Degradation of 2,4,6-Trinitrophenol (TNP) by *Arthrobacter* sp. HPC1223 Isolated from Effluent Treatment Plant

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Received: 20 April 2012 / Accepted: 14 June 2012 / Published online: 17 July 2012
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Abstract *Arthrobacter* sp. HPC1223 (Genebank Accession No. AY948280) isolated from activated biomass of effluent treatment plant was capable of utilizing 2,4,6 trinitrophenol (TNP) under aerobic condition at 30 °C and pH 7 as nitrogen source. It was observed that the isolated bacteria utilized TNP up to 70 % (1 mM) in R2A media with nitrite release. The culture growth media changed into orange-red color hydride-meisenheimer complex at 24 h as detected by HPLC. Oxygen uptake of *Arthrobacter* HPC1223 towards various nitro/amino substituted phenols such as dinitrophenol (1.2 nmol/min/mg cells), paranitrophenol (0.9 nmol/min/mg cells), 2-aminophenol (0.75 nmol/min/mg cells), *p*-aminophenol (0.4 nmol/min/mg cells), phenol (0.56 nmol/min/mg cells) and TNP (2.42 nmol/min/mg cell) was analysed, which showed its additional characteristic of broad substrate catabolic capacity. The present study thus report a novel indigenous bacteria isolated from activated sludge utilized TNP and has broad catabolic potential towards substituted phenols.

Keywords Trinitrophenol · *Arthrobacter* · ETP · Nitrite · Degradation

Introduction

Nitrophenols are among the most widely used industrial organic compounds. They are frequently used as intermediates in the manufacture of pharmaceuticals, dyes,

pesticides and explosives [1, 2]. Because of their widespread use, nitrophenols are found as contaminants in industrial effluents, rivers, ground water and pesticide treated soils [3, 4]. Although the resistance to biodegradation of the aromatic ring is enhanced by the presence of more nitro groups such as trinitrophenol (TNP), still few bacterial strains have been reported which are capable of using as sole carbon or nitrogen source [5].

Nitroaromatics are not found as natural compounds in the environment. Due to the pronounced electron-withdrawing character of the nitro groups, they harbor an electron deficient pi-electron system, generating a xenobiotic character. As a result, the electrophilic attack which is usually the first step in aromatic biodegradation becomes more difficult. Nitroaromatics with few nitro groups such as mononitrophenol and mononitrotoluene, are easily degraded through an oxidative attack [6]. However dinitroaromatic compounds and particularly trinitroaromatic compounds are more resistant to oxidative attack and also mineralization [5, 7, 8].

In the present study, attempts were made to isolate bacteria capable of utilizing 2,4,6-TNP from effluent treatment plant (ETP). The study also report the presence of broad catabolic potential in the indigenous isolate *Arthrobacter* towards substituted phenols.

Materials and Methods

Chemicals

2,4,6-Trinitrophenol of analytical grade was obtained from Sigma-Aldrich. Other chemicals required for mineral salt media preparation and solvents were purchased from Merck (India). Yeast extract, Luria broth used for growth

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of strains were purchased from HiMedia Laboratories Pvt. Ltd, India. Reagents used for PCR reaction such as Amp-liTaq, $MgCl_2$, PCR buffer were used from Applied Biosystems, Life Technologies, India. Double distilled water was used throughout the study.

Selection and Characterization of 2,4,6-TNP Degrading Bacteria

Enrichment and Isolation

For enrichment and isolation of bacteria, activated biomass was collected from an industrial ETP, situated at Gujarat, India, where nitroaromatic contaminated wastewater was treated. Five grams of activated biomass obtained from ETP was inoculated into 500 ml mineral salts medium supplemented with 0.5 mM TNP. After 10 days the acclimatized culture (5 ml) was transferred to fresh mineral salts medium with TNP (0.5 mM) to enrich the bacterial population. After 2–3 successive transfers in mineral salt medium the enriched bacterial culture was transferred to R2A medium using identical growth conditions, except that the TNP concentration was increased stepwise varying from 0.5 mM to final 1 mM. Each fresh medium was incubated for about 48 h. One month later, the final enrichment culture were serially diluted and streaked onto R2A media containing 1 mM TNP. On incubation of plates for 1 week, colonies were picked and re-streaked for purification. All the colonies exhibited the same morphology on agar plates and were tested for their degradation ability on TNP. One bacterial isolate designated HPC1223 strain exhibited TNP utilization showing change from yellow color TNP to orange-red color.

Media

The composition of R2A media used for growing cells comprised of, mineral salts media with 0.1 g/l of yeast extract. Mineral salts medium contained (per litre) 1.53 g of $Na_2HPO_4 \cdot 2H_2O$, 0.76 g of KH_2PO_4 , 0.5 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $CaCl_2$ and 10 ml of trace element solution SL-4 (pH 7.0). Trace element solution SL-4 contained 0.5 g of EDTA, 0.2 g of $FeSO_4 \cdot 7H_2O$, 100 ml of trace element solution SL-6, and 900 ml of water. Trace element solution SL-6 contained 0.1 g of $ZnSO_4 \cdot 7H_2O$, 0.03 g of $MnCl_2 \cdot 4H_2O$, 0.3 g of H_3BO_3 , 0.2 g of $CoCl_2 \cdot 6H_2O$, 0.01 g of $CuCl_2 \cdot 2H_2O$, 0.02 g of $NiCl_2 \cdot 6H_2O$, 0.03 g of $Na_2MoO_4 \cdot 2H_2O$, and 1,000 ml of water. The initial pH of the medium was 7.0. The experimental cultures were incubated on a rotary shaker at 180 rpm and 30 °C during the enrichment and selection of bacteria.

Identification Using 16s rRNA Gene Sequencing

The isolated strain HPC1223 was characterized by 16S rDNA sequence analysis. DNA was extracted from culture grown in LB medium. PCR amplification of the 16S rDNA was performed using 16S rDNA universal primers with a Biorad thermal cycler. The reaction mixture contained 5 µl template, 1x PCR buffer, 200 µM each of dNTP's, 3 mM $MgCl_2$, 50 pmol of each 16S primer (27F5'-AGAGTTTGATCMTG GCTCAG-3'; 1492R5'-TACGGYTACCTTGTTAGACTT-3') and 2.5 units of Amplitaq DNA polymerase from Perkin-Elmer USA, in a final volume of 50 µl. The thermocycling conditions used were as follows: 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C followed by extension at 72 °C for 1 min. Nucleotide sequence similarities were determined using BLAST (NCBI—National Centre for Biotechnology Information Databases).

Biodegradation and Analysis of 2,4,6-TNP

The degradation experiments were carried out with isolated pure culture of *Arthrobacter* HPC1223 strain using R2A medium amended with 1 mM TNP. The cultures were incubated at 30 °C for 24 h on a rotary shaker at 150 rpm. The isolated pure culture was inoculated from glycerol stocks in 5 ml of LB media to get the biomass. LB grown cells were pelleted and washed once with sterile distilled water, suspended in 50 ml R2A medium with TNP (0.5, 0.7, 1 mM) and the initial cell density of 0.1 and 0.2 (OD600 nm) to study the degradation. Growth was monitored by cell density at OD600 nm till 96 h. The experiment was performed using specific controls for comparison, one containing HPC1223 cells without TNP and another with TNP as substrate but without HPC1223 in the media. The degradation experiments were conducted in 250 ml Erlenmeyer flasks with 50 ml culture media.

The optical density (OD) of cell growth were measured by spectrophotometric analysis (Perkin-Elmer double-beam Spectrophotometer, Model Lambda 900) at 600 nm. TNP concentrations were determined by High-performance liquid chromatography (HPLC; Perkin-Elmer series 200 LC system) at 420 nm. Separations were performed on C18 column (Merck, Lichrosphere-100, RP-18 and 5 µm) with acetonitrile:water (20:80 v/v) as the mobile phase, at a flow rate of 0.5 ml/min. To calculate % degradation, peak areas were measured to quantify TNP. The release of nitrite ion concentration in the culture media was analyzed as described earlier [9].

Preparation of Standard Hydride-Meisenheimer Complex (H-TNP) and Metabolite Detection

An authentic standard of TNP degradation product (H-TNP), meisenheimer complex was synthesized chemically

following the procedure described by Singh et al. [8]. H-TNP was prepared by adding 15 μ l sodium borohydride (20 mg/ml) to 500 μ l 20 mM TNP in aqueous solution (pH 10). An orange-red product (H-TNP) of TNP was formed that was used as standard in HPLC analysis for metabolite detection.

Oxygen Uptake (Respirometric Assay) Experiment

The cells of strain HPC1223 were grown in R2A media amended with TNP (1 mM) to get the cell mass. The cells were harvested at 6,000 rpm for 5 min, washed and suspended in 50 mM phosphate buffer. The reactions were carried out in 3 ml phosphate buffer (1 mM, pH 7) in oxygen chamber of a Digital Oxygen system (model 10, Rank Brothers, Bottisham, UK) with 3 OD cells per reaction. The uptake rates were corrected for endogenous oxygen consumption. The final substrate concentration

used for each reaction was 1 mM. The oxygen uptake rates were expressed as nanomoles of oxygen/min/mg of cells. Weight was determined by drying cell pellets at 70 °C until a constant weight was obtained. The catabolic potential of the isolated strain for different nitro and amino substituted phenols were also analysed by recording changes in oxygen uptake rates.

Results

Selection and Characterization of TNP Degrading Bacteria

A bacterial strain able to grow on 2,4,6-TNP was isolated from an activated biomass of ETP. The yellow color of TNP was converted to orange-red color metabolite with time. Isolated colony which showed the best degradation

Fig. 1 Phylogenetic tree of 16S ribosomal RNA gene of the isolated strain *Arthrobacter* HPC1223 and standard ATCC strains obtained from Genbank using 1000 bootstrap values

ability in liquid media was named as HPC1223 strain. The HPC1223 strain was identified by 16S ribosomal gene sequencing as closely related to *Arthrobacter* sp. on phylogenetic analysis (Fig. 1). The partial sequence was deposited in GenBank database under Accession No. AY948280.

Growth and TNP Degradation

The experiments were done to determine the degradation of TNP and growth of HPC1223 strain in R2A media using two different initial inocula viz; 0.1 and 0.2 OD_{600 nm} per milliliter (Fig. 2). The degradation rate was relatively enhanced with 0.2 OD_{600 nm} initial cell concentrations with initial lag period remaining same in both inocula.

In order to evaluate the effect of TNP concentration on microbial growth and TNP degradation, *Arthrobacter* sp. HPC1223 strain was cultivated in R2A media having different TNP concentrations, viz; between 0.5 and 1.5 mM (Fig. 3). At concentrations viz; 0.5, 0.75 and 1 mM, maximum degradation was achieved at 48, 72, 96 h, respectively, indicating the effect of increasing concentration of TNP on growth. Nitrite was released in the media with time, indicating that it is used as nitrogen source. However above 1 mM concentration of TNP, growth of *Arthrobacter* was inhibited. The results revealed that 70 % of TNP (1 mM) was utilized up to 96 h by *Arthrobacter* sp. with removal of nitro group as nitrite.

Metabolite Formation

At 24 h culture supernatant was analysed for metabolite by HPLC. It was observed that the orange-red product formed

Fig. 2 Graph showing growth of *Arthrobacter* HPC1223 in R2A medium (pH 7) in presence of TNP as substrate, utilization of 1 mM TNP and nitrite release (filled square—OD_{600 nm}; filled circle—residual concentration of TNP; filled triangle—nitrite release)

Fig. 3 Graph showing degradation of various concentrations of TNP by *Arthrobacter* HPC1223 (filled circle—0.5 mM, open triangle—0.75 mM, filled square—1 mM, inverted filled triangle—1.5 mM, filled diamond—2 mM)

was hydride-meisenheimer complex (H-TNP). An additional peak appeared along with TNP peak, which showed similar retention time (2.845 min) as that of authentic standard of H-TNP (Fig. 4).

Respirometric Assay

Respirometric analysis revealed that *Arthrobacter* strain HPC1223 consumed oxygen in presence of various substituted phenols such as dinitrophenol (1.2 nmol/min/mg cells), paranitrophenol (0.9 nmol/min/mg cell), 2-aminophenol (0.75 nmol/min/mg cells), *p*-aminophenol (0.4 nmol/min/mg cells), phenol (0.56 nmol/min/mg cells). Oxygen uptake was

Fig. 4 Metabolite (H-TNP) detected during HPLC analysis of culture media at 24 h

Fig. 5 Oxygen uptake rates of strain HPC1223 pregrown on R2A media and R2A media with TNP (1 mM). Oxygen uptake rates are reported as nmol/min/mg weight of cells. Data are means of three independent experiments

relatively more with TNP substrate (2.42 nmol/min/mg cell) in *Arthrobacter*. The results revealed the broad catabolic ability of *Arthrobacter* sp. HPC1223 isolated from ETP to utilize different substituted phenols (Fig. 5).

Discussion

Research in the last few decades has revealed number of microbes capable of degrading nitro-aromatic compounds via aerobic as well as anaerobic pathways [11]. There are only a few examples of microbial conversion of picric acid (TNP). Three bacterial sp., i.e., *Nocardioides* sp. strain CB 22 [10], *Rhodococcus* sp. [7], *Bacillus* sp. [8] have been examined for their efficiency toward TNP (picric acid) degradation. These bacteria utilized TNP as the sole source of nitrogen only. Despite the availability of information on TNP degrading microbes there is lack of information regarding the elimination of the nitrite released from TNP degradation in *Arthrobacter* sp.

In this study we isolated and characterized a strain of *Arthrobacter* HPC1223 (Fig. 1) that was able to use TNP as a sole source of nitrogen. The isolate was capable of growing on 1 mM TNP with release of nitrite (Fig. 2). Since the strain was able to utilize only up to 70 % TNP (1 mM), it was inferred that the strain lose the ability to completely degrade TNP till 96 h. It was observed that, culture media with more than 1 mM concentration, the growth and utilization was inhibited (Fig. 3) due to the toxicity of TNP. In 1 mM concentration, accumulation of nitrite ions (0.2 mM) was observed with time in the culture media, which could be interfering in the further utilization of TNP by *Arthrobacter*. At 24 h a metabolite peak of H-TNP (hydride-meisenheimer

complex) was detected during HPLC analysis (Fig. 4) indicating initial reduction of phenolic ring followed by removal of nitrite group. These complexes are reported as key intermediates of denitration and productive microbial degradation of picric acid [12]. The isolate also showed oxygen uptake in presence of various substituted phenols. The presence of one of the substituted phenol during oxygen uptake was dinitrophenol (1.2 nmol/min/mg cells) giving a clue as an intermediate after denitration (Fig. 5).

The present study reported the isolation of an indigenous bacterial strain capable of utilizing TNP as nitrogen source in aerobic condition. TNP was reported to be transformed by many bacterial strains, however, to our knowledge, the report on the broad catabolic capacity of *Arthrobacter* sp. capable of degrading TNP (1 mM) with nitrite release was first time reported.

Conclusion

Arthrobacter HPC1223 was isolated from activated sludge of ETP, capable of degrading TNP up to 1 mM with release of nitrite ions. An orange-red metabolite detected as hydride-meisenheimer complex of TNP (H-TNP) by HPLC indicated a key intermediate of denitration in *Arthrobacter*. Oxygen consumption studies demonstrated that *Arthrobacter* HPC1223 could utilize various substituted phenolic substrates such as dinitrophenol, paranitrophenol, 2-aminophenol, *p*-aminophenol, phenol and TNP. The broad catabolic potential was observed in *Arthrobacter* sp. HPC1223 capable of utilizing TNP.

Acknowledgments The authors thank Director, National Environmental Engineering Research Institute (NEERI), Nagpur, for providing the facilities for carrying out this work. Funding from CSIR for the network project is also acknowledged.

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