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Biodegradation of 2,6-dinitrotoluene and plant growth promoting traits by *Rhodococcus pyridinivorans* NT2: Identification and toxicological analysis of metabolites and proteomic insights

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ABSTRACT

2,6-dinitrotoluene (2,6-DNT), one of the major priority pollutants, is a common isomer produced during 2,4,6-trinitrotoluene (TNT) synthesis and also frequently used in production of herbicides, dyes, and synthetic foams. While 2,6-DNT may be degraded relatively rapidly under batch liquid culture conditions, very limited biodegradation details have been reported particularly at high initial DNT concentrations. In this study, *Rhodococcus pyridinivorans* NT2 was subjected to 0.5–1.6 mM of 2,6-DNT and shown to grow on 2,6-DNT as a sole carbon and nitrogen source. Although strain NT2 tolerated high concentrations of 2,6-DNT (1.6 mM in 108 h), maximum biodegradation was observed at 0.54 mM of 2,6-DNT (within 48 h) that was described well by both first-order and pseudo-first-order reaction kinetics (R^2, 0.927). Biodegradation of 2,6-DNT suggested a reductive metabolic pathway with the formation of 2-methyl-3-nitroaniline and 2,6-diaminotoluene. A nitroreductase catalyzing nitroreduction of 2,6-DNT was detected in the cell lysate of strain NT2. Phytotoxicity (with *Triticum aestivum* and *Vigna radiata*), cytogenotoxicity (with *Allium cepa* root-tip cells), and microbial toxicity (using *Escherichia coli* DH5α) studies were performed to evaluate the toxicity of metabolites produced after degradation of 2,6-DNT. Besides, strain NT2 possessed important plant-growth promoting traits, both in the presence and absence of 2,6-DNT. Furthermore, proteomic characterization using nano LC-MS/MS identified a total of 516 proteins, of which 75 were up-regulated. These proteins were involved in 2,6-DNT degradation (oxidation-reduction systems related to nitroreductase-like proteins), transport processes, carbon and energetic metabolism, transcription/translational changes and stress tolerance, shedding light on the complexity of 2,6-DNT catabolism by strain NT2.

1. Introduction

The unique and versatile chemistry of the nitro group has led to the use of nitroaromatic compounds in the manufacturing of pharmaceuticals, dyes, pesticides, herbicides, explosives, etc. (Nishino and Spain, 2004; Singh and Ramanathan, 2013; Ye et al., 2004). 2,6-dinitrotoluene (2,6-DNT), for instance, is produced as a major by-product during the synthesis of 2,4,6-trinitrotoluene (TNT) and used as precursor of toluene diisocyanate in the manufacture of polyurethane foams (Lendenmann and Spain, 1998; Zhang et al., 2000). The release of these compounds into the environment is often through accidental spills, industrial waste discharge, military ammunitions, agricultural runoff and/or from other anthropogenic sources (Singh and Ramanathan, 2013). 2,6-DNT is extremely recalcitrant to biological degradation and thus persists in the environment for a very long time. Moreover, 2,6-DNT exhibit acute toxicity, low level carcinogenicity and aquatic toxicity. The 14-day LC_{50} (guppy, *Poecilia reticulata*) and US Environmental Protection Agency (EPA) drinking water as well as industrial waste streams treatment standards are 98 μM, 0.22 μM, and 3.08 μM for 2,6-DNT, respectively (Han et al., 2011; Lendenmann and Spain, 1998). Hence it is listed as priority pollutant by the US EPA (Nishino and Spain, 2004; Shin et al., 2005).

In general, DNTs are removed from the environment abiotically and biotically, for example by volatilization, photolysis, and biodegradation (Gupta and Bhaskaran, 2004; Nipper et al., 2004; O'Sullivan et al., 2010; Prak et al., 2013). Microbial degradation of DNTs is known to occur through both oxic and anoxic conditions (Rieger and Knackmuss, 1995; Nishino et al., 2000). In the oxidative reactions, bacterial monooxygenase or dioxygenase produces a derivative of methyl nitrocatechchol with the loss of one of the nitro groups. The reductive reactions result in the accumulation of aminotoluenes which are catalyzed by oxygen-sensitive or

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insensitive type of non-specific nitroreductases (Ju and Parales, 2010; Nishino et al., 2000; Shin et al., 2005). However, whether the compounds were biodegraded or only transformed remains unclear in many studies because several unidentified metabolites were found (Lendenmann and Spain, 1998). In addition, this knowledge alone seems to be insufficient to explain the low conversion rates of DNTs observed at polluted sites. DNTs biodegradation studies previously reported were conducted in the range of 50 μM to 1 mM (Han et al., 2011). Information regarding the effectiveness and sustainability of DNTs biodegradation at higher concentrations is rather limited because incomplete degradation of 2,6-DNT was observed after inoculation, and low-level degradation activity could not be sustained without repeated bioaugmentation (Zhang et al., 2000).

Several reports on model organisms exist where genomic, transcriptomic, and proteomic tools have been used to explain the mechanism of tolerance to toxic compounds. Till present, most literature data identified only a small subset of predicted proteins in model organisms and these studies are largely confined to xenobiotics like tributyltin, bisphenol A, nonylphenol, polychlorobiphenyl, toluene, ethylbenzene, xylene, benzoate, benzene, cyclohexane, aniline, alachlor, phenanthrene and phthalate (Mujahid et al., 2015; Słaba et al., 2015; Szewczyk et al., 2014; Vandera et al., 2015). However, as yet, proteomic studies underlying mechanisms of toxicity and survival strategy induced by DNTs are largely unknown.

In this study, we report reduction of nitro group of 2,6-DNT to amino group at the ortho position on benzene ring by a previously characterized *Rhodococcus pyridinivorans* strain NT2 (Kundu et al., 2013, 2015a). It could efficiently degrade 2,6-DNT at high concentrations with concomitant production of methylnitroaniline (an intermediate in manufacturing of dyes and pigments) which has possible commercial applications. Strain NT2 possessed in vitro plant growth promoting traits even in the presence of 2,6-DNT and the metabolites formed in biodegradation were less toxic than parent compound. By employing a gel-free quantitative proteomic analysis using nano LC-MS/MS, this study also provides significant details of proteomic changes observed in NT2 cells grown on 2,6-DNT.

2. Materials and methods

2.1. Chemicals, reagents and bacterial strain

2,6-DNT [$CH_3C_6H_3(NO_2)_2$, CAS#606-20-2, 98%] was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). 2-amino-6-NT, 2,6-diaminotoluene and acetone were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were procured from HiMedia, Mumbai (India). All chemicals used were of analytical grade. The bacterial strain (*R. pyridinivorans* NT2) was previously isolated from effluent-sediment of a pesticide manufacturing plant (Kundu et al., 2013). *R. pyridinivorans* NT2 was grown in mineral salt basal (MSB) medium (pH 7.0 ± 0.2) (Kundu et al., 2013) supplemented with filter-sterilized 2,6-DNT (100 mg l^{-1}, i.e. 0.54 mM from stock solution in acetone), unless otherwise mentioned. Acetone was removed by evaporation prior to the addition of the aqueous medium. The biomass was harvested by centrifugation (10,000 rpm for 10 min) and washed twice with sterile MSB medium. The washed cells were then suspended in assay medium to a final density of OD_{600}=0.5 (corresponding to 1.6 mg cdw l^{-1}; cdw: cell dry weight).

2.2. Biodegradation studies

Growth and 2,6-DNT utilization kinetics of strain NT2 was determined in batch flasks at a 2,6-DNT concentration of 0.54 mM. This concentration range was selected based on our preliminary study on mono- and di-nitrotoluenes (Kundu et al., 2015a). A stock solution of 2,6-DNT was prepared by dissolving the required mass of 2,6-DNT in acetone. An appropriate aliquot of filter sterilized (0.2 μm nylon filter) stock solution was transferred into sterile 500 ml conical flasks and the flasks were left for a few hours in the fume hood sealed with cotton wool in order to evaporate the solvent. Liquid MSB medium (100 ml; pH 7.0 ± 0.2) was added after complete evaporation of the solvent. 2,6-DNT grown NT2 strain taken from mid-exponential phase was harvested by centrifugation, washed with 100 ml MSB, and finally, resuspended in 2,6-DNT (0.54 mM) supplemented MSB to obtain a final $0.5OD_{600}$. The flasks were then incubated in a rotary shaker (120 rpm) set at 30 °C. The entire biodegradation experiment for 100 mg l^{-1} (0.54 mM) of 2,6-DNT was monitored for 72 h unless otherwise mentioned. Flasks containing cultures without 2,6-DNT acted as biotic controls and uninoculated flasks containing 2,6-DNT served as an abiotic control. Also, media inoculated with dead cells were used as control by autoclaving at 1×10^5 Pa for 30 min. The degree of 2,6-DNT degradation was calculated from residual substrate in the culture medium using the formula:

$$\text{Degradation efficiency } (\%) = \left(\frac{C0 - Ct}{C0}\right) \times 100 \quad (1)$$

where, C_0 is the initial concentration of 2,6-DNT in the medium and C_t is the concentration at time *t*. 2,6-DNT-supplemented culture samples were removed at periodic intervals for cell growth quantification using a spectrophotometer (UV-1601 Shimadzu, Japan) at OD_{600}.

2.3. Effect of environmental factors on 2,6-DNT degradation

Various abiotic environmental factors influencing bacterial growth and degradation of 2,6-DNT were investigated. These included pH (5, 6, 7, 8, and 9), temperature (20, 25, 30, 35, 40, 45, and 50 °C), salinity (5, 10, 15, 25, and 30 g l^{-1} of NaCl), initial 2,6-DNT concentration (1, 1.2, 1.4, 1.6, 1.8, and 2 mM) and presence of additional substrate (glucose or carboxylic acids at 0.1 and 1.0 g l^{-1}). All experiments were carried out in triplicates.

2.4. 2,6-DNT extraction, quantification and metabolites identification

In order to identify 2,6-DNT catabolism products, aliquots were withdrawn at fixed time intervals during degradation studies, appropriately diluted and biomass was removed by centrifugation at 10,000 rpm. HPLC-grade ethyl acetate was blended with the culture supernatant at a ratio of 1:0.5 and vortex-mixed for 2 min. It was followed by centrifugation at 10,000 rpm for 15 min to separate the aqueous and organic phase. The upper aqueous phase was discarded by pipetting and the combined organic extract obtained from repeated extractions was passed through anhydrous sodium sulfate. Subsequently, ethyl acetate was evaporated to dryness and the extracted samples were dissolved in an equivalent volume of HPLC grade methanol and subsequently analyzed by thin layer chromatography (TLC) on silica gel G plates using the solvent system toluene-ethylacetate-acetic acid (60:30:5, v/v). The metabolites were visualized under ultraviolet (UV) light (at 254 nm) (Mulla et al., 2011). The metabolites were also analyzed by HPLC (Shimadzu, Japan) equipped with SPD-10AVP UV-Detector at 254 nm. Injected sample volume was 20 μl for every run. Analytes were separated by using silica gel-packed C_{18} column [dimension: 4.6 mm (ID) × 250 mm (l)] of particle size (5 μm) (Phenomenex). The mobile phase used was methanol/water (60:40, v/v) with 0.1% TFA in water and the flow rate was fixed