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Isolation and characterization of Nocardiodies nitrophenolicus capable for degradation of P-Nitrophenol

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Abstract

P-Nitrophenol is the potent contaminant in the environment. We have isolated and characterize the *Nocardiodies nitrophenolicus* strain use for degradation of P-Nitrophenol. Greatest degradation of PNP was observed at 35°C and under slightly alkaline pH (pH 79) conditions. Effective degradation rates slowed as the concentration of PNP was increased. Addition of glucose from 0.1% to 0.5% generally enhanced the degradation of PNP at high concentration (325 mg/l) although acidification as a result of glucose metabolism had a negative effect on PNP depletion. On the basis of 16S rRNA gene sequencing the bacterium is a species of *Nocardiodies*, closely related to *Nocardiodies nitrophenolicus.*

Key words : P-Nitrophenol, 16S r-RNA sequencing, *Nocardiodies nitrophenolicus,* Degradation, Glucose

INTRODUCTION

The wide use of nitroaromatics as synthetic intermediates in the manufacture of pharmaceuticals, pigments, dyes, plastics, pesticides and fungicidal agents, explosives, and industrial solvents[1] leads to the accumulation of nitrophenols[2]. P-Nitrophenol (PNP) is probably the most important among the mono-nitrophenols in terms of the quantities used and potential environmental contamination[3]. Several bacterial strains able to utilize PNP as their sole source of carbon and energy have been documented. These include species of Arthrobacter[4], Bacillus[5], Burkholderia[6] ,Ochrobactrum[7], Pseudomonas[8] , Rhodobacter[9] , Rhodococcus[10] , and Strenotrophomonas[11]. Because the toxicity of high concentrations of PNP inhibits its degradation by most microorganisms[12] , the screening of microbes capable of degrading high concentrations of PNP is becoming a priority research objective in the field of PNP biodegradation. To date, very few bacteria have been documented as exhibiting high PNP tolerance.

MATERIALS AND METHODS

1 Isolation and identification of bacteria

One gram of soil sample, Agricultural soil of North Gujarat, India was used as the inoculum for 50 ml soil enrichment medium supplemented with PNP (5 mg/l final concentration). These culture was incubated at 30°C with agitation (150 rpm) for a week and then transferred into MSM supplemented with 5 mg/l PNP as the sole carbon source. After sub-culturing (5%) in MSM with increasing concentrations of PNP at up to 50 mg/l for six generations, pure cultures were obtained by performing appropriate serial dilutions of the enrichment culture in MSM and plating them onto MSM plates containing 50 mg/l PNP. The isolates which grew fastest and rapidly turned the culture from yellow to colourless were selected for further investigation.

2 Characterization and Identification of the isolate.

2.1 Morphological analysis

A dried fixed smear was covered with oxalate crystal Violet reagent for sixty seconds. The stain was then rapidly washed off with clean water. All the water was then tipped off and the smear was covered with Lugol's Iodine for sixty seconds. The Iodine was then washed off with clean water. The smear was then decolorized rapidly for 10 seconds with acetone alcohol and was washed immediately with clear water. Finally, the smear was covered with Safranine for 30 seconds. The slide was then washed thoroughly in water and blotted dry. The smear was examined under microscope by using immersion oil objective.

2.2 Biochemical analysis

The physiological activities of the selected isolate tested through Oxidase, Catalase Motility Indole, Urease (MIU), Methyl red (MR), Acetone production (Voges-Proskauer), Nitrate reduction, Citrate utilization, Hydrogen Sulfide (H_2S) production, Gelatine liquefaction and carbohydrate fermentation methods as demonstrated by [13].

2.3 Genetic analysis

2.3.1 Characterization of isolate using 16S r-RNA sequencing

2.3.1.1 Culture medium and growth conditions

The pure culture was grown in a 100 ml medium of LuriaBertani broth and incubated in an incubator shaker (120 rpm) at 30 °C for 48 hrs. The pellet of culture was obtained by centrifugation of broth.

2.3.1.2 Genomic DNA extraction from isolate

Genomic DNA was extracted from the isolate using a MEDOX-Bio ™ Ultra pure Genomic DNA Spin Miniprep kit, following the manufacturer's instructions. The cell pellet was re-suspended in 200µl cold TE buffer. 400 µl of Digestion solution was added; mixed and 3 µl of proteinase K solution was added and incubated for 5 minutes. Then 260 µl of ethanol was added and thoroughly mixed. The mixture was applied to column placed in 2 ml of collection tube. The mixture was then centrifuged at 8000 rpm for 1 minute. 500 µl of wash solution was added and spin at 8000 rpm for 1 minute. This step was repeated. The flow through and collection tube was discarded. The mini spin column was carefully removed and placed in 1.5 ml micro-centrifuge tube; 30 µl of elution buffer was added and was incubated for 2 minutes at

room temperature and then centrifuged at 1000 rpm for 1 minute. The DNA sample was then stored at -20 °C and thawed at room temperature as required.

2.3.1.3 Determination of DNA concentration, yield and purity

DNA yield was measured by determining the absorption of the elute at 260 nm wavelength. The purity of the DNA was calculated by the ratio of the absorbance at 260 nm and 280 nm, which provided an estimate of the purity with respect to contaminants that absorb UV light, such as protein. Pure DNA has an A260/A280 ratio of 1.8-2.0. DNA concentration was measured using spectrophotometric reading at 260 nm (Shimadzoo).

2.3.1.4 Amplification of 16S r-DNA by polymerase chain reaction (PCR)

In PCR the isolated DNA was amplified by using 16S r-DNA primers (F-5'AGAGTTTGATCCTGGCTCAG 3' and R-5'AAGGAGGTGATTCCAGCC3'), and by using the Taq and GO PCR master mix; which was obtained from MP Bio. The Master mix contained Taq polymerase, Divalent cations Mg^{+2}, dNTPs. The reaction for 50 µl was prepared according to standard protocol provided by MP Biomedicals for amplification. The isolated DNA was added in range of 100pg-100ng. The DNA was amplified by using Thermal cycler (eppendroff) using parameters (viz:- Denaturation temperature : 94 °C for 20 s, Annealing temperature 55 °C for 40s, Extension temperature 72 °C for 2 min) for 32 cycles. The PCR product was analyzed by gel electrophoresis.

3 Gel Electrophoresis of PCR product and photographing of the gel

Standard electrophoresis protocol was used using a Gel electrophoresis device. PCR product 5µl was mixed with 2µl of gel loading dye. The reaction was loaded in to the well of a 1% agarose gel prepared in TAE buffer. Etbr was added in gel. The gel was photographed using a Gel Documentation System.

3.1 Extraction of PCR Product and DNA sequencing

The PCR Product was extracted by a low melting agarose gel method. Sequencing was carried out by Ocimum Biosolution.

4 Biodegradation of PNP

The inoculums for PNP degradation experiments were prepared by growing bacteria in 50 ml of LuriaBertani (LB) medium[14], supplemented with 50 mg/l PNP and incubating for 36 h at 30 °C on a shaker at 150 rpm. The culture was harvested aseptically. The cells were washed thoroughly with MSM medium contained (mg/l) $MgSO_4.7H_2O$ 120, $ZnSO_4.7H_2O$ 5.0, $Na_2MoO_4.2H_2O$ 2.5, KH_2PO_4 400, Na_2HPO_4 $7H_2O$ 700, $CaCl_2$ 14, $FeSO_4$ 0.13, $(NH_4)_2$ SO_4 500 according to Pesce and Wunderlin (2004)[15] and suspended in sterile MSM as the inoculum. Filter-sterilized glucose solution was added into MSM to different initial concentrations. The degradation of PNP was carried out at different temperature and pH. At appropriate intervals, aliquots of samples were removed to determine periodically the amount of PNP and/or nitrite in the media, and also to evaluate microbial growth. All the experiments were performed in triplicate. Un-inoculated controls were maintained in all experiments. Results are reported as the average of three replicates.

5 Analytical method

PNP was quantified by HPLC (Agilent 1100, Agilent Technologies) conducted at room temperature using Agilent Zorbax 300SB-C18 column and acetonitrile:water 15:85 (water contains acetic acid, 650:1, v:v; pH 3.0) as the mobile phase at a flow rate of 0.75 ml/min. The analysis was performed at 290 nm, column pressure of 71 bar, column temperature at 40°C. Sample volume was 5µl. Nitrite ion was quantified on the basis of the standard curves prepared using sodium nitrite according to the method of Montgomery and Dymock (1961)[16].

RESULTS

1 Identification of isolates

Biochemical characterization and carbohydrate utilization of isolate is indicated in Table-1 and 2. The isolate is gram positive filamentous. The 16S r-RNA partial sequence of isolated organism was aligned by using bioinformatics tool BLAST and it was found that the isolate is showing 99.2% similarity with *Nocardiodies nitrophenolicus and the Accession number of this isolate is* JX564633.

Table 1: Selected biochemical tests of isolate after 48 hours of incubation at 37±0.5ºC

Name of the Tests	Reaction
Gram reaction	+
H_2S production	-
Indole production	-
Methyl Red reaction	-
Voges-Proskauer reaction	-
Citrate Utilization	+
Urease activity	-
Catalase	-
Oxidase	+
Gelatin Liquefication	+
Starch hydrolysis	+
Lipid hydrolysis	+
Motility	+

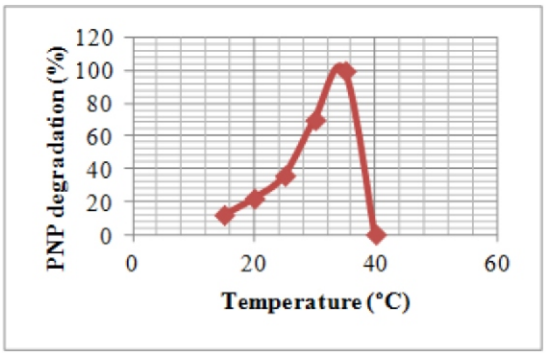

Fig 1: Effect of temperature on PNP degradation by *Nocardiodies nitrophenolicus*

2 Effect of temperature and pH on PNP degradation

The impacts of temperature and pH on PNP degradation were shown in Figs. 1 and 2. PNP degradation occurred at temperatures from 15 to 40ºC (Fig. 1). The greatest release of nitrite was observed at 35ºC. Temperatures over 35ºC or below 20 ºC were unfavourable for PNP degradation (Fig. 2).

PNP degradation was observed over a wide range of pH from 5 to 10 (Fig. 2). The optimal pH for PNP degradation was found to be alkaline (pH 79). A pH lower than 6 or higher than 10 was shown to be less suitable for PNP degradation (Fig. 2).

Table 2: Selected carbohydrate utilization test of isolate after 48 hours of incubation at 37±0.5 ºC.

Carbohydrate test	Reaction
Glucose	+
Maltose	+
Lactose	-
Mannitol	+
Mannose	+
Sucrose	+

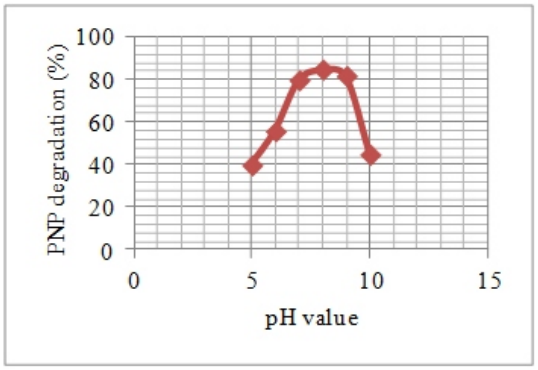

Fig 2: SEffect of pH on PNP degradation by *Nocardiodies nitrophenolicus*

DISCUSSION

PNP is water-soluble, so the main obstacle to PNP degradation is its toxicity. It has been well known that PNP is highly toxic to most microorganisms [9], and that the toxicity of PNP at high concentrations limits its degradation [12]. Microbes with high tolerance to PNP have undoubted advantages for the clearance of high concentrations of PNP. However, to date, few bacteria have been documented to be PNP-tolerant[8]. *Nocardiodies nitrophenolicus* tolerance of PNP up to 325 mg/l, a concentration proven to be toxic to most microorganisms[8], and its ability to degrade PNP effectively over a relatively wide pH range (Fig. 2), make this isolate a realistic candidate for bioremediation.

It has been suggested that the toxicity of PNP increases with a decrease in pH [17] .The initial pH value and the potential range of pH in the processing system are therefore important parameters and must be considered when constructing a PNP remediation system [18]. Our results of PNP degradation at varying pH values suggest that a slightly alkaline pH is favorable for PNP degradation by *Nocardiodies nitrophenolicus* (Fig. 2). Other studies on different bacteria[8] have also reported faster degradation of PNP at high pH, probably due to the increased bioavailability and decreased toxicity of PNP, and optimal metabolic activity of the bacterial cells [19].

Glucose is often used as a carbon source for bacteria in order to enhance their growth and metabolism. We assessed the effect of different glucose concentrations on PNP degradation. Our results show that the addition of glucose at 0.10.5% enhances the PNP degradation. The enhancement effect is not positively related to the glucose concentration, better degradation occurs at 0.1% glucose than that at 0.3% or 0.5%. In contrast,[8] examined the impact of 0.4 g/l glucose on PNP degradation at 300 mg/l by *P. putida* and concluded that the higher concentration of glucose in the minimal medium did not favor PNP degradation. In the presence of glucose, the pH of the medium dropped from pH 7.0 to pH 3.0 within 6 h, resulting in the inhibition of PNP degradation by *P. Putida*[8]. In our biodegradation system, the pH remained at a value greater than pH 6.8 for 36 h, which was favorable for PNP degradation. Although additional carbon source such as glucose usually accelerate PNP biodegradation activity, it is worth noting that the metabolism of additives may acidify the environment, depending on the amount added, and this may contribute to a decrease of PNP degradative efficiency [7].

The findings concerning *Nocardiodies nitrophenolicus* may have direct relevance to bioremediation of PNP and related pollutants in contaminated water. Given that toxicity is the main obstacle to PNP degradation, the addition of compounds which alleviate this may improve PNP degradation.

CONCLUSION

A isolated soil bacterium, *Nocardiodies nitrophenolicus*, which utilizes PNP as a carbon and energy source and which tolerates PNP up to 325 mg/l. The pH affects on the rate of PNP degradation. The addition of glucose from 0.1% to 0.5% enhances the PNP degradation. *Nocardiodies nitrophenolicus* may have direct relevance to bioremediation of PNP and related pollutants in contaminated water.

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