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Full Length Research Paper

A study of metabolism of *para*-nitrophenol in *Arthrobacter* sp. SPG

Akash Mukul Jyothi

Department of Environmental Sciences, School of Life Sciences, University of Hyderabad, P.O Central University, Hyderabad-500 046. Email : akash_jyothi@gmail.com

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***Arthrobacter* sp. SPG utilized *p*-nitrophenol (*p*NP) as the sole carbon, nitrogen, and energy source. The stoichiometric amounts of nitrite ions were released from *p*NP. The pathway of *p*NP degradation was studied for strain SPG. On the basis of thin layer chromatography, high performance liquid chromatography and gas chromatography-mass spectrometry, hydroquinone (HQ) was identified as major intermediate product. HQ dioxygenase activity was found in crude extract of *p*NP induced cells of strain SPG that suggested the cleavage of HQ into gamma-hydroxymuconic semialdehyde. This study clearly showed that strain SPG degraded *p*NP via HQ pathway. *Arthrobacter* sp. SPG degrades various nitroaromatic compounds including *p*NP, 2-chloro-4-nitrophenol, 3-methyl-4-nitrophenol, 2-nitrocatechol and 2-nitrobenzoate. This strain would be a suitable candidate for bioremediation of nitroaromatic compounds contaminated sites.**

Keywords: *Arthrobacter* sp. SPG, Biodegradation, Bioremediation, hydroquinone, metabolism, *p*-nitrophenol, nitroaromatic compounds, sole carbon and energy source

INTRODUCTION

p-nitrophenol (*p*NP) is an environmental pollutant that is used for the manufacturing of dyes, explosives, pesticides, herbicides and drugs (Spain, 1995). It is also a hydrolytic product of the pesticide parathion (Munnecke and Hsieh, 1974). It may cause a blood disorder that reduces the ability of the blood to carry oxygen to tissues and organs due to methemoglobinemia (ATSDR, 1992). It is a potent uncouple of oxidative and photosynthetic phosphorylation. Further, it is also considered as mutagen (Cooper et al., 1997). Due to its high toxicity, United State Environmental Protection Agency (USEPA) has listed it as a priority pollutant.

Bacteria that use *p*NP as sole carbon and energy source have been isolated and characterized (Mitra and Vaidyanathan, 1984; Hanne et al., 1993; Spain, 1995; Wan et al., 2007). Aerobic degradation of *p*NP was occurred either formation of hydroquinone (Spain and Gibson, 1991) or formation of nitrocatechol (Kadiyala and Spain, 1998). Previously, it has been supposed that Gram-positive bacteria degrade *p*NP via nitrocatechol pathway whereas Gram-negative utilize *p*NP via hydroquinone pathway. Literature studies showed that both Gram-positive and Gram negative bacteria can degrade *p*NP either via hydroquinone pathway or via nitrocatechol pathway (Jain et al., 1994; Hanne et al., 1993; Spain, 1995; Pakala et al., 2006; Chauhan et al., 2007). In this communication, we reported the degradation of *p*NP via hydroquinone pathway by *Arthrobacter* sp. SPG

MATERIALS AND METHODS

Isolation Of A *pNP* Degrading Bacterium

Strain SPG was isolated from the soil collected from a pesticide contaminated site, India by enrichment method using 4NP as a substrate. Strain SPG utilized *p*NP as sole carbon and energy source. Strain SPG was screened to find out its degradation capacity for various

nitroaromatic compounds. For screening, strain SPG was streaked on minimal agar plates containing 0.3 mM test compound as sole carbon and energy source. Minimal agar plates were prepared by dissolving the followingcompounds in 100 mL of double distilled water: 0.4 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.08 g $(NH_4)_2SO_4$, 0.08 g $MgSO_4.7H_2O$, 0.1 mL trace element solution and 1.8 g agar. The composition of trace element solution was exactly same as described previously (Arora and Jain, 2011a). The media was autoclaved at 15 lbs for 20 min. After autoclave, the desired concentration (0.3 mM) of the filter sterilized test compound was added to the media and media was allowed to cool at room temperature and poured into petri plates. 2-Nitrophenol, 2-chloro-4-nitrophenol, 3-methyl-4-nitrophenol, 3-nitrophenol, 2-amino-4-nitrophenol, 4-nitrocatechol and 2-nitrobenzoate were used as test compounds. Decolourization and/or growth of strain SPG on minimal agar plates was/were considered as positive results.

Identification Of Bacteria

Strain SPG was identified by the 16S rRNA gene sequencing using the universal primers as described previously (Arora et al., 2011).

Growth Of Strain SPG On PNP

Strain SPG was grown on 1L Erlenmeyer flask containing 250 mL minimal media and 0.3 mM *p*NP as sole carbon and energy source. The flask was incubated at 30°C under shaking condition at 200 rpm. Samples were collected at regular intervals and growth of microorganism was measured by taking optical density at 600 nm. For estimation of nitrite release, samples were centrifuged at 8000 rpm and supernatant was used for nitrite detection by classical method (Spain and Gibson, 1991).

Identification Of Metabolites

Samples collected at different intervals were centrifuged and supernatants were extracted with ethyl acetate. Extracted samples were analyzed by thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS).

TLC was performed using pre-coated silica gel 60 F_{254} plates (20 X 20 cm, 0.25 mm; Merck, Germany) with solvent (toluene: ethyl acetate: glacial acetic acid, 60:30:5). The compound(s) was/were visualized under UV radiation and sprayed with Folin-Ciocalteu's reagent. HPLC analysis was carried out using a Waters 600 model high performance liquid chromatography (HPLC) equipped with a photodiode array detector system. The compounds were separated on a C_{18} reverse-phase silica column using 1% glacial acetic acid in methanol and 1% glacial acetic acid in HPLC grade water at a ratio of 80:20 as the mobile phase. Flow rate was 1.0 mL/min; injection volume was 15 µL, and the compounds were detected at 280 nm and 300 nm.

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 Da. The column temperature was initially increased from 90°C to 180°C at the rate of 5°C/min and then from 180°C to 280°C at the rate of 10°C/min. The carrier gas (nitrogen) flow rate was 10 mL/min.

CRUDE EXTRACT PREPARATION

Strain SPG was grown on 200 mL minimal media, 10 mM sodium succinate and 0.3 mM *p*NP. Cells of strain SPG were centrifuged just prior to decolourization and washed twice with phosphate buffer (20 mM, 7.4 pH) and resuspended in the same buffer. The cells were sonicated in a sonicator by twenty 30s burst with intermittent 30s cooling on ice. The cell extracts were centrifuged (12000 rpm) at 4°C for 15 minutes to remove cell debris and the supernatant was used for enzyme assay. Protein contents were estimated according Bradford method (Bradford, 1976).

Enzyme Assay

Hydroquinone dioxygenase activity was determined by measuring decreased the absorbance of substrate at 289 nm and increased the absorbance of product at 320 nm. The reaction mixure contanined (in a final volume of 1mL) 20 mM phosphate buffer, 0.1 mM hydroquinone, 0.1 mM ferrus sulphate and 0.5-1.0 mg crude extract of the proteins.

RESULTS

Isolation And Identification Of Strain SPG

A *p*NP degrading bacterium, strain SPG was isolated from a pesticide contaminated site, India and utilized following compounds as sole carbon and energy source:

*p*NP, 2-chloro-4-nitrophenol (2C4NP), 2-nitrobenzoate, 3-methyl-4-nitrophenol and nitrocatechol strain SPG was

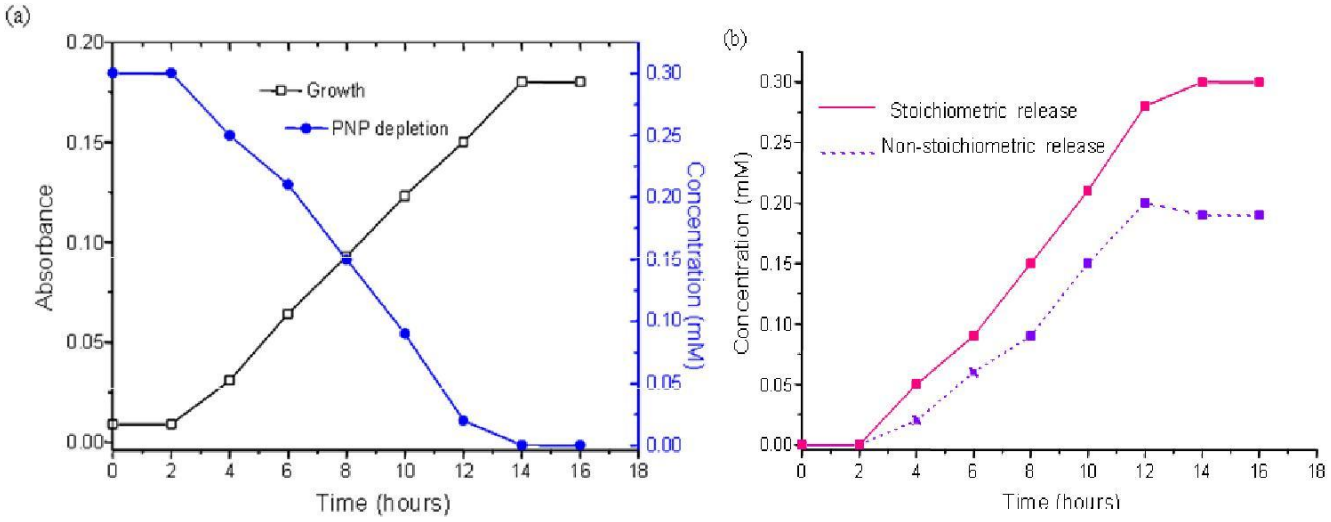

Figure 1. (a) Growth of the strain SPG on *p*NP and depletion of the *p*NP by strain SPG. (b) Stoichiometric release of nitrite ions when strain SPG grown on minimal salt media and 0.3mM *p*NP and non-stoichiometric release of nitrite ions when strain SPG grown on nitrogen free minimal media and 0.3mM *p*NP.

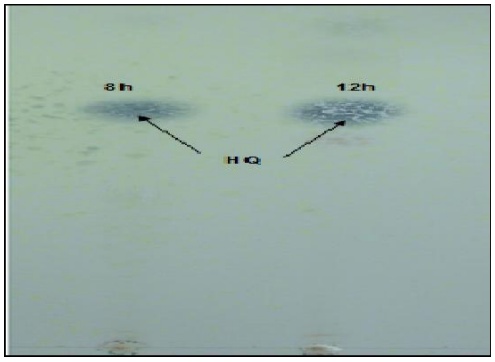

Figure 2.TLC analysis of samples of *p*NP degradation by *Arthrobacter* sp. SPG. TLC showing the presence of hydroquinone in blue colour spots after spraying with Folin-Ciocalteu's reagent.

identified as *Arthrobacter* sp. on the basis of the 16S rRNA gene sequencing. The 16S rRNA gene sequence of strain SPG has been deposited in the

Growth Of Strain SPG On *p*NP:

When strain SPG was grown on minimal media containing 0.3 mM *p*NP as sole carbon and energy source, the yellow color of *p*NP (0.3 mM final concentration) changed to colourless indicating its utilization by the microorganism. Microbial growth was measured by the increased in optical density (OD) at 600 nm and depletion of *p*NP was measured by taking the OD at 420 nm (Figure. 1a). The microorganism released nitrite ions in the medium, suggesting the involvement of an initial oxidative step in the degradation of *p*NP. The stoichiometric amounts of nitrite ions was observed when strain SPG was grown on minimal media containing *p*NP as sole carbon and energy source. When strain SPG was grown on nitrogen free minimal media (without GenBank database under the accession number HM027882.ammonium sulphate) containing 0.3 mM *p*NP, non-stoichiometric release of nitrite ions was observed (Figure. 1b). This is due to the rapid utilization of nitrite ions as a nitrogen source in the absence of ammonium sulphate. This data suggested that strain SPG utilize *p*NP as sole carbon, nitrogen and energy source.

Metabolic Pathway For *p*NP Degradation:

TLC analysis indicated the presence of HQ (Rf 0.65) and *p*NP (Rf 0.75) in samples drawn from 8 and 12 h intervals. However, the samples drawn after 16 hours growth did not show the presence of *p*NP or another intermediate indicating the whole degradation of *p*NP. Furthermore, when TLC plates were sprayed with Folin-Ciocalteu's reagent an immediate blue coloration was apparent in case of suspected HQ spot since dihydroxylated benzene gives an immediate blue coloration with this reagent (Figure 2). HPLC analysis also showed the presence of HQ along with *p*NP in the samples drawn from 8 and 12 h period (Figure. 3).

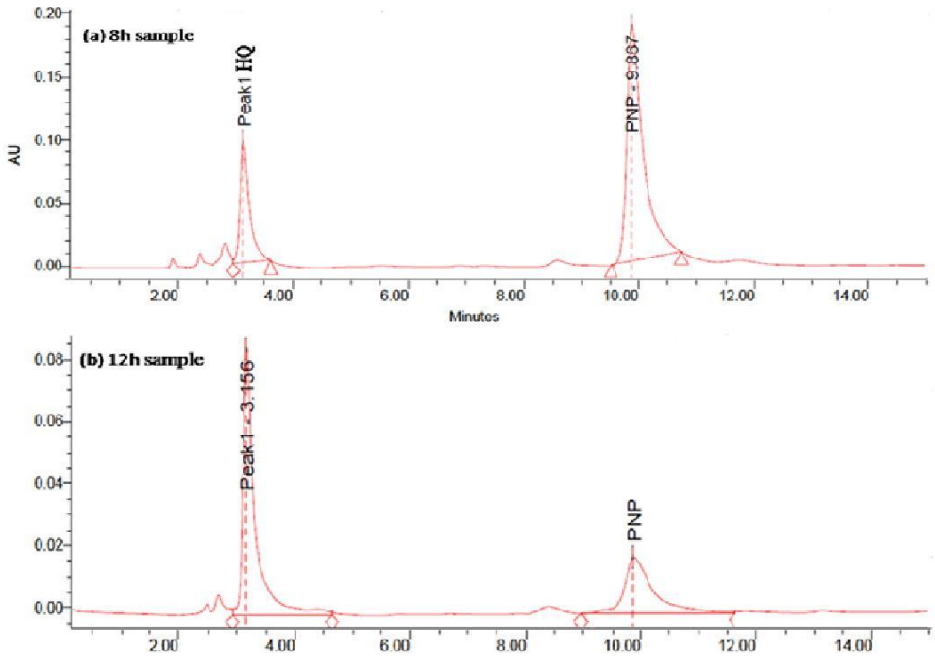

Figure 3. HPLC analysis of samples showing degradation of *p*NP by *Arthrobacter* sp. SPG with appearance of intermediate hydroquninone.

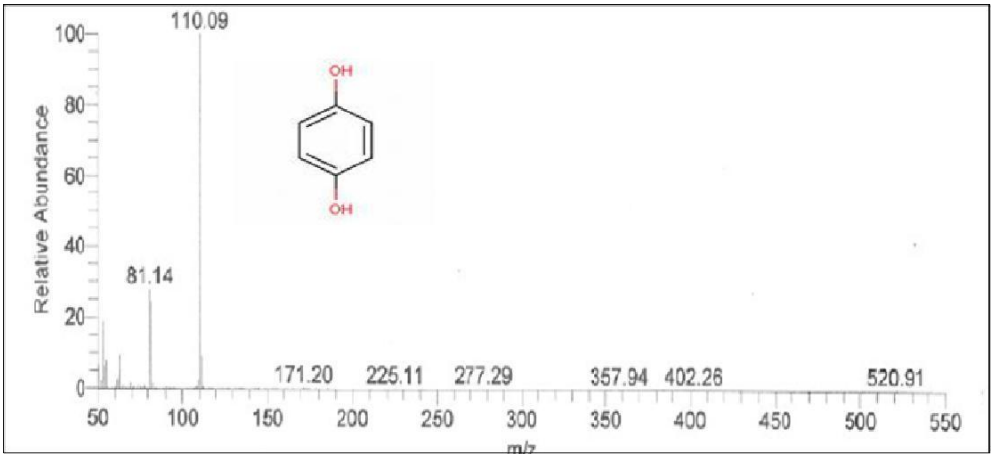

Figure 4. Mass fragement of metabolite hydroquinone.

However, no *p*NP or HQ peaks were observed in 16 h drawn samples, again suggesting the whole degradation of *p*NP. GC-MS analysis was also carried out to confirm the presence of HQ in the degradation pathway of *p*NP. GC-MS(Figure 4). showed that mass fragment of the metabolite was observed at 110 m/z that was exactly matching that of the standard HQ (Figure. 3). Inhibition studies were also carried out using 2,2'-dipyridyl since the presence of this compound in the growth mediumchelates ferrous ions required for the ring-cleavage of aromatic compounds. Strain SPG was grown on minimal media containing *p*NP, sodium succinate and 2,2'-dipyridyl and samples were taken at regular interval and extracted with ethyl acetate and extracted samples were analyzed by TLC and HPLC. Results of this experiment showed that there was accumulation of HQ even after 48 h of growth,

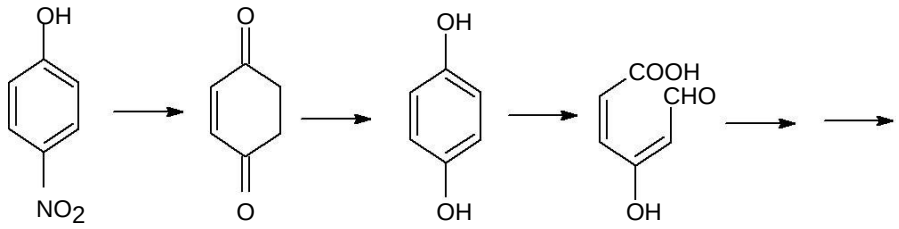

Figure 5. Proposed pathway of *p*NP degradation by *Arthrobacter* sp. SPG

suggesting the inhibition of the ring cleavage of HQ (data not shown). To identify the ring cleavage product, enzyme assay for HQ dioxygenase activity was carried out. The spectrophotometric analysis of HQ dioxygenase assay showed that the peak of the HQ at289 nm disappeared gradually and a transient peak of 4-hydroxymuconic semialdehyde around 320 nm appeared.

DISCUSSION

Arthrobacter sp. SPG is a versatile bacterium that able to degrade various nitroaromatic compound including *p*NP, 2C4NP, 3-methyl-4-nitrophenol, nitrocatechol and 2-nitrobenzoate. This strain would be a suitable candidate for bioremediation of a nitroaromatic contamitaned site.

Several members of the genus *Arthrobacter* are known to degrade phenolic compounds. *Arthrobacter chlorophonolicus* A6 was characterized to degrade mixture of phenol, *p*NP and 4-cholrophenol (Westerberg et al., 2000). *Arthrobacter ureafaciens* CPR706 has been reported to degrade 4-chlorophenol via HQ pathway (Bae et al., 1996). The degradation of phenol by immobilized cells of *Arthrobacter citrus* has also been achieved (Karigar et al., 2006). *Arthrobacter* sp. SJCon utilized 2-chloro-4-nitrophenol as the sole carbon and energy source (Arora and Jain, 2011b). Furthermore,

Arthrobacter protophormiae RKJ 100 (Chauhan et al, 2007), *Arthrobacter* sp. JS443 (Jain et al., 1994), and *Arthrobacter aurescens* TW17 (Hanne et al., 1993) have been reported to degrade *p*NP. This study also report degradation of *p*NP by *Arthrobacter* sp. SPG.

The members of genus *Arthrobacter* degrade *p*NP either by HQ pathway (Jain et al., 1994) or nitrocatechol pathway (Chauhan et al., 2007). In nitrocatechol pathway, *p*NP was converted to nitrocatechol by monooxygenation at ortho position. Nitrocatechol was further converted to 1,2,4-benzentriol (BT) by monooxygenation at para position with release of nitrite ions (Chauhan et al., 2007). BT then cleaved into maleylacetate by ring cleavage activity of BT dioxygenase (Chauhan et al., 2007). In HQ pathway, *p*NP was first converted to 1,4-benzoquinone (BQ) by a monooxygenase and BQ was further reduced to hydroquinone (HQ) that was further cleaved to gamma-hydroxymuconic semaldehyde by HQ dioxygenase (Jain et al., 1994). This study clearly showed that *Arthrobacter* sp. SPG degraded *p*NP via HQ pathway (Figure. 5).

CONCLUSION

A *p*NP degrading bacterium *Arthrobacter* sp. SPG was isolated from soil collected from a pesticide contaminated site. Strain SPG utilized *p*NP as sole carbon, nitrogen and energy source and degraded it via formation of HQ. Strain SPG was also able to degrade 2-chloro-4-nitrophenol (2C4NP), 2-nitrobenzoate, 3-methyl-4-nitrophenol and nitrocatechol. This strain may be used for bioremediation of nitroaromatic compounds contaminated sites.

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REFERENCES

Arora PK, Chauhan A, Pant B, Korpole S, Mayilraj S, Jain RK (2011). Description of *Chryseomicrobium imtechense* gen. nov., sp. nov., a new member of the family Planococcaceae. Int J Sys Evol Microbiol 61:1859–1864.

Arora P K, Jain RK (2011a). Biotransformation of 4-chloro-2-nitrophenol into 5-chloro-2. methylbenzoxazole by a marine *Bacillus* sp. strain MW-1. Biodegradation., doi: 10.1007/s10532-011-9512-y.

Arora PK, Jain RK (2011b). Pathway for degradation of 4-chloro-2-nitrophenol by *Arthrobacter* sp. SJCon. Curr Microbiol, 63:568-573.

ATSDR (1992). Toxicological Profile for Nitrophenols: 2-Nitrophenol, 4-nitrophenol, US DHHS, PHS, Agency for Toxic Substance and Disease Registry (ATSDR), Atlanta, GA.

Bae HS, Lee JM, Lee ST (1996). Biodegradation of 4-chlorophenol via a hydroquinone pathway by *Arthrobacter ureafaciens* CPR706, FEMS Microbiol Lett, 45:125-129.

Bradford MM (1976). Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem, 72: 248–254.

Chauhan A, Chakraborti AK, Jain RK (2007). Plasmid-encoded degradation of p-nitrophenol and 4-nitrocatechol by *Arthrobacter protophormiae*. Biochem Biophys Res Commun, 270:733-740.

Cooper AR, Overholt L, Tillquist H, Jamison D (1997). p-nitrophenol . In Cooper's Toxic Exposures (A. R. Cooper, L. Overholt, H. Tillquest, and D. Jamison, Eds.), CRC Press, Inc, New York, NY, pp. 1610–1619.

Hanne LF, Kirk LL, Appel SM, Narayan AD, Bains KK (1993). Degradation and induction specificity in Actinomycetes that degrade p-nitrophenol. Appl Environ Microbiol, 59, 3505-3508.

Jain RK, Dreisbach JH, Spain JC (1994). Biodegradation of p-nitrophenol via 1,2,4-benzenetriol by an *Arthrobacter* sp. Appl Environ Microbiol, 60:3030-3032.

Kadiyala V, Spain JC (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. Appl Environ Microbiol, 64: 2479-2484.

Karigar C, Mahesh A, Nagenahalli M, Yun DJ (2006). Phenol degradation by immobilized cells of *Arthrobacter citreus.* Biodegradation, 17:47-55.

Mitra D, Vaidyanathan CS (1984). A new p-nitrophenol 2-hydroxylase from a *Nocardia* sp. Biochem Int. 8:609-615.

Munnecke DM, Hsieh DP (1974). Microbial decontamination of parathion and p-nitrophenol in aqueous media. Appl. Microbiol, 28:212-217.

Pakala SB, Gorla P, Pinjari AB, Krovidi RK, Baru R, Yanamandra M, Merrick M, Siddavattam D (2006). Biodegradation of methyl parathion and p-nitrophenol: evidence for the presence of a p-nitrophenol 2-hydroxylase in a Gram-negative *Serratia* sp. strain DS001. Appl Microbiol Biotechnol, 73:1452-62.

Spain JC, Gibson DT (1991). Pathway for biodegradation of p-nitrophenol in *Moraxella* sp. Appl. Environ. Microbiol, 57:812-819.

Spain JC (1995). Biodegradation of nitroaromatic compounds. Annu Rev Microbiol, 49: 523-555.

Westerberg K, Elväng AM, Stackebrandt E, Jansson JK (2000). *Arthrobacter chlorophenolicus* sp. nov., a new species capable of degrading high concentrations of 4-chlorophenol. Int J Syst Evol Microbiol, 6:2083-2092.

Yamamoto K, Nishimura M, Kato D, Takeo M, Negoro S (2011). Identification and characterization of another 4-nitrophenol degradation gene cluster, nps, in *Rhodococcus* sp. strain PN1. J Biosci Bioeng, 111:687-94.