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IDENTIFICATION OF A NEWLY ISOLATED P-NITROPHENOL DEGRADING STRAIN CHARACTERIZED AS *Rhodococcus* SP. BUPNP1

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ABSTRACT

In an attempt to study biodegradation of p-Nitrophenol (PNP), several bacterial strains from soil of agricultural field and landfill area were isolated by enrichment culture technique. Among many isolates, one designated as BUPNP1 was identified as *Rhodococcus* sp., based on 16S rDNA sequence analysis along with phenotypic characteristics such as gram-staining, motility, catalase, oxidase, starch hydrolysis and sugar utilization ability. The Minimum Inhibitory Concentration (MIC) was determined and it was found that 1.8mM PNP is detrimental for the strain but it can tolerate up to 1mM concentration of PNP. Spectrophotometric analysis of residual PNP during degradation showed that the strain was capable of degrading 0.5mM of PNP up to 95% within 33 h of time interval. It was found to produce 4-Nitrocatechol (4-NC) as an intermediate product during PNP degradation. Strain BUPNP1 have high potential for the purpose of bioremediation of PNP contaminated soil, wastewater and other anthropogenic habitats where PNP contamination is very common.

Key Words : Biodegradation, p-Nitrophenol (PNP), *Rhodococcus* sp., 4-Nitrocatechol (4-NC), Bioremediation

INTRODUCTION

A nitrophenolic compound p-Nitrophenol (PNP) is frequently used in manufacture of pesticides (Parathion and Methyl parathion), dyes, pharmaceuticals and explosives.[1,2] PNP is also produced as bye product from plastics and petrochemical industries[3] The United States Environmental Protection agency (U.S. EPA) enlisted PNP as a priority pollutant.[4] Due to its high water solubility and chemical stability it persists in the contaminated environmental niches for longer period. The release of PNP into various ecological niches has led to contamination of ground water through which it has entered the food web ultimately leading to unestimated destabilization of several ecosystems. Burdwan district (West Bengal, India) is the feeding bowl of Bengal. It is mainly known for cultivation of paddy, potato, pulses and vegetables. Indiscriminate use of pesticides, herbicides has resulted in bioaccumulation of many toxic xenobiotic compounds. Restoration of polluted environmental niches by microbial bioremediation has gained popularity mainly due to its cost effectiveness and better pollution free approach. Some aerobic microorganisms that are able to degrade PNP have been found to exist in environmental niches. Two major pathways have been characterized so far among PNP degrading bacteria, one degradation pathway leads to the formation of 4-Nitrocatechol (4-NC) has been reported in Gram-positive bacteria such as *Arthrobacter* spp., *Bacillus* spp. and *Rhodococcus* spp.[5-9] and other leads to formation of Hydroquinone (HQ) has been reported in Gram-negative bacteria such as *Burkholderia* spp., *Moraxella* spp. and *Pseudomonas* spp.[10-12] In *Burkholderia* sp. SJ98[13] and *Pseudomonas* sp. 1-7[14] both the branches of the degradation pathway has reported. Among these PNP degrading bacteria a very few can degrade higher PNP concentration. The present manuscript reports isolation, characterization, identification, biodegradation and determination of MIC of PNP by a bacterial isolate designated as BUPNP1 and was identified as *Rhodococcus* sp.

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AIMS AND OBJECTIVES

Isolation, characterization, identification of PNP degrading bacteria, determination of MIC of PNP for the strain, growth and biodegradation study in PNP containing medium and preliminary identification of intermediate formed during degradation.

MATERIAL AND METHODS

Strain isolation and taxonomic characterization

The strain BUPNP1 was isolated from a soil sample collected from landfill area of Burdwan, West Bengal, India by enrichment culture technique on minimal salt medium (11). The pH of the medium was adjusted (7.0 ± 0.2) before autoclaving at 15 lbs for 15 min. Filter sterilized PNP (0.22 µm, Millipore) was added after autoclaving the minimal medium. The morphological, physiological and biochemical tests of isolate, were performed by using standard methods.[15] Carbohydrate utilization test were performed by using KB009 HiCarbohydrate^{TM} kit, HIMEDIA.

Phylogenetic analysis

The genomic DNA was extracted by using Genomic DNA isolation kit (HiPurA^{TM} Bacterial and Yeast Genomic DNA Miniprep Purification spin kit, HIMEDIA) as per manufacturer's instructions. Amplification of 16S rRNA gene and its purification was performed according to Saha and Chakrabarti, 2006.[16] For sequencing, three separate primers were used namely 27f (5´AGAGTTTGATC CTGGCTCAG 3´), 530f (5´ GTGCCAGCM GCCGCGGTAA 3´), and 1492r (5´ TACGGY TACCTTGTTACGACTT 3´). The GenBank Accession number for 16S rRNA gene of the strain BUPNP1 is KF652059. Phylogenetic analysis and tree was constructed according to Saha et al.[16]

Determination of MIC of PNP for the strain

MIC was determined by growing the isolate on different concentrations of PNP made in minimal salt medium and started with a fixed cell number (2.5 x 10^7 CFU/ml) for each PNP concentration. After 24 h of incubation at 37°C with 120 rpm, the CFU counts were determined on PNP (0.5mM) agar plates to detect viability of the cell. All the experimental sets were performed in triplicate and Standard Deviation (SD) were calculated for the CFU counts. MIC was determined as PNP concentration where least CFU counts were detected.

Biodegradation studies of PNP during growth

The isolate was cultured using minimal salt medium with PNP (0.5mM) as sole source of carbon. The culture was grown on Tryptic soya broth (TSB) with PNP, centrifuged and cell pellet was washed twice in normal saline. The washed cell pellet was re-suspended in minimal salt media supplemented with PNP (0.5mM) and incubated at 37°C with 120rpm. Growth of the culture was monitored by measuring optical density (OD) of the culture at 600nm while the residual PNP concentration from filtered (0.22 µm, Millipore) culture supernatant was detected at 400nm using a UV-VIS spectrophotometer (VARIAN, Cary50 Bio UV-VIS spectrophotometer) at 3 hours of regular time interval. A standard curve was prepared and molar extinction coefficient (ε) of PNP was determined 10.63$mM^{-1}cm^{-1}$ for given sets of experiment. All readings were measured in triplicate.

Identification of intermediates in the PNP degradation pathway

The isolate was grown on minimal medium with PNP as sole C source in 37°C at 120 rpm. 25ml of culture was collected and centrifuged to remove cell pellet. The supernatant was vigorously shaken with equal volume of ethyl acetate and the two layers were allowed to separate in a separating funnel. The ethyl acetate layer was collected and was referred as neutral extract. The pH of aqueous phase set to approx 2 with 5N HCl and was extracted again with equal volume of ethyl acetate. After separation of two layers, the ethyl acetate layer is collected which was referred as acidic extract. The neutral and acidic extracts were mixed together and evaporated to dryness at 50°C. The residue was dissolved in 1ml of methanol. All standard intermediate compounds (Sigma Aldrich and Fluka) of known PNP degradation pathway were also dissolved in methanol. TLC was performed using pre coated silica plates (F_{254}, 20 x 20 cm; Merck) and was developed using Toluene: ethyl acetate : acetic acid, (60:30:5) as solvent

system.[16] Spots were visualized under UV illumination and R_f value was recorded for each spots.

RESULTS AND DISCUSSION

Isolation and characterization of PNP degrading bacteria

The strain BUPNP1 was decolorizing the color of PNP when grown on minimal medium containing 0.5mM PNP **(Fig.1)**. It completely decolorized PNP after 30 hour of incubation by forming some intermediate compounds (reddish yellow in color).The strain was Gram positive, non-motile, non-spore forming and Scanning Electron Microscope (SEM) image revealed typical rod-coccus cycle **(Fig. 2)** of growth pattern, usually reported for the genus *Rhodococcus*. The phenotypic properties of the strain are listed in **Table. 1**.

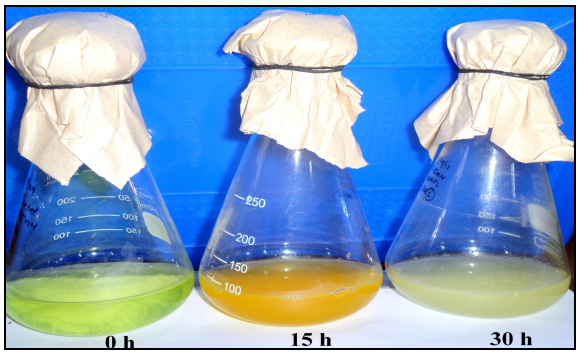

Fig. 1 : Decolorization of PNP in culture medium by Strain BUPNP1

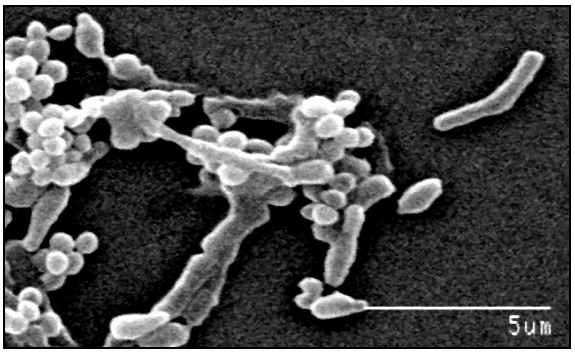

Fig. 2 : SEM image (8000X) of strain BUPNP1 shows rods and *Cocci*

Phylogenetic analysis

Based on 16S rDNA gene sequence analysis, carried out using various online tools available at website of Ribosomal Data project (RDP), Release-10 (*www.rdp.cme.msu*), the strain was classified as a member of the genus *Rhodococcus* **(Fig. 3)**. The strain BUPNP1 showed closest sequence similarity with *Rhodococcus rhodochrous*^{T} (99.5%), followed by *Rhodococcus pyridinovorans*^{T} (99.1%) and *Rhodococcus gordoniae*^{T} (98.3%). The phylogenetic analysis shows that the strain is a member of *Rhodococcus* sp. However in absence of overall genome relatedness data (+) the species status of the strain remains undetermined.

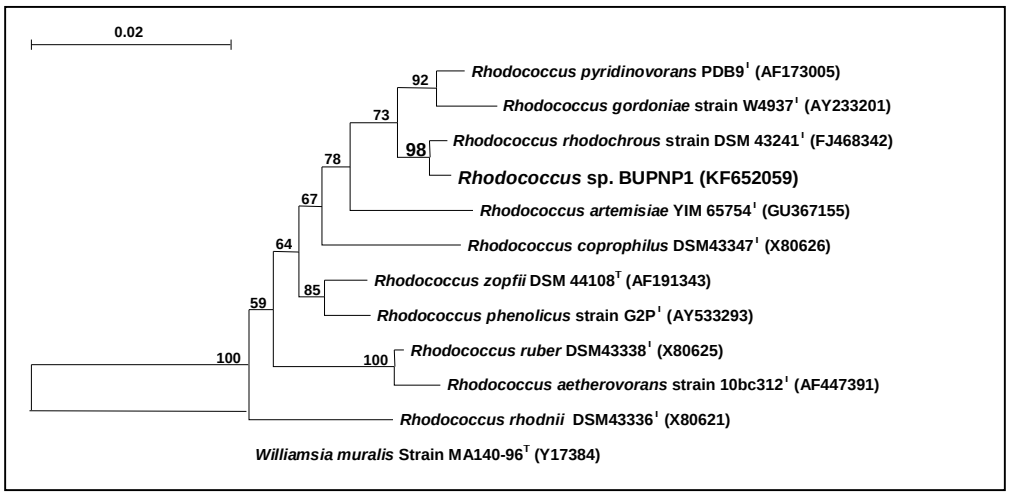

Fig. 3 : Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence analysis

Fig. 3 is showing the relative position of strain BUPNP1 among various representative members of the genus *Rhodococcus* (having greater than 96% sequence identity). Bootstrap values (as percentages of 500 replications) are shown at nodes. Bar, 0.02 substitutions per

site. The sequence of *Williamsia muralis* strain MA140-96^T was used as an out group. The tree was generated using TREECON software using NJ method (Kimura model).

Table 1 : Morphology, biochemical tests and various sugar utilization tests performed for strain BUPNP1

Morphology and biochemical tests		**Sugar utilization profile**			
Gram's character	+	Lactose	-	Sorbitol	+
Motility	-	Xylose	-	Mannitol	-
Endospore formation	-	Maltose	+	Adonitol	-
Catalase	+	Fructose	+	Arabitol	-
Oxidase	-	Dextrose	+	Erythriol	-
Methyl red test	+	Galactose	+	α-Methyl-D-glucoside	-
Voges-Proskaur test	-	Raffinose	+	Rhamnose	-
Nitrate reduction	+	Trehalose	-	Cellobiose	-
Indole production	+	Melibiose	-	Melezitose	+
Phosphate solubilization	-	Sucrose	+	α-Methyl-D-mannoside	-
Caesin hydrolysis	-	L-Arabinose	+	Xylitol	-
Starch hydrolysis	-	Mannose	-	o-Nitrophenyl β-galactoside	-
		Inulin	-	Esculin hydrolysis	-
		Sodium gluconate	-	D-Arabinose	+
		Glycerol	+	Citrate	-
		Salicin	-	Malonate	-
		Dulcitol	-	sorbose	-
		Inositol	+		

MIC for the strain

Cells remained viable up to 1mm PNP which indicated that the strain was able to tolerate up to 1mM PNP. Above 1mM PNP, cell number decreased (as indicated by decrease in viable count, **Fig.4**) and beyond 1.8 mM PNP no cell survival could be detected, indicating, 1.8mM being MIC of PNP for the strain BUPNP1.

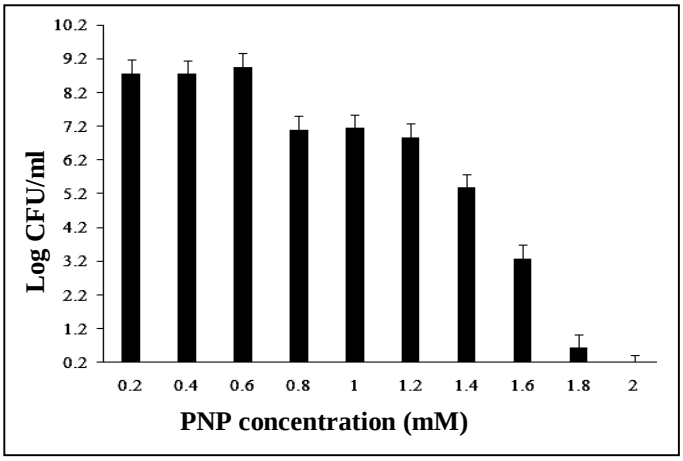

Fig. 4 : MIC for strain BUPNP1, Log CFU/ml with standard error shown with increasing PNP concentration (mM)

Biodegradation of PNP during growth

Exponential growth on minimal medium supplemented with 0.5mM PNP indicates that it utilized PNP as sole source of carbon and degraded up to 95% after 33 h incubation **(Fig. 5)**. The viable cell count of *Rhodococcus* strain BUPNP1 increased from 2.5 x 10^7 cells/ml to 1.8 x 10^8 cells/ml after 24 h incubation, indicating that the strain could grow by mineralizing PNP. Generation time of the strain was determined to be 8.5 h when growing on 0.5mM PNP. No abiotic

degradation of PNP was observed in controls although the auto hydrolysis of PNP was measured in control sets (without inoculum) which was detected very low.

Identification of intermediates in the PNP degradation pathway

Results obtained from TLC detected a spot of intermediate product present in the solvent extract from *Rhodococcus* BUPNP1 culture with R_f value 0.68 **(Fig. 6)**, by comparing with the R_f value of standards it was identified as 4-Nitrocatechol (4-NC). Solvent extract from control was found to contain only PNP which indicated that degradation of PNP was carried out by the strain.

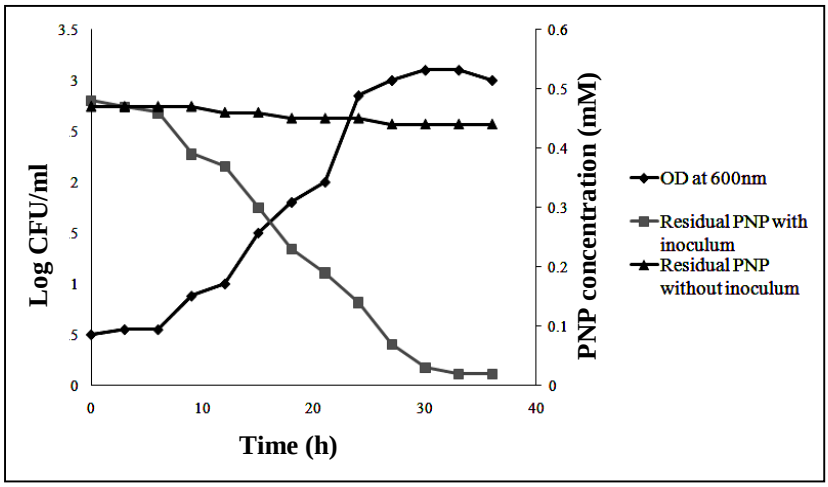

Fig. 5 : Degradation of PNP by strain BUNP1 during its growth in minimal medium supplemented with 0.5mM PNP

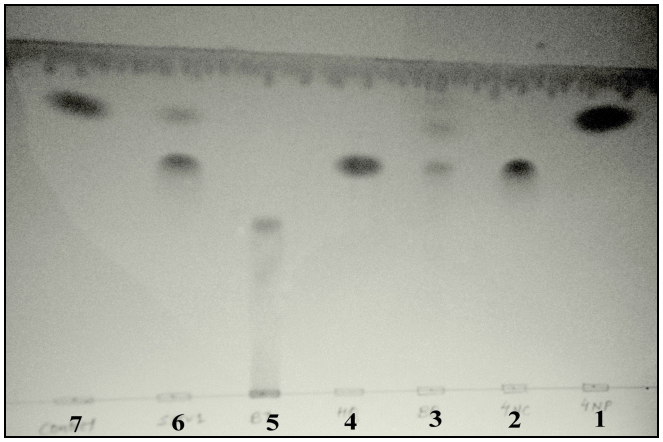

Fig. 6 : Spots of extracted intermediates on TLC visualized under UV illumination, (1) PNP (R_f =0.82), (2) 4-NC (R_f =0.68), (3)BQ (R_f =0.69), (4) HQ (R_f =0.71), (5) 1,2,4- BT (R_f =0.52), (6) Culture extract of BUPNP1 (R_f =0.68), (7) Control (R_f =0.82)

CONCLUSION

A Gram positive, non-motile 4-NP degrading bacterium was isolated from a soil sample collected from a landfill site in Burdwan (West Bengal, India). The strain was designated as *Rhodococcus* sp. BUPNP1 which showed 99.5% similarity with *R. rhodochrous*^{T}. It can grow profusely on medium with PNP as sole source of carbon and can degrade up to 95% of 0.5mM PNP within 33hr. Reports on PNP degradation by Gram-positive bacteria shows that they follow a PNP degradation pathway with 4-NC as an intermediate product. *Rhodococcus* sp. BUPNP1 also found to produce 4-NC as an intermediate product during the PNP degradation which was detected by TLC. This result is supported by the reported PNP degradation pathway in other

strains of *Rhodococcus* sp. The strain BUPNP1 also showed a diverse sugar utilization capacity. Strain BUPNP1 can tolerate very high PNP concentration (1mM) which is rarely tolerable concentration by other bacteria although 1.8mM was found detrimental for the strain BUPNP1 and thus its use for bioremediation of environmental niches contaminated by PNP is applicable.

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