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Bioremediation of Hexanoic Acid and Phenanthrene in Oil Sands Tailings by the Microbial Consortium BioTiger™

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) and naphthenic acids (NAs) are toxic contaminants of environmental concern found in process water and tailings from the oil sands industry. BioTiger™ (BT), a patented microbial consortium, was found to cometabolically biodegrade the NA hexanoic acid (HA) and the PAH phenanthrene (PH). BT also biodegrades both HA and PH when present in a mixture as well as biodegrading HA in the presence of phenanthrene and mature fine tailings (MFT). Hexanamide was produced and consumed during cometabolism of HA. Also, three of the BT strains were found to generate biosurfactants with the bacterial adhesion to hydrocarbons assay, seven with the MBAS assay, and nine with a hemolysis assay. Serial transfers of the BT consortium demonstrated the stability of HA degradation over several generations. The results demonstrate that BT cometabolically biodegrades various combinations of PH HA and that some components in the consortium produce biosurfactants in concert.

Key Words:
Biosurfactants, BioTiger™, hexanamide, hexanoic acid, phenanthrene, mature fine tailings

Introduction

Oil sands are deposits of sand, clay, water and bitumen, and their processing produces wastewater that is toxic to wildlife and ecosystems (Alberta Government, n.d; Dalmia, 2013; Yergeau et al., 2013). A major challenge of the oil extraction process is the varying compositions of the oils sands, especially the bitumen proportion (Brigmon et al., 2016). Additionally, there are environmental toxicity concerns regarding byproducts of the oil sands industry, such as mature fine tailings (MFT) ponds, runoff from the MFT, and wastewater from the facilities, collectively known as oil sands process-affected water (OSPW) (Demeter et al., 2015; Herman et al., 1994). The oil extraction process produces large volumes of slurry wastes contaminated with various byproducts, including naphthenic acids (NAs) and polycyclic aromatic hydrocarbons (PAHs) that are toxic to aquatic life and can readily enter into local water sheds (Bordenave et al., 2010).

NAs are a family of alkyl substituted and unsubstituted acyclic, monocyclic, polycyclic, and aromatic carboxylic acids with the general chemical formula $C_nH_{2n+Z}O_2$, where *n* indicates the carbon number and *Z* is zero or a negative even integer specifying the hydrogen deficiency resulting from ring formation (Herman et al., 1994; Johnson et al., 2011). NAs are considered the primary toxic compounds in oil refinery wastewaters since they are the most water-soluble (Dalmia, 2013). The aquatoxicity of NAs on rainbow trout was demonstrated using single-ion monitoring GC-MS, which showed that NAs tend to bioaccumulate in the fish tissue (Young et al., 2008). Frank et al. (2008) confirmed that low molecular weight NAs, like hexanoic acid (HA), are more potent than high molecular weight NAs, for example, when using EC_{50} values.

Microbial bioremediation is a potential environmentally favorable strategy, which can render harmful materials, such as the NAs, innocuous with less processing. Previous studies have shown that Pseudomonads, such as *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Pseudomonas* sp., are capable of biodegrading NAs, particularly via cometabolism (Horvath, 1972; Johnson et al., 2011). Similarly, Shuttleworth & Cerniglia (1996) showed that *Acidovorax delafieldii* and *Sphingomonas paucimobilis* biodegrade environmental concentrations of phenanthrene (PH), a PAH, which corroborates previous research showing the biodegradation of PH by Sphingomonads (Brigmon et al., 2016)., NAs have, however, been found to be only weakly biodegradable (Dalmia, 2013; Frank et al.; 2008; Johnson et al., 2011), with mixed microbial cultures shown to be more effective at NA biodegradation than single microorganism cultures (Demeter et al., 2015; Frank et al., 2008, Johnson et al. 2011). Therefore, bioremediation of NAs as well as PAHs may be possible using a consortium of bacteria.

BioTiger™ (BT), U.S. Patent 7,472,747, is a consortium of twelve aerobic bacteria extracted from the acidic sludge of the Katowice oil refinery in Poland. The sludge was highly contaminated with asphaltics, which are generally non-toxic to microbes, and PAHs (Brigmon et al., 2016; Yergeau et al., 2013). Due to its harsh source, BT could prove to be effective at bioremediating the NAs and PAHs in the tailings ponds.

Some hydrocarbon biodegradation is due to biological surface active agents, known as biosurfactants (BS). These amphipathic compounds reduce surface tension at the oil-water interfaces (Youssef et al., 2004). This can facilitate desorption of hydrocarbons from MFT particles, increasing bioavailability. Desorption from the MFT matrix transfers the pollutants into the organic-phase liquid, which is composed mostly of water-insoluble organics (Christofi & Ivshina, 2002). BSs have historically been used in microbially-enhanced oil recovery, cleaning oil contaminated vessels, and transporting heavy crude oil in pipelines (Carrillo, et al., 1996). Presently, BSs are mainly used for enhancement of oil recovery and hydrocarbon bioremediation

(Reis et al., 2013; Youssef et al., 2004; Carrillo et al., 1996). The scale and severity of OSPW makes artificially introducing BS complex. Strains of bacteria that normally produce BSs could provide an alternative. However, at the industrial scale there are numerous issues including cost of feed, processing costs, and low output (Reis et al., 2013; Carrillo et al., 1996).

One particularly important feature of BT is BS production. It has been shown by Plaza et al. (2007) via the methylene blue active substances (MBAS) assay that BT strains BPZ and BP-20 both produce BSs. Moreover, a mixture of different surfactants often presents better properties than individual surfactants because of a synergistic effect (Reis et al., 2013). In the present study, the ability of BT to cometabolically biodegrade HA was investigated in several combinations, especially in the presence of phenanthrene and tailings. Additionally, BS production by the consortium and the individual components were studied to begin to classify the mechanism by which the consortium biodegrades and consequently bioremediates.

Methods and Materials

Microbiological Preparation

BT component cultures and cells were prepared as previously published (Plaza et al, 2007; Brigmon et al. 2016). Serial transfers (1% v/v) of BT were grown in R2A.

Single Carbon Source (SCS) Biodegradation Experiments

SCS biodegradation was performed as previously stated in Brigmon et al. (2016), and for cometabolism, 0.2% yeast extract (YE) was added to cultures. A range of HA was tested from 5-25mM; Hexanamide (Ham) was tested at 10mM although no carbon balance was calculated. Cultures were incubated in varying temperatures ranging from 4-60°C. Dead cells for the experiments were obtained by autoclaving cultures as stated in Brigmon et al. (2016).

Analytical Procedure

PH and HA concentrations were determined with triplicate cultures using a method modified from Brigmon et al. (2016). When studying hexanoic acid biodegradation products, the analysis was performed as stated above except the oven temperature was held at 90 °C for two minutes and ramped at a rate of 10 °C/minute for the duration of the 8-minute runtime in Scan mode. HA and Ham calibration standards were extracted, and retention times were verified.

Bacterial Adhesion to Hydrocarbons (BATH) Assay

Adapted from Thavasi et al. (2011), the BATH assay was performed by creating a buffer solution with cells diluted to an OD ≈ 0.5 to which 100 µL of crude oil was added.
The percentage of cell adherence is represented in the table by a number of "plus" signs where +++ corresponds to >90% adhesion, ++ to 60 – 89%, and + to 40-59%. "-" indicates <40%.

Methylene Blue Active Substances (MBAS) Assay

Adapted from Plaza et al. (2007) and Gunther et al. (2005), the anionic-selective MBAS BS assay was performed. A positive result for BS production is a color transformation from light to dark blue within 48 hours.

Hemolysis Assay

BBL™ TSA II 5% sheep blood agar plates were used. A positive result of BS production is a color change in the zone around the inoculation sites (Thavasi et al., 2011). *B. subtilis* (ATCC# 6051) was used as a positive control (Mulligan, 2005).

Results and Discussion

Microbial BS Analysis

BS investigation was initiated by Plaza et al. (2007), who assessed only isolates BP-Z and BP-20 with the methylene blue active substances assay (MBAS) and hemolytic assay. In this study, the

BATH assay results indicate C, S, and Z are BS-producing (Table 1), and the MBAS assay demonstrates that -E, -I, -K, -L, -S, -Z, and 20 produce anionic BS. Finally, the hemolysis assay shows BPB, -F, -H, - J, - K, -L, -S, -Z, and 20 are BS producing by beta hemolysis. The consortium BT tests positively for BS production by all 3 assays.

Table 1. Biosurfactant Production and Biodegradation Data

Bact#	BATH Assay	MBAS Assay	Hemolysis Assay	HA degrad.	PH degrad.
BPB	-	-	+++	-	-
BPC	+	-	-	-	+
BPE	-	+	-	-	-
BPF	-	-	+	-	-
BPH	-	-	++	-	+
BPI	-	+	-	-	-
BPJ	-	-	++	+	+
BPK	-	+	++	+	-
BPL	-	+	+	-	-
BPS	++	++	++	+	+
BPZ	++	++	++	+	+
BP-	-	+	+	-	-
Bsub	-	+	+	ND	ND
K12	ND	ND	ND	-	-
BT	+	+	++	+	+

The addition of crude BS to PAH-contaminated environments has been shown to increase the mass transfer rate of NAs and PAHs from their binding matrices, increasing their bioavailability's (Amodu et al., 2013; Shuttleworth & Cerniglia, 1996). The present study has shown BT produces BS, and this enhances the appeal for BT's use in bioaugmentation because BT enhances the bioavailability of certain chemicals of interest, i.e., pseudosolubilization. BS production is desirable for hydrocarbon-degrading microorganisms (Plaza et al., 2007).

In soil and aquatic environments, removal of hydrophobic recalcitrant compounds usually requires detergents or dispersants because the contaminants must be available for microbial degradation. Microbes preferentially degrade solubilized chemicals; some recalcitrant compounds, like PAHs, are poorly soluble (Mulligan, 2005; Reis et al., 2013). Since the multiple strains comprising BT are shown to be BS-producing, then in accordance with a synergistic effect, BT might be well suited to make pollutants bioavailable (Reis et al., 2013).

Microbial Biodegradation.

In the SCS experiments, a concentration of 10 mM HA, which approximates the low aqueous concentrations found in oil sands wastewaters, was used as a typical substrate concentration, and 24-hour incubations were implemented because of the high value of rapid biodegradation (Table 1). Brigmon et al. (2016) showed PH biodegradation by BT without another carbon source being provided; but no bioremediation of HA was detected in the present study without the addition of 0.2% wt/v yeast extract. After 48-hours of incubation, the original

10 mM HA was rendered below the detection limit of 0.5 mM with biostimulation, defined here as the addition of 0.2% yeast extract, at 30°C, with washed cells in Bushnell-Haas medium. Another experiment tested the ability of the BT consortium to retain the ability to cometabolically biodegrade HA as the consortium is serially transferred. The results showed positive biodegradation of HA, under the conditions listed above, during and after 3 serial transfers of the consortium.

When assessing the twelve BT components, only J, K, S, and Z exhibit HA biodegradation. Moreover, of the 4 HA-biodegrading isolates, J exhibits the greatest bioremediation activity, reducing the concentration of 10 mM HA by approximately 40% over 24-hours, whereas S exhibits the least activity, reducing the concentration by approximately 10% over 24-hours. Additionally, solely C, H, J, S, and Z are capable of biodegrading phenanthrene in a saturated solution over 24-hours (Table 1).

Investigation of the biodegradation of both HA and PH compounds was performed. The multiple carbon source (MCS) experiments were completed with 10 mM in HA and medium-saturated PH. Within 24-hours, biostimulated BT was able to biodegrade HA and PH by approximately 55% and 80%, respectively (See Figure 1). A control of killed-BT cells biostimulated with 0.2% YE did not biodegrade either carbon source.

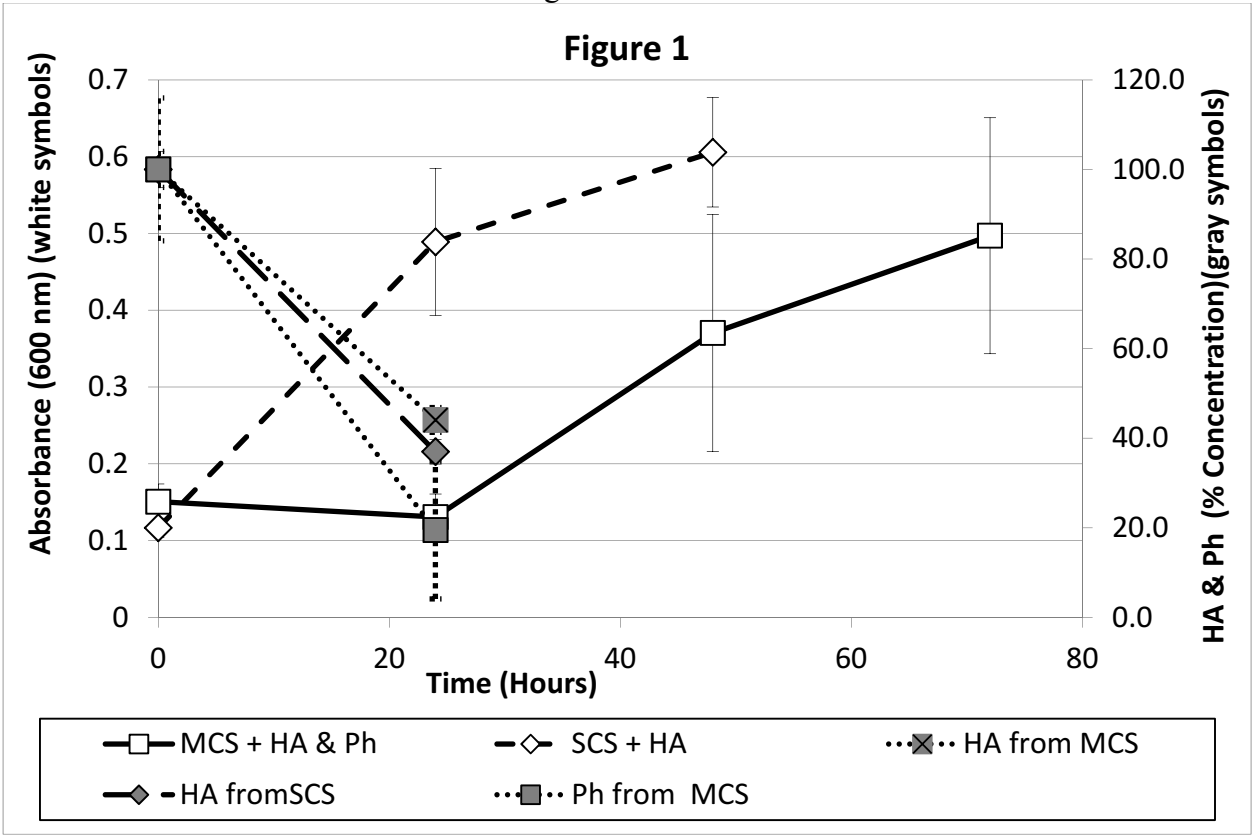

Figure 1. Cometabolic growth of BioTiger™ on Hexanoic acid (HA).

In Figure 1, cometabolic growth of BT on HA is demonstrated. The white markers represent growth curves, and the gray markers represent substrate utilization. White diamonds represent growth on 10 mM HA; white squares represent growth on 10 mM HA and saturated PH as MCS; gray diamonds represent the concentration of 10 mM HA when utilized as SCS;

gray-X squares represent the concentration of 10 mM HA when utilized in a MCS condition; gray squares represent the concentration of PH when utilized in a MCS condition.

With the HA-only amended group, the culture grows cometabolically over the first 24 hrs as the HA is degraded. Figure 1 shows a 24 hour lag in the cultures that had both HA and PH. Only when the concentration of PH is decreased, do the cultures begin to increase in optical density, cometabolically growing. The decrease in HA does not seem to be linked to the limitation in growth as the rates are similar in all the cultures. They appear to grow to the same density at a similar rate after the lag where PH is eliminated.

The SCS and MCS experiments were repeated with the addition of 40g of tailings per liter culture. Biodegradation in tailings more closely mimics the conditions in which BT could be utilized for bioaugmentation in OSPW. Beginning with 10 mM HA, biostimulated BT decreased the concentration of HA by approximately 55% in the presence of tailings over 24-hours. The killed-cell BT control demonstrated no significant decrease in HA concentration. Additionally, a control culture with no BT amended including yeast extract and tailings did not display HA biodegradation. (data not shown)

In additional experiments, concentration ranges of 5mM to 30 mM HA, which is near the maximum solubility of HA in water, were investigated. Over the course of 120 hours, the biostimulated BT consortium was incapable of biodegrading HA at any concentration over 20 mM (Data not shown).

Similarly, incubation temperatures were assessed from 4 °C to 60 °C. The biostimulated BT consortium was incubated for several days, but no biodegradation of 10 mM HA occurred at these temperature extremes including 4, 15, 45 and 60 °C. Cometabolic biodegradation was observed at 20, 30, and 35 °C. (data not shown) Also, the ability of BT to biodegrade the combination of multiple carbon sources, PH and HA, in tailings was tested. Over 24 hours, BT reduced the concentration of 10 mM by approximately 45%. Detection of PH in this condition was not possible with the currently employed method. A biostimulated, killed-cell control served as the negative control and did not exhibit HA biodegradation.

Hexanamide or hexanoamide (HAM) was produced as 10 mM HA was biodegraded by the BT consortium. HAM was confirmed by a retention time standard, and a calibration curve was run to determine the concentration of HAM after 24 and 48h. The amount of HAM did not seem to increase after the initial amount of 0.5 mM HAM was detected at 24h. Decreases in HAM, and HA, was not seen in any of the appropriate killed-cell controls. For every mole of HA that was transformed, 0.1 mole of HAM was generated (data not shown). It is possible that the cells are utilizing a glutamate-to-glutamine type enzymatic process, and the production of natural amides is not common in the bacterial world, but has been observed before this study (Gottschalk, 1986; Whitby, 2010).

HAM was tested as a sole carbon source as previously described. Briefly, as with the other SCS interrogations, BT was grown in R2A for 3 days, then cells were washed in Bushnell-Hass medium amended with 0.2% YE. Some of the preparation was autoclaved to be used for killed-cell controls. HAM was found to be biodegraded below detection limits of 0.1 mM in 2 days. While the results of the investigation of HAM cometabolism are brief, they may have major implications for the fate of the OSPW remediation. HAM is more toxic to organisms (based on LD50) and less soluble in water; moreover its presence as an intermediate cometabolic byproduct may suggest that BT could render an aquatic environment temporarily more dangerous as intermediates may become far less aqueous and therefore less bioavailable.

NAs and PAHs are important components of oil sands because of environmental toxicity. Though there are chemical and physical means of remediating aromatic hydrocarbons and naphthenic acids, microbial biodegradation could be an effective route for long-term, significant pollution removal. An important consideration for studies of NAs and PAHs is how to administer the carbon source to the bacteria, i.e., as individual compounds or as a mixture. Another consideration is whether or not the microbial consortium requires biostimulation, which is the addition of electron acceptors, nutrients, oxygen, and/or other factors to aid the biodegradation process (Horvath, 1972; Plaza et al., 2007). Microbially-produced BSs are especially appealing due to their low-cost, decreased invasiveness, and minimal environmental toxicities as compared to physico-chemical means of remediation (Amodu et al., 2013). BSs help release hydrocarbons from macroparticles in the tailings matrix, thereby making the hydrocarbons bioavailable in the aqueous layer for microbial biodegradation (Christofi & Ivshina, 2002; Reis et al., 2013). Our results indicate that several strains comprising the BT consortium produce BSs, which may aid in their application in bioremediation of NA and PAHs. It is known that even in the presence of excess carbon sources, microbes will not grow if there is insufficient nitrogen and phosphorous (Yergeau et al., 2013). These considerations indicate the possible need for a bioreactor to facilitate BT as a viable bioremediator. It has also been shown that microbes can biodegrade organic pollutants via cometabolism, or co-oxidation (Horvath, 1972). Additionally, Plaza et al. (2007) found that the rate of consumption of one hydrocarbon is affected by the presence of another hydrocarbon, although the degree of influence varies widely. That is, microbial biodegradation of one substrate can be inhibited by another substrate and can sometimes be considered competitive (Stringfellow & Aitken, 1995). While our studies show the BT can biodegrade both HA and PAHs, careful consideration must be taken when generalizing the efficacy of biodegradation in the complex MFT environment because of the interactive effects of toxicity, inhibition, and bioavailability.

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