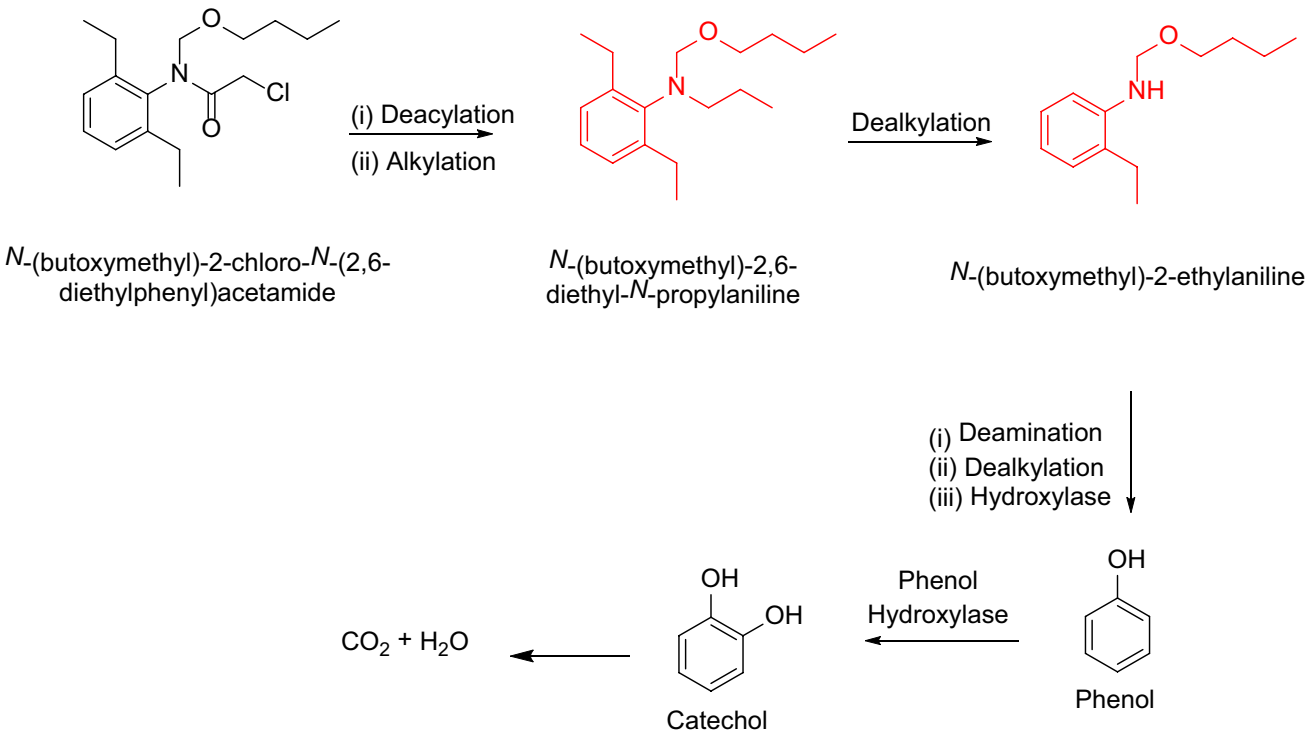

Fig. 5 Proposed degradation pathway of butachlor by strain *Ammoniphilus* sp. JF

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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