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ORIGINAL PAPER

Biotransformation of 4-chloro-2-nitrophenol into 5-chloro-2-methylbenzoxazole by a marine *Bacillus* sp. strain MW-1

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Abstract Decolourization, detoxification and biotransformation of 4-chloro-2-nitrophenol (4C2NP) by *Bacillus* sp. strain MW-1 were studied. This strain decolorized 4C2NP only in the presence of an additional carbon source. On the basis of thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS), 4-chloro-2-aminophenol, 4-chloro-2-acetaminophenol and 5-chloro-2-methylbenzoxazole were identified as metabolites. Resting cells depleted 4C2NP with stoichiometric formation of 5-chloro-2-methyl benzoxazole. This is the first report of the formation of 5-chloro-2-methylbenzoxazole from 4C2NP by any bacterial strain.

Keywords 4-chloro-2-nitrophenol · 4-chloro-2-acetaminophenol · 5-chloro-2-methylbenzoxazole · Biotransformation · Detoxification

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Introduction

4-Chloro-2-nitrophenol (4C2NP) is an anthropogenic compound that is present in industrial effluents due to its wide range application in pharmaceuticals, dyes and pesticides (Saritha et al. 2007). This compound is toxic to human beings as well as animals and is considered recalcitrant to microbial degradation due to electron withdrawing properties of chloro and nitro groups. Few reports have been published dealing with degradation of 4C2NP using physical methods. Priya and Madras (2006) reported degradation of 4C2NP by photocatalysis. Effective degradation of 4C2NP has been achieved by combination of advanced oxidation processes (Saritha et al. 2007). The major drawback of these physical methods is that they are not suitable for in situ bioremediation and are not eco-friendly. Microbial degradation is cost effective and efficient technology for remediation of xenobiotic compounds. Unfortunately, very little information is available for microbial degradation of 4C2NP. Beunink and Rehm (1990) reported degradation of 4C2NP by mixed co-culture of *Enterobacter cloaceae* and an *Alcaligenes* sp. under coupled reductive and oxidative processes. *E. cloaceae* initially acted upon 4C2NP under anaerobic conditions to transform 4C2NP to 4-chloro-2-aminophenol (4C2AP) as major transformation product. Degradation of 4C2AP was carried out under aerobic conditions by the *Alcaligenes* sp. with release of chloride and ammonia. An important feature of this study was anaerobic

conversion of nitro group of 4C2NP to an amino group, suggesting the involvement of putative oxygen sensitive nitroreductase that could not be carried out in oxygen rich aerobic conditions.

In a similar study, a 4C2NP assimilatory bacterium was constructed by bringing together the degrading properties of *Pseudomonas* sp. strain NW31 and *Ralstonia eutropha* JMP 134 (Bruhn et al. 1988). *Pseudomonas* sp. strain NW31 used 4C2NP as a nitrogen source and transformed 4C2NP to 4-chlorocatechol with release of nitrite. This strain was unable to further degrade chlorocatechol. The second strain *Ralstonia eutropha* JMP134 degraded chlorocatechol with release of chloride ion. For constructing a 4C2NP mineralizing bacterium, a plasmid encoding chloro aromatic compound degrading sequence of strain JMP134 was transferred into the strain NW31. The constructed strain now was able to use 4C2NP as sole source of carbon and energy.

Previous studies (Beunink and Rehm 1990; Bruhn et al. 1988) showed that 4C2NP is so toxic to microbes that no single 4C2NP mineralizing bacteria could be isolated. Microbes may adapt alternative mechanisms to detoxify the 4C2NP. The present communication describes such a mechanism for decolourization and detoxification of 4C2NP by *Bacillus* sp. strain MW-1.

Materials and methods

Chemicals

4-Chloro-2-nitrophenol (4C2NP) and 4-chloro-2-aminophenol (4C2AP) were purchased from Aldrich (Milwaukee, Wis.). 4-Chloro-2-acetaminophenol (4C2AAP) was synthesized by acetylation of 4-chloro-2-aminophenol (Katz and Cohen 1954). 5-Chloro-2-methylbenzoxazole (5C2MBZ) was purchased from Across Organics (Belgium). All other chemicals were used of purity grade.

Media and growth conditions

Minimal media (MM) for growth was prepared by dissolving the following compounds in a liter of double distilled water: 4.0 g Na_2HPO_4, 2.0 g KH_2PO_4, 0.8 g $(NH_4)_2SO_4$, 0.8 g $MgSO_4{\cdot}7H_2O$. Trace element solution (1 ml) was added to the solution after above mentioned compounds were dissolved completely; one liter of the trace element solution contained: 0.10 g $Al(OH)_3$, 0.05 g $SnCl_2{\cdot}2H_2O$, 0.05 g KI, 0.05 g LiCl, 0.08 g $MgSO_4$, 0.05 g H_3BO_3, 0.10 g $ZnSO_4{\cdot}7H_2O$, 0.01 g $CoCl_2$, 0.01 g $NiSO_4{\cdot}6H_2O$, 0.05 g $BaCl_2$, 0.05 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$. The pH of MM was adjusted to 7.0 before autoclaving at 15 lbs for 15 min. The stock solution of 4C2NP was prepared in methanol at 50 mM concentration and the desired concentration of the compound was added after filter sterilization (0.22 μm, Millipore). Filter sterilized glucose (10 mM) was added as an additional carbon and energy source.

Collection of the sample

Sample collection was performed by scuba diving team from Clearance and Collection Divers of the Indian Navy, at a depth of 30 m in the Bay of Bengal, India. The samples included marine water, sponge particles, sea floor sediments. The samples were placed in sterile bags and transported to the laboratory in a cool-box.

Isolation of marine microbes

Marine sea water samples were serially diluted and dilutions were spread-plated on different media including Zobell marine agar, 1/10 diluted Zobell marine agar, tryptone soya agar and 1/10 diluted tryptone soya agar and plates were incubated at 30°C for 2–10 days. Sponge and sediment samples were crushed, grinded and washed with artificial sea water. The residue is diluted and then spread-plated on above mentioned media and plates were incubated at 30°C for 2–10 days. Viable counts of the bacteria on different media were calculated at every 2 days and about 82 morphologically different colonies were purified by streaking. The pure cultures were maintained at −70°C as 10% glycerol stocks for further use.

Screening of bacterial strains

Marine bacteria (82) isolated from marine samples were streaked on minimal media agar plates containing 0.3 mM 4C2NP as a sole source of carbon and energy. The microbes were also streaked on minimal media agar plates containing 0.3 mM 4C2NP and

10 mM glucose or sodium succinate as additional carbon source. Positive strains were selected by decolourization of 4C2NP on agar plates. No marine bacterium was found that utilized 4C2NP as a sole source of carbon and energy. However one marine bacterium designated MW-1 showed decolourization of 4C2NP on the agar plates containing 4C2NP along with additional carbon source. This strain was selected for further study.

Identification of a 4C2NP decolourizing strain

Marine strain MW-1 was identified on the basis of 16S rRNA gene sequencing using the universal primers 27f and 1492r (Arora et al. 2011).

Identification of metabolites

Marine strain MW-1 was grown in 1 liter Erlenmeyer flask containing 250 ml minimal media, 0.3 mM 4C2NP and 10 mM glucose. Samples (50 ml) were collected at regular intervals, centrifuged at 8000*g* for 15 min and extracted with ethyl acetate. The extracted samples were analyzed by TLC, HPLC and GC-MS analysis. TLC and HPLC were performed as described by Ghosh et al. (2010). GC-MS analysis was carried out using a GC-MS-QP5000 instrument (Shimadzu, Tokyo, Japan) equipped with quadrupole mass filter and DB-1 capillary column with ionization of 70 eV, scan interval 1.5 s and mass range of 50–550 (*m*/*z*). The column temperature was initially increased from 80°C to 160°C at the rate of 5°C/min and then from 160°C to 260°C at the rate of 10°C/min. The carrier gas (nitrogen) flow rate was 20 ml/min.

Resting cell study and detection of ammonia and chloride

Resting cell study was carried out as described by Ghosh et al. (2010). Ammonia was detected by Nessler's reagent (McClure and Venables 1986) and chloride ion was detected by mercuric thiocyanate method (Bergmann and Sainik 1957).

Preparation of the crude extract

Strain MW-1 was grown on 100 ml minimal media, 10 mM glucose and 0.3 mM 4C2NP. After decolourization, the cells were centrifuged, washed twice with phosphate buffer (20 mM, pH 7.4) and resuspended in the same buffer. The cells were sonicated in a sonicator by twenty 30 s burst with intermittent 30 s cooling on ice. The cell extract were centrifuged at 4°C for 20 min to remove cell debris and the supernatant was used for enzyme assay.

Enzyme assay

The activity of 4C2NP reductase was measured spectrophotometry by monitoring the decreased absorbance at 340 nm due to the oxidation of NADPH. The reaction mixture contained 0.2 µmol 4C2NP, 0.6 µmol NADPH, 50 µmol phosphate buffer (pH-7.4) and 50–100 mg cell extract in a final volume of 1 ml. Reaction was initiated by the addition of the 4C2NP.

Results

Identification of the 4C2NP decolourizing strain

Marine strain MW-1 was identified as *Bacillus* sp. based on 16S rRNA gene sequence analysis. The 16S rRNA gene sequence of strain MW-1 was deposited in the GenBank under the accession number HM027879. Strain MW-1 decolorized 4-chloro-2-nitrophenol only in the presence of an additional carbon source.

Decolourization of 4-chloro-2-nitrophenol

Marine MW-1 was grown on minimal media containing 10 mM glucose and 0.3 mM 4C2NP. Growth of strain MW-1 was measured by increase of the absorbance at 600 nm and the decolourization was measured by decrease of the absorbance of 4C2NP at 425 nm. Strain MW-1 decolorized 4C2NP very efficiently and showed complete decolourization within only 12 h (Fig. 1). To check the effect of glucose concentration on decolourization, strain MW-1 was grown on minimal media containing 0.3 mM 4C2NP with different concentrations of glucose (5 mM, 10 mM, 15 mM, 20 mM). Glucose concentration did not significantly effect on decolourization. When strain MW-1 was grown 10 mM, 15 mM and 20 mM concentration of glucose, the

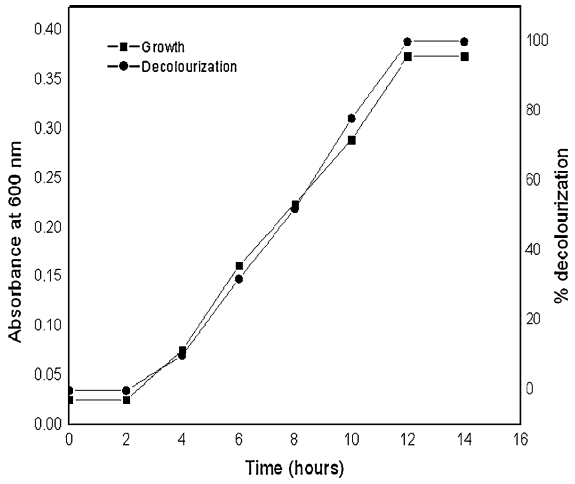

Fig. 1 Growth of strain MW-1 and decolourization of minimal media containing 0.3 mM 4C2NP and 10 mM glucose

complete decolourization was observed within 10–12 h. However, when the strain MW-1 was grown on 5 mM glucose, the decolorization was observed at 24 h due to the low growth of strain MW-1 in the media (data not shown).

Identification of metabolites

To identify the metabolite of 4C2NP degradation, culture samples were collected, centrifuged and extracted with ethyl acetate. The extracted samples were analyzed by TLC, HPLC and GC-MS. TLC studies indicated the presence of the three metabolites. The Rf values of the metabolites were 0.69, 0.63 and 0.78 and matched with authentic 4-chloro-2-aminophenol (4C2AP), 4-chloro-2-acetaminophenol (4C2AAP) and 5-chloro-2-methylbenzoxyzole (5C2MBZ), respectively.

HPLC studies showed the presence of the three metabolites of 4C2NP. The retention times of metabolites were 10.69, 14.43 and 11.74 min, respectively (Fig. 2). The retention times of these metabolites were exactly matched to that of authentic 4C2AP, 4C2AAP and 5C2MBZ, respectively.

GC-MS analysis showed the mass spectrum of the first metabolite was almost identical to that to 4C2AP (Fig. 3a). The molecular ion peak of authentic 4C2AP was observed as *m*/*z* 143. The mass spectrum of the metabolite II matched to that to 4C2AAP (Fig. 3b). The molecular ion peak of metabolite II appeared at *m*/*z* 185 due to acetylation of metabolite

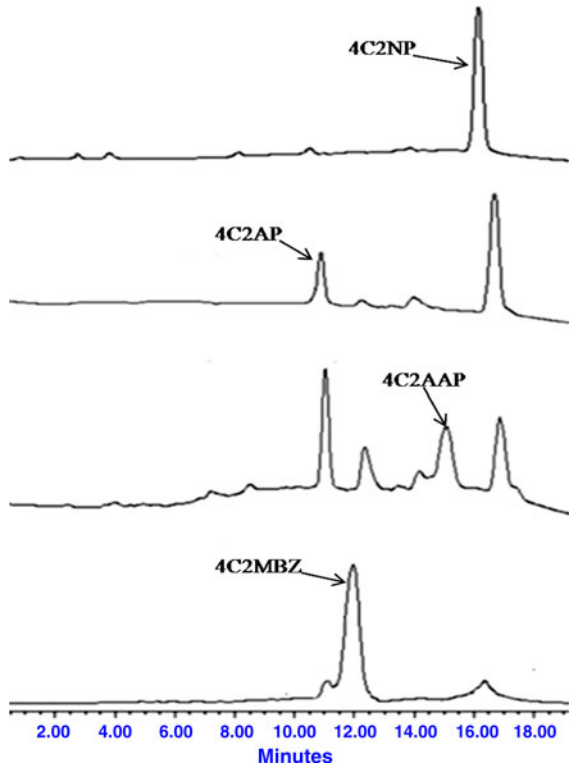

Fig. 2 HPLC elution profiles of the samples collected from different time intervals (0, 4, 8 and 12 h) during the degradation of 4C2NP by marine strain MW-1. All chromatograms have been recorded at 290 nm

I. The mass spectrum of the metabolite III exactly matched to that to 5C2MBZ (Fig. 3c). The molecular ion peak of metabolite III appeared at *m*/*z* 167 due to loss of the water molecule from metabolite II.

Resting cell study and chloride and ammonia release

4C2NP induced cells of strain MW-1 decolourized 0.3 mM 4C2NP within 30 min. In contrast, no decolourization was observed in non-induced control cells at the same time (data not shown). These results showed that enzyme system catalyzing decolourization is inducible. Further, HPLC analysis of the samples collected from resting cells of strain MW-1 showed complete depletion of 4C2NP with stiochiometric formation of 5C2MBZ (Fig. 4). There was no chloride and ammonia release from 4C2NP by marine strain MW-1 (data not shown).

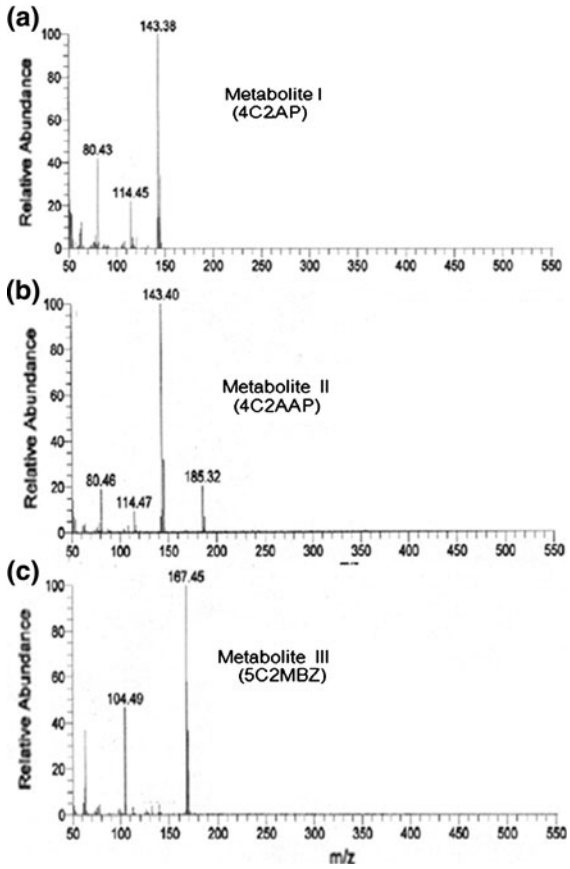

Fig. 3 Mass spectra of metabolites obtained in the cultivation of the strain MW-1

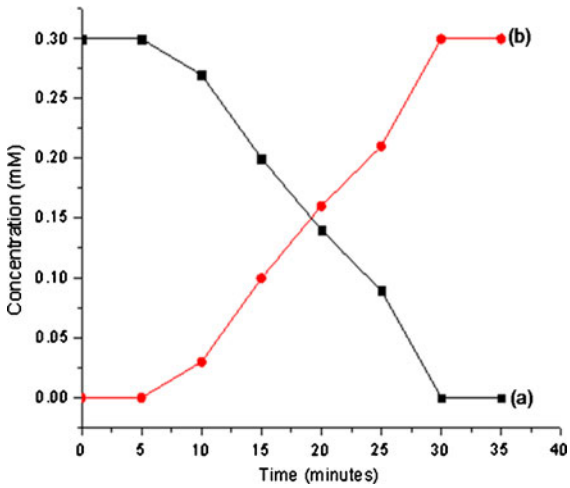

Fig. 4 (a) Depletion of 4C2NP and (b) formation of 5C2MBZ by resting cells of strain MW-1

Enzyme assay

The 4C2NP nitroreductase activity was observed in the crude extract of 4C2NP induced cells of strain MW-1 that suggested the reduction of the 4C2NP to 4C2AP. However, no attempt was made to carry out the enzyme assays for acetylation of 4C2AP and formation of 5C2MBZ.

Discussion

Bacillus sp. MW-1 decolourized 4C2NP only in the presence of additional carbon source. When strain MW-1 was grown on nitrogen free minimal media containing 10 mM glucose and 0.3 mM 4C2NP, no decolourization was observed, suggesting that strain MW-1 could not use 4C2NP as a nitrogen source. Strain MW-1 depleted 4C2NP to minimize the toxic effects of this compound and transformed highly toxic 4C2NP to less toxic products.

Our studies clearly showed formation of 4C2AP in the biotransformation pathway of 4C2NP by *Bacillus* sp. MW-1. Studies carried out by Donlon et al. (1995) showed that the aromatic amines are 500-fold less toxic than their corresponding nitroaromatics. Furthermore, the reduction of electron withdrawing nitro group to electron donating amino group make aromatic ring susceptible to electrophilic attack. Lendenmann and Spain (1996) showed that ring cleavage of 4C2AP is mediated by 2-aminophenol-1,6-dioxygenase purified from *Pseudomonas pseudoalcaligenes* JS45. Beunink and Rehm (1990) also reported degradation of 4C2AP with release of ammonia by *Alcaligenes* sp. TK-2. The reduction of nitro to ammonia group generally occurred under anaerobic conditions and this reduction is catalyzed by an oxygen sensitive nitroreductase (Williams et al. 1993). Beunink and Rehm (1990) reported the formation of 4C2AP from reduction of 4C2NP under anaerobic conditions. The reduction of nitro group under aerobic conditions was initiated by an oxygen-insensitive nitro-reductase through the formation of hydroxylamines or amines (Reinado et al. 2005; Peterson et al. 1979; Spain 1995).

Park et al. (1999) reported formation of 4C2AP in the reductive pathway of degradation of 3-chloronitrobenzene under aerobic condition. This study reported the formation of 4C2AP in the biotransformation pathway of 4C2NP under aerobic condition by

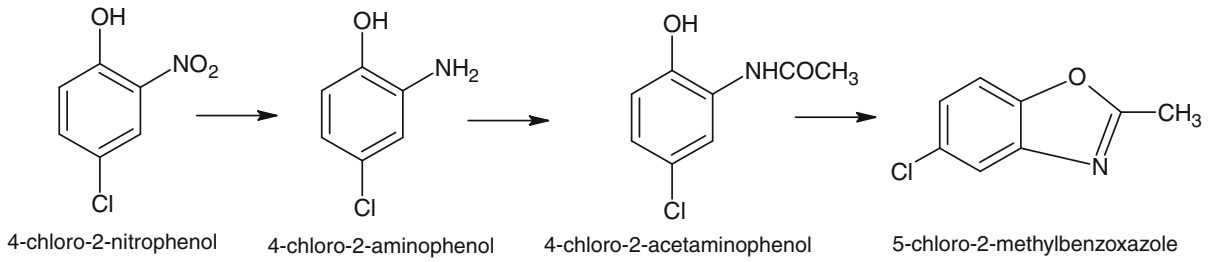

Fig. 5 Proposed pathway for decolourization of 4C2NP by strain MW-1

strain MW-1. 4C2AP was acetylated to 4C2AAP rather than further degraded. Park et al. (1999) also reported 4C2AAP as the acetylation product of 4C2AP in the degradation pathway of 3-chloronitrobenzene.The detoxification mechanism through acetylation has also been reported in the degradation of 4-chloronitrobenzene (Corbett and Corbett 1981), 2,4-dinitrotoluene (Noguera and Freedman 1996), 3-nitrophenol (Schenzle et al. 1997) and 2,4,6-trinitrotoluene (Gilcrease and Murphy 1995). The further degradation of acetylated compounds is a complex process that is not easy to understand. Park et al. (1999) reported degradation of 4C2AAP by strain *Rhodococcus* sp. HS51 with release of chloride and ammonia. In our case, neither chloride nor ammonia is released indicating the biotransformation product has both chlorine and nitrogen. In this study, acetylated product 4C2AAP transformed into novel intermediate 5-chloro-2-methyl benzoxazole (5C2MBZ) with release of water molecule by marine strain MW-1.

Based on identification of intermediates formed by *Bacillus* sp. MW-1, we propose detoxification mechanism for 4C2NP. Our studies showed formation of 4C2AP through partial reduction of 4C2NP. Further, acetylation of the amino group leads to formation of 4C2AAP. Due to dehydration and cyclization, novel intermediate 5-chloro-2-methyl benzoxazole (5C2MBZ) is formed (Fig. 5).

The molecular structure of 5C2MBZ is complex than the parent compound 4C2NP; therefore, it is not easy to be biodegraded/biotransformed and the biodegradation of 5C2MBZ is subject of further investigation.

Conclusion

Marine bacterium, *Bacillus* sp. strain MW-1 decolorized and detoxify 4C2NP in the presence of the additional carbon source. This strain completely degraded 4C2NP with stoichiometric formation of 5-chloro-2-methylbenzoxazole (5C2MBZ). This is the first report of biological formation of 5-chloro-2-methylbenzoxazole. Strain MW-1 may be used to detoxify 4C2NP contaminated site.

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