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Enhancement of 2,4-dinitrotoluene biodegradation by *Burkholderia* sp. in sand bioreactors using bacterial hemoglobin technology

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Abstract

Continuous flow sand column bioreactor experiments were conducted to investigate the effect of 2,4-dinitrotoluene (DNT) concentration (i.e. DNT loading rate) and influent dissolved oxygen (DO) concentration on aerobic biodegradation of DNT by wild type (DNT) and recombinant (YV1) *Burkholderia* sp., the latter containing plasmid pSC160 which carries the gene (*vgb*) encoding the hemoglobin (VHb) from the bacterium *Vitreoscilla* . The experiments were conducted in two continuous flow packed bed sand column bioreactors, one growing the wild type strain and the other growing YV1. Under oxygen-rich feed conditions (6.8 mg DO/L in the feed) with an influent DNT concentration of 99.6 mg/L (DNT loading rate $\cong$ 9.2 mg/m^2/day), the effluent DNT concentration from the wild type bioreactor reached 0.7 mg DNT/L in 40 days whereas it was less than 0.2 mg DNT/L for the YV1 bioreactor in about 25 days. When influent DNT concentration was increased to 214 mg/L (DNT loading rate $\cong$ 20.3 mg/m^2/day) while maintaining the same influent DO level of 6.8 mg/L, the effluent DNT concentration increased to about 5 mg/L for the wild type bioreactor whereas it was maintained at less than 0.2 mg/L for the YV1 bioreactor. Additionally, when influent DO was reduced from 6.8 mg/L to 3.1 mg/L while the influent DNT concentration remained at 214 mg/L, the effluent DNT concentration increased to more than 20 mg/L for the wild type bioreactor but up to only 1.7 mg/L for the YV1 bioreactor. A subsequent increase of influent DO back to 6.6 mg/L reduced the effluent DNT concentration to about 5 mg/L for the wild type bioreactor and to 0.10–0.19 mg/L for the YV1 bioreactor. These results confirm the utility of *vgb* technology to enhance biodegradation of aromatic compounds under hypoxic conditions and also that this enhancement can be maintained over extended periods of time as evidenced by plasmid stability in *Burkholderia* YV1.

Introduction

Two key aspects of soil and groundwater bioremediation still challenge its full potential, namely, identification and isolation of microorganisms capable of degrading specific contaminants, and ability to provide nutrients such as N, P, and dissolved oxygen (DO) to the microorganisms in the subsurface environment. The use of genetic engineering to produce bacteria capable of degrading specific contaminants has been receiving strong research interest as a way to increase the efficiency of bioremediation (Esteve-Nunez et al. 2001). Once the initial bacteria are found, the mechanisms of contaminant degradation are determined, and the specific enzymes responsible for degradation are isolated. With the amino acid sequence of the enzyme known, the gene encoding the enzyme can be isolated and transferred to heterologous hosts. Alternatively, since sequence data from the genetic material of many different types of bacteria has been collected into DNA databases, these databases can be screened for homologous enzymes, thereby allowing new organisms to be discovered that have the potential ability to degrade the desired contaminants. By selection of a particular species of bacteria that uses a favorable mechanism, the end products of degradation can also be controlled.

Furthermore, in soil and groundwater bioremediation, the combination of low oxygen solubility and mass transport limitations in the soil pores increases the cost of providing DO to the bacteria. Mechanical methods such as aeration or bioventing are often used to supply oxygen into the subsurface, but these methods are limited to shallow depths to be cost effective (Billings et al. 1995; and Lords et al. 1995). Hydrogen peroxide, which readily breaks down to water and oxygen is also used as an oxygen source. Low levels of H_2O_2 are insufficient for aerobic biodegradation due to poor oxygen solubility in water, and high levels of H_2O_2 inhibit microbial growth by acting as an inhibitor under unacclimated conditions. Hence, aerobic bioremediation of the subsurface environment occurs under hypoxic conditions, and is therefore very slow. One way of overcoming this problem could be by developing aerobic bacteria capable of better growth and organic contaminant breakdown under hypoxic conditions. In fact, there are known naturally occurring bacteria that have the capability to survive and grow under hypoxic conditions such as in soil and high altitude conditions. The bacteria, *Vitreoscilla*, are strict aerobes but have a tendency to grow in oxygen-poor environments (Webster 1987). They have adapted the mechanism, novel for bacteria, to grow under these conditions by synthesizing a bacterial hemoglobin (VHb) in response to hypoxic conditions (Webster 1987; Dikshit et al. 1992). The putative function of hemoglobin is to transfer and transport oxygen to the membrane terminal oxidases for respiration and metabolism, particularly under oxygen limited conditions (Ramandeep et al. 2001; Park et al. 2002). Recent evidence suggests that the presence of hemoglobin in heterologous hosts also facilitates efficient supply of oxygen to monoxygenases and dioxygenases in the degradation pathway of aromatic compounds (Urgun-Demirtas et al. 2003).

Attempts have been made to overcome the oxygen limitation problem by genetically engineering microorganisms to enhance their survival, growth and ability to degrade aromatic compounds under oxygen limiting conditions (Liu et al. 1995, 1996; Fish et al. 2000; Patel et al. 2000; Nasr et al. 2001; Urgun-Demirtas et al. 2003). Specifically, success has been achieved by transformation of the gene (*vgb*) encoding VHb into *Burkholderia* sp. to enhance DNT degradation. This is thought to be due to the VHb-related increase in oxidative phosphorylation, and thus ATP production, and perhaps direct stimulation of the DNT catabolic pathway. This pathway requires oxygen addition at three steps to break the aromatic ring structure (Spanggord et al. 1991). This strategy of enhancing oxygen supply by VHb has also shown promise in the degradation of benzoic acid by *P. aeruginosa* (Liu et al. 1996) and 2-chlorobenzoic acid by *Burkholderia* (Urgun-Demirtas et al. 2003). To this point, the work with *vgb*/VHb in *Burkholderia* has been performed in liquid medium, such as in shake flasks and chemostat reactors. In order to establish the potential of this system for engineered bioremediation, however, it is necessary to perform experiments with bioreactors under conditions that approximate those in the field. To that end in the research reported here, we have tested the ability of *vgb*/VHb to enhance DNT biodegradation in continuous flow sand column bioreactors, particularly under low oxygen conditions and for extended periods of time.

Methods and materials

Bacterial strains and plasmid

Wild type *Burkholderia* sp. (formerly known as *Pseudomonas*) strain DNT was obtained from J. Spain of Tyndall Air Force Base, FL (Spanggord et al. 1991) in March, 1995. A pUC8 recombinant plasmid bearing the hemoglobin gene (*vgb*) from the bacterium *Vitreoscilla* strain C1 (Dikshit & Webster 1988) was cloned into the EcoRI site of vector pKT230 to produce plasmid pSC160 (Liu et al. 1995). Plasmid pSC160 confers resistance to both ampicillin and kanamycin. *Burkholderia* sp. strain DNT was transformed with pSC160; the transformant is denoted as YV1. Both strains DNT and YV1 were maintained on agar plates that contained DNT (100 mg/L) and tryptic soy broth (TSB). The agar plates with YV1 also contained 40 mg/L of kanamycin and 100 mg/L of ampicillin to ensure the plasmid stability of YV1.

Chemicals

Kanamycin and ampicillin were from Sigma Chemical Co. (St. Louis, MO). Yeast extract was from Difco Laboratories (Detroit, MI). Mineral salts medium consisted of the following chemicals (in grams per liter): K_2HPO_4, 0.7; KH_2PO_4, 0.3; $(NH_4)_2SO_4$, 0.5; $NaCl{\cdot}7H_2O$, 0.05; $CaCl_2{\cdot}2H_2O$, 0.1; $FeSO_4{\cdot}7H_2O$, 0.003; and 1.0 ml of trace elements solution. The trace elements solution contained the following (per liter): 0.1 g of H_3BO_3, and 0.05 g (each) of $CaSO_4{\cdot}5H_2O$,

$ZnSO_4 \cdot 7H_2O$, and $Na_2MoO_2 \cdot 6H_2O$. All other chemicals were obtained from Fisher Scientific (Fair Lawn, NJ).

Batch experiments

To investigate the biodegradability of DNT as the sole carbon source by strains DNT and YV1, batch experiments with three different concentrations of DNT were conducted. First, 1.2 liter of 217 mg/L DNT solution was prepared in three 2 liter Kimax glass reactors. Each reactor was sealed with a rubber stopper with three openings. A glass pipe (5 mm inner diameter) was installed in each opening. Two of them were used for inflow and outflow of air. Air was supplied into the batch reactor headspace at 5 psi and surface turbulence created by magnetic mixing facilitated the oxygen transfer to the liquid. To prevent contamination, two Whatman membrane filters (0.2 μm pore size $\times$ 20 mm diameter) were installed in series at the air inflow tube. Air outlet tubes were submerged in a 70% H_2O_2 solution to inspect the airflow in the reactors and to prevent contamination. The one remaining opening was used to collect samples. Reactors were autoclaved for 30 minutes at 121 °C prior to inoculation.

Each of strains DNT and YV1 was acclimated for 72 hours in 400 ml solution containing 100 mg/L of DNT and 1000 mg/L of yeast extract in the Purifier Class II Safety Cabinet (Labconco Corp., Kansas City, MO) at 25 $\pm$ 2 °C. The solution for YV1 contained antibiotics (40 mg/L kanamycin and 100 mg/L ampicillin) to ensure the growth of YV1 only. After acclimation, each solution was poured into a 50 ml tube separately and centrifuged for 10 minutes at 3000 rpm. The supernatant solution was discarded and then each reactor was inoculated with the contents of one tube. The reactor that was not inoculated was used as a control reactor. Samples were collected periodically (18–25 hours) and analyzed for dissolved organic carbon (DOC) and DNT. The experiment was repeated with two different DNT concentrations (i.e. 55 mg/L and 110 mg/L) in addition to the 217 mg/l tried earlier following the same procedures as described above.

Sand column preparation

Two polycarbonate columns containing well-characterized (size distribution and specific surface area) sand were prepared to serve as bioreactors. Each column measured 45 cm in height, 2.54 cm in inner diameter, and 187 ml in volume. Medium-grade sand was obtained from a local hardware store, and the grain sizes passing through US No. 10 sieve (2 mm Ø) and retained on No. 40 sieve (0.6 mm Ø) were used in the sand column reactors. The sand was water cleaned and ashed at 550 °C to remove attached organic matter. The particle size analysis of sand was conducted according to the American Standard for Testing and Material (ASTM D 421; D 422, 1991), and based on the sand particle size analysis, the specific surface area of sand was determined. Total sand weight in the column bioreactor was 329 g for strain DNT and 327 g for YV1, while the specific surface area was found to be about 22.7 cm^2/g sand for both columns.

The sand was well mixed and poured into each column until it was full. The final porosity of the sand in the column was determined to be 0.3, with a total pore volume of 70 ml in the sand media. Both ends of the column were sealed with polycarbonate rod screws and were installed with polycarbonate fittings. Rubber rings were installed with polycarbonate rod screws to prevent any leakage. Sand columns were placed vertically and two layers of wire nets (Fisher Scientific, Fair Lawn, NJ) with 20 $\times$ 20 mesh count were installed at the bottom of each column to retain the sand. Columns filled with sand were autoclaved at 121 °C for 30 minutes prior to the experiment.

Experimental set-up and procedure

The experimental setup for the column bioreactors is shown in Figure 1. There were two differences between the two setups. First, prior to entering the bioreactor, the influent solution for YV1 passed through a 30 ml column where a mixture of ampicillin (100 mg/L) and kanamycin (40 mg/L) solution was injected during the initial stage of the experiment up to 25 days to ensure the plasmid stability in YV1. Second, the effluent from the YV1 bioreactor passed through a 25 ml column where the samples were collected for the Most Probable Number (MPN) test to investigate the stability of pSC160 in YV1 with time. All other experimental conditions, such as influent concentration of DO, DNT and yeast extract, flow rate, and reactor volume were identical for both columns. Both bioreactors were fed from the same feed stock bottle. The bottle containing feed stock solution was fitted with a rubber stopper with 5 openings. Two openings were used for inflow and outflow of air/nitrogen to control dissolved oxygen concentration in the feed stock solution, and the other two openings were used to feed solution to both bioreactors. The remaining one was used for withdrawal of

stock solution to measure DO. Additionally, an air inlet equipped with a 0.2 μm cartridge filter allowed supply of air into each bioreactor from the inlet side of the bioreactor. The effluent pump was operated at a higher speed than the feed pump to draw air into each bioreactor. To prevent contamination, three Whatman membrane filters (0.2 μm pore size $\times$ 20 mm diameter) were installed in series at the air/nitrogen inflow tubes into the feed bottle. The air/nitrogen outlet tube was submerged in H_2O_2 solution to observe the air/nitrogen flow in the reactor and to prevent contamination. Therefore, the DO to the column bioreactors was supplied via the influent and via direct addition to from the inlet air tube.

The continuous flow sand column bioreactor experiments were conducted according to the following procedure. Strains DNT and YV1 were grown in chemostat reactors with mineral salts medium and 1,000 mg/L of yeast extract at a dilution rate of 0.5 day^{-1} for 6 days. The chemostat reactors were operated at near neutral pH (7.0 $\pm$ 0.1) and at 25 °C. Once both strains reached a steady state condition (change in the biomass concentration less than 10% over 3 days), the contents of the chemostats were pumped over 5 days from the inlet side into the respective soil columns. The sand column bioreactors were operated in a 25 °C temperature-controlled laboratory.

Following the inoculation of strains DNT and YV1 into soil columns, stock solution (mineral salts medium with 1,000 mg/L yeast extract) containing various concentrations of DNT (i.e., 99.6, 148.8, or 214 mg DNT/L) was pumped through each column at a rate of 71 ml/day. All stock solutions were autoclaved at 121 °C for at least 1.5 hours prior to feeding the column. The pH and temperature of the stock solution were maintained at 7.0 and 25 °C, respectively. To change and maintain the DO concentration in the stock solution, air/nitrogen mixture was fed into the feed stock bottle at 5–10 psi and the surface turbulence created by magnetic mixing was relied on to transfer oxygen into liquid. The purpose of nitrogen was to reduce the DO concentration when the stock solution had higher than desired influent DO level.

Analytical methods

High Pressure Liquid Chromatography (HPLC) analysis with a Spherosorb C18 column (Alltech, Deerfield, IL) was performed to measure DNT concentration; acetonitrile and trifluoroacetic acid (13.5 mM) constituted the mobile phase. The isocratic elution was 50% acetonitrile and 50% trifluoroacetic acid over a period of 6 minutes with a flow rate of 1.5 ml/min. All compounds were detected at 230 nm with an HP1040A diode array detector (Hewlett-Packard Corp., Palo Alto, CA). Dissolved oxygen was measured with a YSI 5100 DO meter (YSI Inc., Yellow Springs, OH). The dissolved organic carbon (DOC) measurements were made using a Dohrmann 80 total organic carbon (TOC) analyzer (Dohrmann, Santa Clara, CA).

Results and discussion

DNT as the sole carbon source

Previous studies suggested that DNT can be biodegraded only by co-metabolic processes (Vanderloop et al. 1999; Boopathy et al. 1998; Maloney et al. 1998; Cheng et al. 1996; Noguera & Freedman 1996, 1997). However, other studies also suggested that *Burkholderia* sp. can degrade DNT as the sole carbon source (Nishino et al. 2000; Spanggord et al. 1991). Therefore, a series of batch experiments was conducted to investigate whether strains DNT and YV1 can utilize DNT as the sole carbon source. In order to understand co-metabolic DNT biodegradation by strains DNT and YV1, initial experiments were conducted with DNT as the sole carbon source. The DNT concentrations were 55 mg/L, 110 mg/L, and 217 mg/L. Since DNT was the only substrate that contributes to the carbon source, DOC was measured to quantify the remaining DNT.

Spanggord et al. (1991) reported that when *Burkholderia* sp. was inoculated in a solution containing mineral salts with DNT as the sole carbon source, 100 μM of DNT was completely degraded in 120 minutes. However, these cultures were maintained in media containing yeast extract and could have contained intracellular stored carbon. More recent studies also suggested that both 2,6-DNT and 2,4-DNT can be biodegraded as the sole carbon source by *Burkholderia* sp. (Nishino et al. 2000), but these strains were identified much later than the cultures used in this research. It can be seen from Figure 2 that no degradation of DNT occurred over 6 days of the experiment by either strain DNT or YV1 when DNT was the sole carbon source; thus, it was predicted that additional carbon source may enhance aerobic DNT degradation by *Burkholderia* sp. by co-metabolism. It was shown previously that carbon sources such as yeast extract, succinate, casamino acids, and tryptic soy broth could

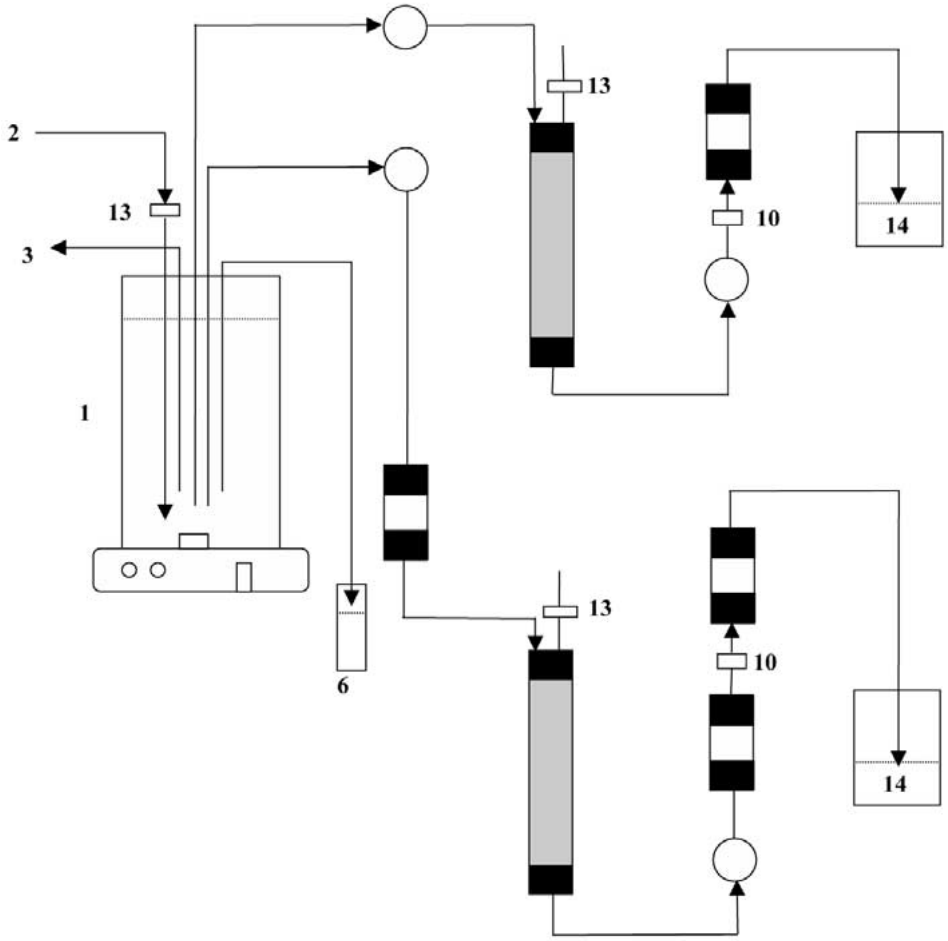

Figure 1. Schematic Diagram of Sand Column Experiment: (1) feed stock bottle; (2) air/nitrogen inlet; (3) dissolved oxygen sampling syringe; (4) magnetic stirrer; (5) magnetic bar; (6) air/nitrogen outlet; (7) antibiotic injection column; (8a) sand column bioreactor for strain DNT; (8b) sand column bioreactor for strain YV1; (9) sample collection column for MPN test; (10) filter; (11) DO column; (12) peristaltic pumps; (13) air filter; (14) effluent.

enhance DNT degradation by both strains DNT and YV1 (Nasr et al. 2001). When yeast extract was used as primary substrate, both strains were able to degrade DNT up to 217 mg/L; the rate of DNT degradation increased with the yeast extract concentration from 220 to 2,000 mg/L. However, there was no significant difference in DNT degradation rate at yeast extract levels higher than 1,000 mg/L. The summary of DNT levels degraded and the corresponding yeast extract concentrations for the work reported here is presented in Table 1. Therefore, in the subsequent continuous flow sand column bioreactor experiment, 1,000 mg/L yeast extract was added as a primary substrate for cometabolic degradation of DNT.

Sand column bioreactor

The influent DO concentration in the feed stock of the bioreactors ranged from 3.1 to 6.8 mg DO/L. The DNT concentration was varied from 99.6 mg/L to 214 mg/L (corresponding DNT loading rates were determined to be 9.2 to 20.3 mg/m^2/day). The effluent DNT concentrations of the bioreactors are shown in Figure 3. Initial influent DNT concentration was 99.6 mg/L (DNT loading rate $\cong$ 9.2 mg/m^2/day) and the initial influent DO concentration was 6.8 mg/L. The effluent DNT concentration increased from about 1 mg/L to more than 20 mg/L in both bioreactors during the initial period of the experiment. This is likely due to the initial adsorption of DNT on the sand medium, and subsequent breakthrough into the effluent. After about

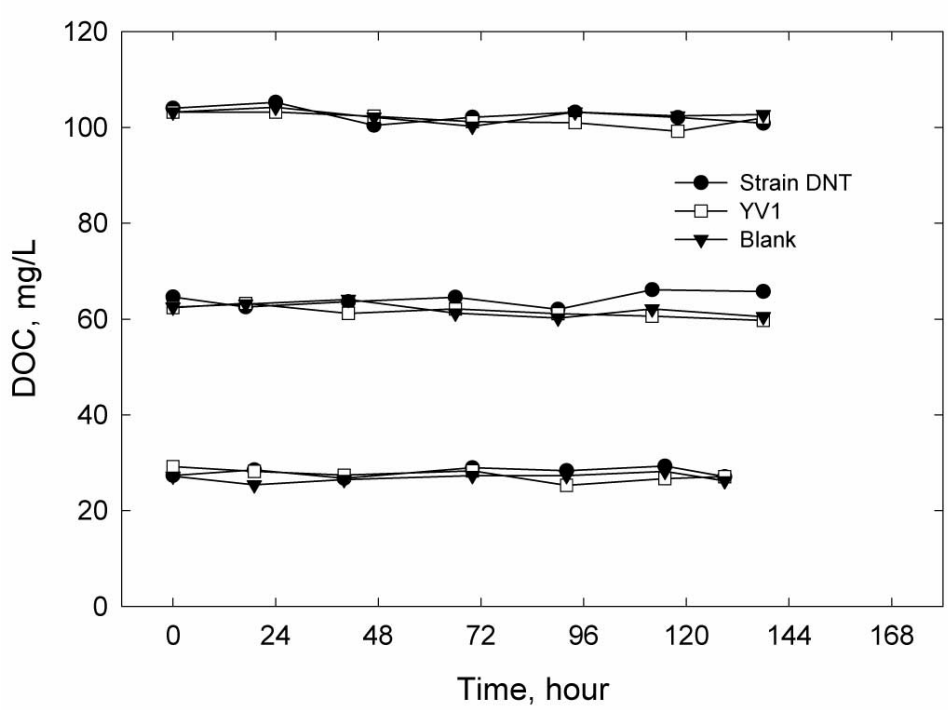

Figure 2. Degradation of DNT by *Burkholderia* sp. (DNT and YV1) with DNT as the Sole Carbon Source. Initial DNT concentrations in the Batch Reactor were: (a) 55 mg/L, (b) 110 mg/L, (c) 217 mg/L.

Table 1. Yeast extract requirements for degradation of DNT by cometabolism

Initial concentration mg/l	DNT removed mg/l	Total DOC removed mg/l	DOC contributed by DNT mg/l	DOC contributed by yeast extract mg/l	Yeast extract DOC/DNT-DOC ratio mg/mg
DNT = 198 YE = 1000	172	230	78	152	1.95
DNT = 198 YE = 1700	197	380	91	289	3.2
DNT = 217 YE = 2000	215	430	99	331	3.4

Note: DNT was measured directly by HPLC, and DNT-DOC was calculated assuming complete DNT mineralization.

25 days, the effluent DNT concentration in the YV1 bioreactor decreased to 0.08–0.19 mg/L; the strain DNT bioreactor, however, took over 40 days to reach 0.7 mg/L. On the 41st day, the influent DNT concentration was increased to 148.8 mg/L (DNT loading rate $\cong$ 14.0 mg/m^2/day), while the influent DO concentration was kept constant at 6.8 mg/L. Under these conditions, the YV1 bioreactor effluent DNT concentration continued between 0.10–0.18 mg/L, while that of the strain DNT bioreactor increased to between 0.7 mg/L and 4.1 mg/L. On the 52nd day, with the influent DO concentration maintained at 6.8 mg/L, the influent DNT concentration to both bioreactors was further increased to 214 mg/L, corresponding to a DNT loading rate of 20.3 mg/m^2/day. The effluent DNT concentration increased over the next few days from less than 0.18 mg/L to as high as 3.7 mg/L for YV1 and from 0.7 mg/L to 8.2 mg/L for strain DNT.

Subsequently, YV1 produced effluent with 0.11–0.16 mg/L DNT while strain DNT could reduce efflu-

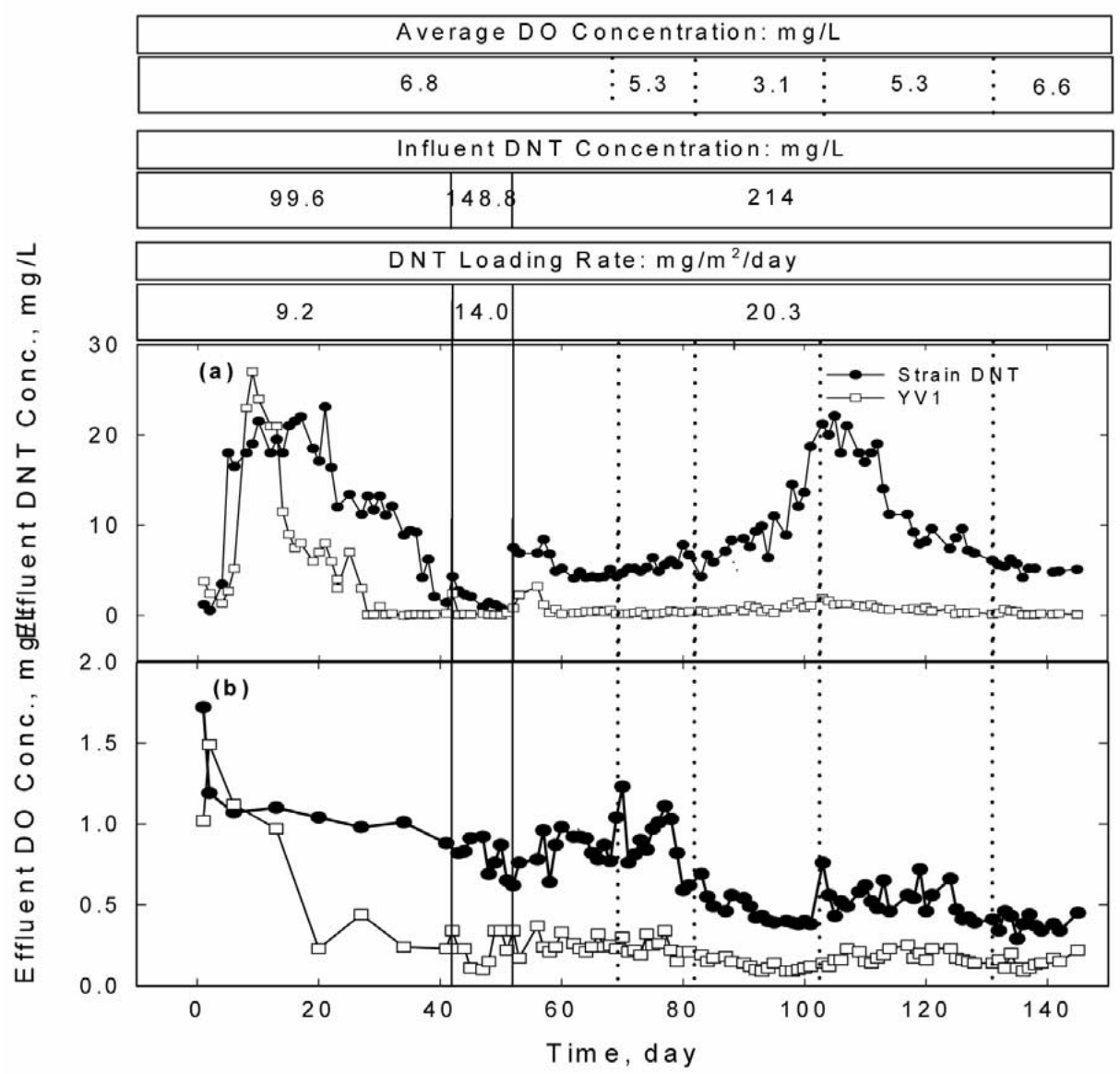

Figure 3. (a) Effluent DNT concentration as a function of time. (b) Effluent dissolved oxygen concentration as a function of time.

ent DNT down to no less than 4.7 mg/L. These results demonstrate that YV1 is more effective than the wild type strain DNT in terms of DNT degradation over a range of DNT concentrations when the DO levels are kept constant and at the same level for both strains.

In order to determine the influence of influent feed DO concentration on degradation of DNT by the two strains, the influent DO was decreased from 6.8 mg/L to 5.3 mg/L on day 68 while keeping the influent DNT concentration at 214 mg/L. The effluent DNT concentration of the YV1 bioreactor did not show any significant change whereas DNT concentration increased to about 8 mg/L for the strain DNT bioreactor. When influent DO concentration was further reduced to 3.1 mg/L on the 82nd day, the effluent DNT of the strain DNT bioreactor increased to over 20 mg/L whereas that of the YV1 bioreactor increased only up to 1.7 mg/L. Thus, as the influent DO decreased, strain DNT was unable to degrade DNT to the lower levels it reached when influent DO was at high levels; hence YV1 has a particular advantage over the non-recombinant strain regarding DNT degradation at

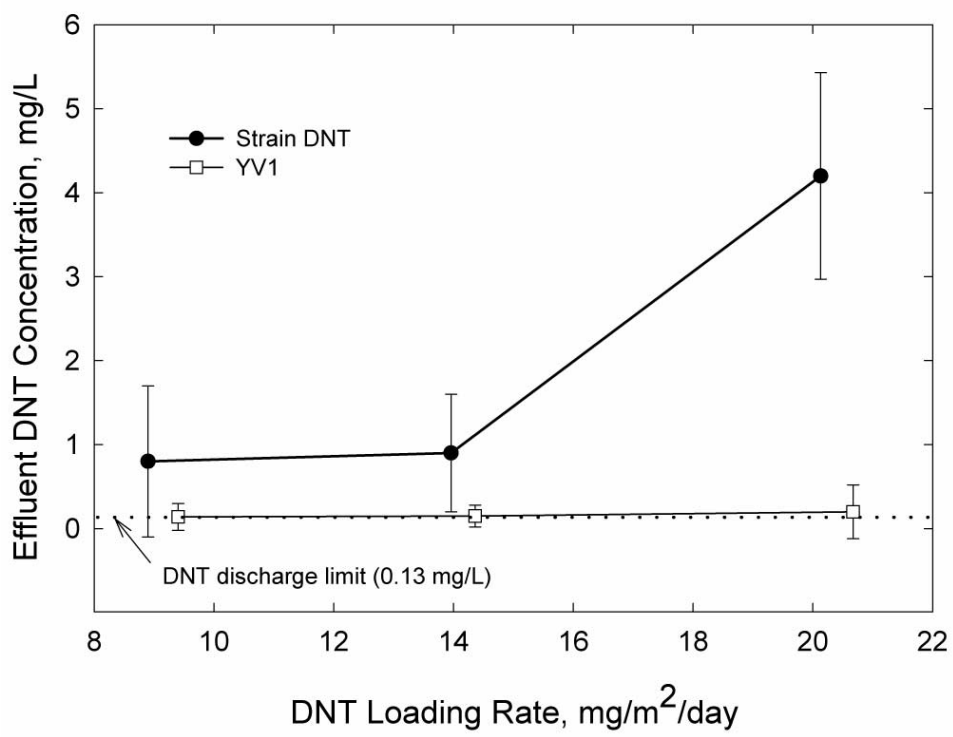

Figure 4. Effluent DNT concentration as a function of DNT loading rate (influent dissolved oxygen concentration at 6.8 mg/L).

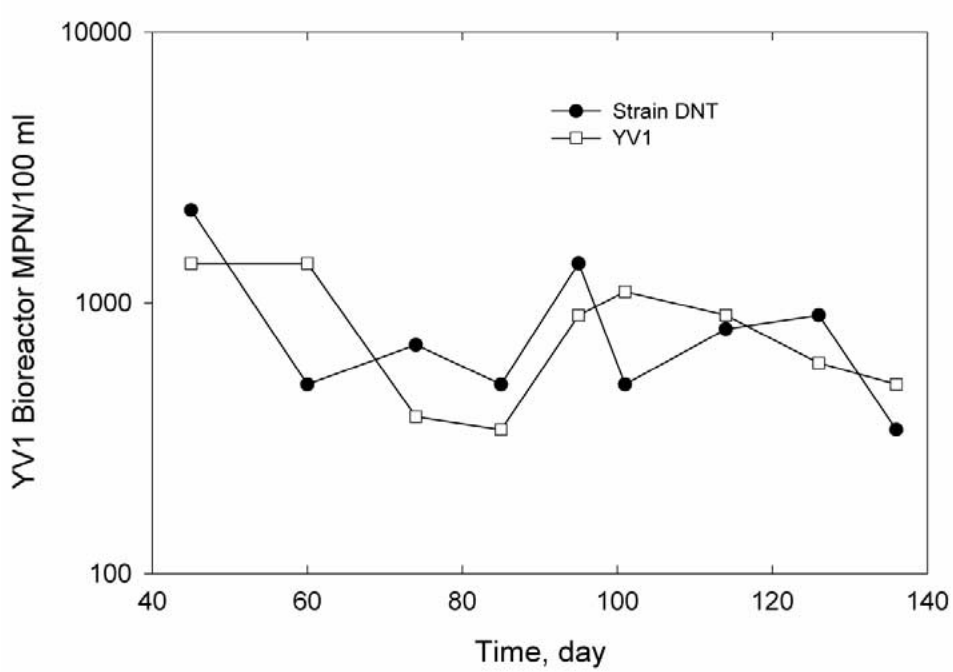

Figure 5. Most Probable Number (MPN)/100 ml of YV1 as a Function of Time (the series with no antibiotics (total cells) and kanamycin + ampicillin (pSC160 bearing cells) are the same, indicating that all cells are plasmid-bearing).

low influent DO and high DNT concentrations. When influent DO was subsequently increased back to 5.3 mg/L (day 102) and 6.6 mg/L (day 131), the effluent DNT concentration of the strain DNT bioreactor decreased to about 5 mg/L, whereas that of the YV1 bioreactor decreased to about 0.11–0.19 mg/L. The results from day 102 onwards confirm that influent DO level influences the effluent DNT concentration, possibly due to the effect on the oxygenases. They also confirm that YV1 is significantly better than strain DNT in degrading DNT over a range of influent DO levels, but especially at lower influent DO.

The effluent DO concentrations of the two bioreactors are shown in Figure 4. Here the difference between the two strains in terms of oxygen utilization can be clearly seen. YV1 bioreactor effluent DO was about 0.2–0.3 mg/L, whereas, the strain DNT bioreactor effluent contained much higher DO levels (about 0.3 to 1.2 mg/L). The ability of YV1 to utilize DO efficiently at low levels, and correspondingly to degrade DNT to much lower levels is presumably due to the presence of VHb in YV1. It is thought that the role of VHb is to uptake and transport oxygen in the cell, increasing ATP produced by oxidative phosphorylation, thus leading to greater cell growth and production of catabolic enzymes (Webster 1987; Fish et al. 2000). Another possibility is that the increase in oxygen supply may also directly enhance the oxygen-requiring pathways of aromatic compound catabolism by providing increased oxygen to the oxygenase reactions (Liu et al. 1996; Stark et al. 1999; Urgun-Demirtas et al. 2003).

Regarding the effluent DNT concentration, both DNT loading rate and influent DO level need to be taken into account in these results. The average effluent DNT concentration as a function of DNT loading rate at an influent DO level of 6.8 mg/L is re-plotted in Figure 5. The average values were calculated after excluding the start-up period of first 25 days, and the error bars represent one standard deviation. In spite of the high oxygen level in the influent and significant levels in the effluent, the effluent DNT concentration from the strain DNT column bioreactor was much higher than the U.S. EPA discharge limit (0.13 mg/L) even at a low DNT loading rate ($\cong$ 9.2 mg DNT/m^2/day). Furthermore, when the DNT loading rate was higher than 14 mg DNT/m^2/day, the effluent DNT concentration dramatically increased. In the YV1 column bioreactor at the same influent DO level and total oxygen supply as strain DNT bioreactor, the DNT effluent concentration was lower than or close to the U.S. EPA discharge limit, exceeding it by less than 0.1 mg/L even at the highest DNT loading rate of 20.3 mg DNT/m^2/day.

The effluent samples were checked for presence of possible degradation product formation during the biodegradation process during HPLC analyses of DNT. Expected DNT biodegradation products including 2-amino-4-nitrotoluene, 4-amino-2-nitrotoluene, 2,4-diaminotoluene, 2-aminio-6-nitrotoluene, and 2,6-diaminotoluene were not detected on the chromatogram of the effluent samples. Similar results were reported by Lendenmann et al. (1998) in mixed culture experiments degrading DNT in fluidized bed biofilm reactors.

Stability of plasmid pSC160 in YV1

The presence of plasmid pSC160 in YV1 is expected to improve the growth and survival of YV1 due to the presence of *vgb*. It is possible, however, that pSC160 was not stably maintained in YV1 in the experiments reported here, especially since the injection of ampicillin and kanamycin solution into the YV1 influent solution stopped on the 25th day of the experiment. The Most Probable Number (MPN) experiments were conducted to investigate this possibility.

Two sets of tubes were used in the MPN test, one with antibiotics (kanamycin and ampicillin) and the other without. YV1 is resistant to kanamycin and ampicillin only in the presence of pSC160 so that if plasmid pSC160 is not stable with time, the MPN count should be lower in the tubes with antibiotics than in those without. Figure 6, however shows that there was no overall significant difference between the two sets of tubes (*paired t-test*, $\alpha = 0.05$). Therefore, it can be concluded that pSC160 in YV1 can be stable for 140 days under low DO conditions, the last 115 of which are in the absence of antibiotics. These results do not agree well with the results from a previous chemostat experiment under high DO conditions where more than 20% of pSC160 was lost in 82 days. It is possible that under oxygen rich conditions where *vgb*/VHb may provide little or no advantage, plasmid-free cells may compete favorably against plasmid-bearing cells. The opposite may occur when oxygen is limited.

Conclusions

Based on the results from batch and continuous flow sand column bioreactor experiments, the following conclusions were made.

1. A primary substrate carbon source such as yeast extract is needed to degrade high DNT concentrations by *Burkholderia* strain DNT used in this research.
2. Under high influent DO conditions (≅ 6.8 mg DO/L), at levels of influent DNT from 99.6 mg/L to 214 mg/L (DNT loading rate: 9.2 mg/m2/day to 20.3 mg/m^2/day), the YV1 bioreactor produced effluent concentrations less than 0.2 mg DNT/L. However, effluent DNT concentration was 0.7 to 4.7 mg/L for the non-recombinant bioreactor under the same DO and DNT conditions. These results suggest that *vgb*/VHb can provide an advantage with respect to DNT degradation even at high influent DO levels, possibly by supplying oxygen to the oxygenases more efficiently.
3. The benefit of *vgb*/VHb in YV1 is much greater under oxygen limiting conditions (influent DO concentration ≅ 3.1 mg/L) with a high DNT loading rate (≅ 20.3 mg/m^2/day). Under these conditions, the YV1 bioreactor was able to degrade DNT to 1.7 mg/L whereas the effluent concentration from the non-recombinant bioreactor was more than 20 mg/L.
4. DO concentration in the YV1 bioreactor effluent was about 0.2–0.3 mg/L whereas the non-recombinant bioreactor effluent contained much higher DO levels under the same oxygen supply conditions. Presumably, the presence of VHb in YV1 is responsible for higher oxygen uptake (shown by lower effluent DO levels), which, in turn, is responsible for its greater ability to degrade DNT to very low levels.
5. The advantages in DNT degradation afforded by engineering with *vgb* can be maintained over relatively long time periods without the use of antibiotics.

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