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Isolation and Characterization of a *Pseudomonas aeruginosa* Strain DN1 Degrading p-Nitrophenol

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Abstract: A bacterial strain, DN1, degrading p-nitrophenol (PNP) was isolated from garden soil by selective enrichment in M63 medium. Repeated subculturing in Nutrient Agar (NA) plates, NA slants and Basal Salts Medium (BSM) containing PNP (BSM+ PNP) led to isolation of pure colonies. The organism is Gram negative, aerobic, catalase positive, oxidase positive and rod shaped with mostly single arrangement. It shows bluish green pigmentation on various specialized media such as Pseudomonas P medium, Pseudomonas F medium, Modified F medium, Pseudomonas Isolation Agar (PIA) and HiFluoro Pseudomonas Agar. DN1 gave positive results with motility, citrate utilization, urease, Nitrate Reduction (NR) and gelatin liquefaction tests but negative results with Methyl Red (MR),Voges Proskauer (VP) and indole tests. It was casein hydrolysis and lipase positive but starch hydrolysis negative. Acid production from carbohydrates tested (glucose and lactose) was negative. It can grow at 42°C but not at 4°C and tolerates <5% NaCl concentration. Optimum pH for PNP degradation was found to be 7.0. Among several media tested such as M9, M63 and BSM, BSM was found to be the optimum medium for biodegradation of PNP. DN1 could degrade upto 100 mg L^{-1} PNP using the xenobiotic as sole carbon or carbon and nitrogen sources. On the basis of gross morphological, micromorphological, physiological and biochemical tests DN1 was definitively identified as *Pseudomonas aeruginosa* strain DN1. To our knowledge this is the first report of a *Pseudomonas aeruginosa* strain able to degrade p-nitrophenol (PNP).

Key words: p-nitrophenol, biodegradation, xenobiotics, *Pseudomonas aeruginosa*, isolation, characterization, nitroaromatics

INTRODUCTION

Nitroaromatics are environmentally significant xenobiotics widely used as or in the production of dyes, explosives, pesticides, herbicides, polymers, plasticizers and solvents (Kulkarni and Chaudhari, 2007; Ye *et al*., 2004; Spain, 1995; Marvin-Sikkema and de Bont, 1994; Spain and Gibson, 1991; Bruhn *et al*., 1987). They pose serious health and environmental risks as majority of them are highly toxic to human beings, animals, plants and microorganisms. Several nitroaromatic compounds are powerful carcinogens (Kulkarni and Chaudhari, 2007) and several of them are listed as priority pollutants (US EPA, 2007). Nitroaromatics are important industrial chemicals, with estimated annual production of 10^8 tons.

p-Nitrophenol (PNP) is a nitroaromatic compound widely used as raw material in the manufacture of pesticides, pharmaceuticals and dyes etc. It is also a breakdown product of the degradation of parathion and methyl parathion, organophosphate pesticides widely used as agricultural insecticides in developing countries including India. The US EPA (2007) has listed PNP along with several other nitroaromatics as priority pollutants. Thus biodegradation studies of PNP are of prime importance (Ningthoujam, 2005).

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Microbial degradation of PNP has been reported for several bacteria including strains of *Arthrobacter* and *Nocardia* (Hanne *et al*., 1993), *Arthrobacter* sp. (Jain *et al*., 1994), *Arthrobacter protophormae* (Chauhan *et al*., 2000), *Bacillus sphaericus* (Kadiyala and Spain, 1998), *Brevibacterium linens* (Ningthoujam, 2005), *Moraxella* (Spain *et al*., 1979), *Nocardiodes nitrophenolicus* (Yoon *et al*., 1999), *Pseudomonas* sp. (Munnecke and Hsieh, 1974), *Pseudomonas cepacia* (Prakash *et al*., 1996), *Pseudomonas putida* (Loser *et al*., 1998; Kulkarni and Chaudhari, 2006), *Rhodobacter capsulatus* (Roldan *et al*., 1998), *Sphingomonas* sp. (Zablotowicz *et al*., 1999). Towards the aim of isolating robust and novel PNP degrading strains we have set up enrichment cultures from several pristine and contaminated sites. We have recently isolated a new p-nitrophenol degrading *Pseudomonas* strain DN1 from garden soil by selective enrichment in M63 medium. The present study deals with the isolation and characterization of this PNP degrading strain.

MATERIALS AND METHODS

Enrichment

The bacterial strain was recently isolated from a local garden soil. Soil sample was collected from the Biochemistry Department Campus, Manipur University, Canchipur, India. Aerobic shake flask cultures were set up in M63 medium containing (L^{-1}), 5.8 g Na_2HPO_4, 3.0 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl, with 20 mg L^{-1} PNP. It was adjusted to pH 7.0 and inoculated with filtered soil suspension (10% v/v) derived from 10 g garden soil mixed with 100 mL distilled water and shaken (170 rpm) for 1 h. The enrichment culture was incubated at ambient temperature.

Isolation of PNP Degrading Species

After 2 to 3 months of incubation, 0.1 mL inoculum from the enrichment flask was taken and spread on sterile NA plates and incubated at 30°C (24-48 h). If the growth of the colony was slow, it was kept for further incubation. Visible colonies were then picked and subcultured on NA plates and slants. We obtained four different isolates exhibiting whitish, transparent colourless, white (branching filaments) and bluish green colonies.

On further investigation, the bluish green strain (DN1) was found to be a promising PNP degrader. Degradation was monitored by visible turbidity and/or disappearance of characteristic yellow colour of PNP. PNP depletion was also monitored by following the absorbance of alkalinized culture supernatants at 405 nm (data not shown). The biodegrading strain was then subcultured for several generations in BSM (L^{-1}), 0.25 g $FeCl_3$. $6H_2O$, 22.5 g $MgSO_4.7H_2O$, 27.5 g $CaCl_2$, 40.0 g $(NH_4)_2SO_4$ and Phosphate buffer (pH 7.0) containing various concentrations of PNP. It was maintained on NA slants with or without PNP and on BSM slants with or without PNP.

Morphological, Biochemical and Physiological Tests

Gross morphology was observed by visual inspection. Micromorphology was observed by light microscopy (PRIOR, UK). Motility was determined using hanging drop method (Gunasekaran, 2000). Catalase activity was determined by bubble production in H_2O_2 solution. Oxidase activity was determined by oxidase discs (HiMedia, India) as well as by oxidation of 0.2% 2, 6- Dichlorophenolindolphenol in 0.1% ascorbic acid. Hydrolysis of casein and starch was determined as per standard procedures (Gunasekaran, 2000). Citrate utilization, Indole, MR, VP, NR, Gelatin Liquefaction tests, Urea Broth test for production of urease, Peptonization of Ulrich milk etc. were also determined (Gunasekaran, 2000; Cappuccino and Sherman, 2004). Growth with or without the production of pigmentation was determined with various growth media such as LB (Luria Bertani), Pseudomonas F medium (proteose peptone; 20 g L^{-1}, tryptone; 10 g L^{-1}, K_2HPO_4; 1.5 g L^{-1}, $MgSO_4.7H_2O$; 0.73 g L^{-1}, glycerol; 10.0 g L^{-1}, agar; 15.0 g L^{-1}, pH 7.0), Pseudomonas P medium (Atlas, 1997), Modified F medium (peptone; 20 g L^{-1}, K_2HPO_4; 1.5 g L^{-1}, $MgSO_4.7H_2O$; 1.5 g L^{-1}, agar; 15.0 g L^{-1}, pH 7.2), Pseudomonas Isolation Agar (PIA) [HiMedia (http://www.himedialabs.com),

India], HiFluoro Pseudomonas Agar (HiMedia, India). Growth on succinate medium with or without $FeCl_3$ and acetamide agar medium were also tested to see if this strain can utilize succinate and acetamide as carbon sources with production of greenish blue pigmentation. All other tests, when not specifically mentioned, are as per Bergey's Manual of Determinative Bacteriology and other standard procedures.

Optimization of Medium and Substrate Tolerance Limits

Various culture media (M63, M9 and BSM) were tested to find out the most suitable medium for PNP degradation. For study of substrate tolerance limits, various acclimation studies at different concentrations of PNP were determined with or without added nutritional supplements e.g., Yeast Extract (YE). Effects of various inoculum sizes for further optimization of degradation were also studied.

Nitrite Assay

Nitrite (NO^-_2) liberation during PNP degradation was assayed colorimetrically according to standard protocols (Montgomery and Dymock, 1961; Ningthoujam, 1998).

RESULTS AND DISCUSSION

Morphological, Biochemical and Physiological Tests

DN1 forms round, smooth, bluish green colonies with entire margins and convex elevations. The organism is gram negative, exhibiting rods with mostly single arrangements. The gram negative property was further confirmed by growth on MacConkey's agar and Eosin Methylene Blue (EMB) agar and also positive result in 3% KOH (String) test. It was motile, non-spore former and can grow at 42°C but not 4°C. Various biochemical and physiological tests (Table 1) showed that DN1 is catalase, urease, casein hydrolysis, NR, citrate utilization, gelatin liquefaction, oxidase and lipase positive and starch hydrolysis, lactose fermentation, MR, VP and indole negative. Based on these tests, PNP degrading DN1 isolate is definitively identified as *Pseudomonas aeruginosa* which was further confirmed by culturing it on media specific for *Pseudomonas* species (Table 2) such as Pseudomonas P medium, Pseudomonas F medium, Modified F medium, Pseudomonas Isolation Agar (PIA) medium, HiFluoro Pseudomonas Agar. The organism has been designated as *Pseudomonas aeruginosa* strain DN1. It can use succinate, acetamide and phenol as carbon sources as shown by growth tests with succinate medium, acetamide agar medium and BSM containing phenol.

Table 1: Biochemical and physiological tests of the PNP degrading isolate *Pseudomonas aeruginosa* DN1

Tests	Results
Catalase	+
Casein hydrolysis	+
Starch hydrolysis	-
MacConkey's agar	+ (growth as nonfermenter)
EMB (Eosine Methylene Blue) agar	+
Citrate	+
Motility	+
Glucose fermentation semi solid medium	-
Lactose broth	-
Indole	-
MR	-
VP	-
Gelatin liquefaction	+ (rapid liquefaction)
Urea broth	+
NR	+
Ulrich Milk broth	Deep red (Peptonization)
Oxidase	+
Lipase	+ (showing clear halo)

MR: Methyl Red, VP: Voges Proskauer, NR: Nitrate Reduction

Table 2: Growth on various media

Media	Growth results
Luria-Bertani (LB)	Growth with no pigmentation
Nutrient agar	Growth with bluish green pigmentation
Muller Hinton agar	Growth with bluish green pigmentation
Pseudomonas medium F	Growth with greenish pigmentation
Pseudomonas medium P	Growth with fluorescent bluish green pigmentation
Modified F medium	Growth with fluorescent bluish green pigmentation
Pseudomonas isolation agar	Growth with fluorescent bluish green pigmentation
Acetamide agar	Slow growth with bluish green pigmentation in 24 h
Succinate medium with $FeCl_3$	Growth with slow development of bluish green pigmentation after 24 h
Succinate medium without $FeCl_3$	Growth with much more development of bluish green pigmentation in 24 h
Hi Fluoro Pseudomonas agar	Growth with fluorescent greenish pigmentation

Table 3: Degradation time (h) of 20-100 mg L^{-1}, PNP with and without 0.02% Yeast Extract (YE)

PNP conc. (mg L^{-1})	-YE	+YE
20	15.0	36.0
50	38.0	40.0
100	43.0	66.0

Table 4: Effect of inoculum size on degradation of PNP (20 mg L^{-1})

Inoculum density (%)	Degradation time (h)
1	26.0
2	43.0
5	25.5

Table 5: Nitrite released at different time intervals during complete degradation of 50 mg L^{-1}

Time (h)	NO_2^-N release (μg L^{-1})	%
0	200	3.9
4	360	7.0
8	440	8.7
17	560	11.0
21	880	17.3
25	1000	19.7
37	1280	25.2
41	1720	33.8
45	1760	34.6
49	2760	54.3
62	4560	90.0

Optimization and Substrate Tolerance Limit

Among various culture media tested such as M63 (Gunasekaran, 2000), M9 (Ford *et al*., 1994) and BSM (Ningthoujam, 1998); BSM was found to be the most satisfactory medium for degradation studies. Initial acclimation studies indicated that degradation time for 20-100 mg L^{-1} PNP took about five days to one week. On further acclimation, degradation occurred in 24 to 72 h when PNP was used as sole carbon and energy source (Table 3). That prior exposure to the xenobiotic (PNP) leads to accelerated biodegradation has been shown earlier (Shinozaki *et al*., 2002; Labana *et al*., 2005). To further optimize PNP degradation, studies were undertaken to establish the effects of various inoculum sizes (Table 4) on the degradation rate.

Nitrite Liberation

Nitrite was released almost stoichiometrically during degradation of 50 mg L^{-1} of PNP by DN1 (90% of nitrite were released at 62 h, Table 5 and Fig. 1). Aerobic degradation of PNP is accompanied with oxygenolytic removal of nitro groups in the first or subsequent steps with hydroquinone (HQ) or nitrocatechol (NC) as intermediates of degradation (Spain and Gibson, 1991; Jain *et al*., 1994; Kadiyala and Spain, 1998; Chauhan *et al*., 2000). Present study also confirms oxygenolytic removal of the nitro group during PNP degradation. However, detailed characterization of the degradative pathway awaits further experimentation.

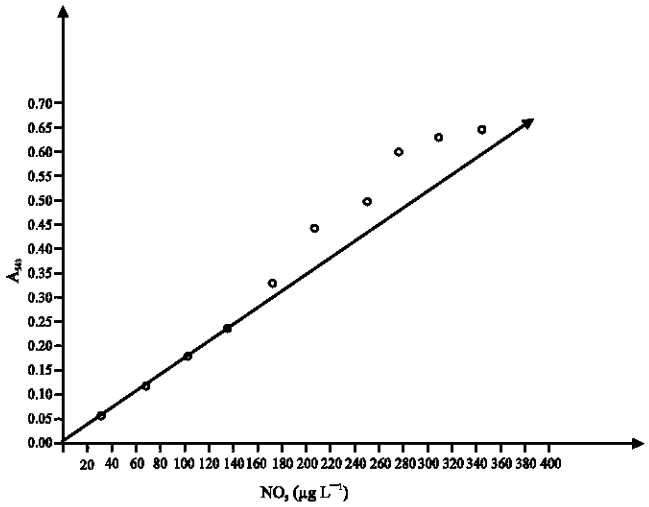

Fig. 1: Standard graph for NO_2^- -N assay

Present studies showed that DN1 can degrade PNP upto 100 mg L^{-1} though there was increasing lag periods corresponding with increasing substrate concentrations. It can use PNP, succinate, phenol and acetamide as C sources but not m-nitrophenol, o-nitrophenol and other higher nitrophenols. It can also use PNP as sole nitrogen source. DN1 thus seems to be an interesting strain of *Pseudomonas aeruginosa* degrading PNP. Though *Pseudomonas aeruginosa* strain has been reported earlier as a PNP degrader in the context of a biodegrading consortium (Daughton and Hsieh, 1977), this is the first report-to our best knowledge of a pure culture of *Pseudomonas aeruginosa* that can biodegrade PNP.

DN1 seems to be a promising addition to the repertoire of PNP degrading microbial isolates and may be a potential agent for biodegradation of nitroaromatic xenobiotics and for possible production of value-added compounds of biotechnological significance (e.g., catechols or siderophores). Studies are now being undertaken to optimize PNP biodegradation and analyze substrate range, optimum pH and metabolic pathways for PNP degradation. Detailed biochemical and genetic characterizations of DN1 will form the target of our further studies, towards the aim of exploiting it for bioremediation or production of biotechnological products (DN1 produces a blue-green siderophore with potential antimicrobial activity, data not shown here).

Further studies on biodegradation of PNP (kinetics, substrate range and xenobiotic tolerance limits etc.) or PNP as part of mixtures with other co-contaminant xenobiotics are necessary as PNP may exist in the environment in various concentration ranges as well as mixtures with other nitroaromatic or non-nitroaromatic xenobiotics.

REFERENCES

Atlas, R.M., 1997. Handbook of Microbiological Media. 2nd Edn. CRC Press, Boca Raton, Florida.

Bruhn, C., H. Lenke and H.J. Knackmuss, 1987. Nitrosubstituted aromatic compounds as nitrogen sources for bacteria. Applied Environ. Microbiol., 53: 208-210.

Cappuccino, J.G. and N. Sherman, 2004. Microbiology: A Laboratory Manual, Pearson Education (Singapore), Indian Branch. New Delhi.

Chauhan, A., A.K. Chakraborti and R.K. Jain, 2000. Plasmid encoded degradation of p-nitrophenol and 4-nitrocatechol by *Arthrobacter protophormae*. Biochem. Biophys. Res. Commun., 270: 733-740.

Daughton, C.G. and D.P.H. Hsieh, 1977. Parathion utilization by bacterial symbionts in chemostat. Applied Environ. Microbiol., 34: 175-184.

Ford, K.G., A.J. Whitmarsh and D.P. Hornby, 1994. Methods in Molecular Biology. Humana Press, Totowa, NJ., 30: 185-198.

Gunasekaran, P., 2000. Laboratory Manual in Microbiology. New Age. International, New Delhi.

Hanne, L.F., L.L. Kirk, S.M. Appel, A.D. Narayan and K.K. Bains, 1993. Degradation and induction specificity in actinomycetes that degrade p-nitrophenol. Applied Environ. Microbiol., 59: 3505-3508.

Jain, R.K., J.H. Dreisbach and J.C. Spain, 1994. Biodegradation of p-nitrophenol via 1, 2, 4-benzenetriol by an *Arthrobacter* sp. Applied Environ. Microbiol., 60: 3030-3032.

Kadiyala, V. and J.C. Spain, 1998. A two-component monooxygenase catalyzes both the hydroxylation of p-nitrophenol and the oxidative release of nitrite from 4-nitrocatechol in *Bacillus sphaericus* JS905. Applied Environ. Microbiol., 64: 2479-2484.

Kulkarni, M. and A. Chaudhari, 2006. Biodegradation of p-nitrophenol by *P. putida*. Bioresour. Technol., 97: 982-988.

Kulkarni, M. and A. Chaudhari, 2007. Microbial remediation of nitro-aromatic compounds: An overview. J. Environ. Manage., 85: 496-512.

Labana, S., O.V. Singh, A. Basu, G. Pandey and R.K. Jain, 2005. A microcosm study on bioremediation of p-nitrophenol-contaminated soil using Arthrobacter protophormiae RKJ100. Applied Microbiol. Biotechnol., 68: 417-424.

Loser, C., M. Ait Oubelli and T. Hertel, 1998. Growth kinetics of the 4-nitrophenol degrading strain *Pseudomonas putida* PNP1. Acta Biotechnol., 18: 29-41.

Marvin-Sikkema, F.D. and J.A.M. de Bont, 1994. Degradation of nitroaromatic compounds by microorganisms. Applied Microbiol. Biotechnol., 42: 499-507.

Montgomery, H.A.C. and F.J. Dymock, 1961. The determination of nitrite in water. Analyst, 86: 414-416.

Munnecke, D.M. and D.P.H. Hsieh, 1974. Microbial decontamination of parathion and p-nitrophenol in aqueous media. Applied Environ. Microbiol., 28: 212-217.

Ningthoujam, D., 1998. Biodegradability of xenobiotics in hazardous wastes using biotechnological routes. Ph.D Thesis, NEERI, Nagpur-440020, India.

Ningthoujam, D., 2005. Isolation and identification of a brevibacterium linens strain degrading p-nitrophenol. Afr. J. Biotechnol., 4: 256-257.

Prakash, D., A. Chauhan and R.K. Jain, 1996. Plasmid encoded degradation of p-nitrophenol by *Pseudomonas cepacia*. Biochem. Biophys. Res. Commun., 224: 375-381.

Roldan, M.D., R. Blasco, F.J. Caballero and F. Castillo, 1998. Degradation of p-nitrophenol by the phototrophic bacterium *Rhodobacter capsulatus*. Arch. Microbiol., 169: 36-42.

Shinozaki, Y., N. Kimura and T. Nakahara, 2002. Differences in degrading p-nitrophenol between indigenous bacteria in a reactor. J. Biosci. Bioeng., 93: 512-514.

Spain, J.C., O. Wyss and D.T. Gibson, 1979. Enzymatic oxidation of p-nitrophenol. Biochem. Biophys. Res. Commun., 88: 634-641.

Spain, J.C. and D.T. Gibson, 1991. Pathway for biodegradation of p-nitrophenol in a *Moraxella* sp. Applied Environ. Microbiol., 57: 812-819.

Spain, J.C., 1995. Biodegradation of nitroaromatic compounds. Annu. Rev. Microbiol., 49: 523-555.

US EPA., 2007. US EPA Environmental Protection Agency. (http://oaspub.epa.gov/wqsdatabase/wqsi_epa_criteria_rep_parameter).

Ye, J., A. Singh and O.P. Ward, 2004. Biodegradation of nitroaromatics and other nitrogen containing xenobiotics. World J. Microbiol. Biotechnol., 20: 117-135.

Yoon, J.H., Y.G. Cho, S.T. Lee, K.I. Suzuki, T. Nakase and Y.H. Park, 1999. *Nocardiodes nitrophenolicus* sp. nov. a p-nitrophenol degrading bacterium. Int. J. Syst. Bacteriol., 49: 675 - 680.

Zablotowicz, R.M., K.T. Leung, T. Alber, M.B. Cassidy, J.T. Trevors, H. Lee, L. Veldhuis and J.C. Hall, 1999. Degradation of 2, 4-dinitrophenol and selected nitroaromatic compounds by *Sphingomonas* sp.UG30. Can. J. Microbiol., 45: 840-848.