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Utilization of Monocyclic Aromatic Hydrocarbons Individually and in Mixture by Bacteria Isolated from Petroleum-Contaminated Soil

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Summary

Fate of benzene ethylbenzene toluene xylenes (BTEX) compounds through biodegradation was investigated using two different bacteria, R*alstonia picketti* (BP-20) and *Alcaligenes piechaudii* (CZOR L-1B). These bacteria were isolated from extremely polluted petroleum hydrocarbon contaminated soils. PCR and Fatty Acid Methyl Ester (FAME) were used to identify the isolates. Biodegradation was measured using each organism individually and in combination. Both bacteria were shown to degrade each of the BTEX compounds. *Alcaligenes piechaudii* biodegraded BTEXs more efficiently while mixed with BP-20 and individually. Biosurfactant production was observed by culture techniques. In addition 3-hydroxy fatty acids, important in biosurfactant production, was observed by FAME analysis. In the all experiments toluene and m+p- xylenes were better growth substrates for both bacteria than the other BTEX compounds. In addition, the test results indicate that the bacteria could contribute to bioremediation of aromatic hydrocarbons (BTEX) pollution increase biodegradation through the action by biosurfactants.

Keywords: aromatic hydrocarbons, biodegradation, biosurfactant, bioremediation

Introduction

BTEX compounds (benzene, ethylbenzene, toluene, and three isomers of xylene) are classified as environmental priority pollutants. They are volatile monoaromatic hydrocarbons which are commonly found together in crude petroleum and petroleum products such as gasoline and diesel fuel. BTEX hydrocarbons make up a significant percentage of petroleum products and make up about 18% (w/w) in standard gasoline (Budavari 1996).

They are also produced on the large scale as bulk chemicals for industrial use as solvents and starting materials for the manufacture of pesticides, plastics etc. (Budavari 1996). BTEXs are considered a major cause of environmental pollution because of widespread occurrences of leakage from underground petroleum storage tanks and spills at petroleum production wells, refineries, pipelines, and distribution stations (Brigmon et al., 20002). The presence of these hydrocarbons in the environment is a hazard to public health and ecological concern due to their toxicity and ability to bioaccumulate through the food chain (Brigmon *et al*. 2002). For BTEX compounds, the principal concern is their migration away from the source areas. All are soluable to some degree and are found in the water-soluble fraction and consequently these compounds are some of the most common contaminants found in groundwater.

Remediation of BTEX compounds is affected by volatilization, dissolution, sorption and degradation by microorganisms. While physical/chemical routes may remove some BTEX groundwater contamination, only microbial degradation leads to a long term removal of BTEX associated with a significant mass removal (Fischer et al. 2006).

The natural bacterial flora in soil has an ability to aerobically degrade the BTEX (Baggi *et al*. 1987; Yeom & Daugulis 2001; Arofa 2003). The biodegradation of these compounds has been and continues to be studied extensively. Laboratory microcosms and field studies reported in the literature have shown that aerobic BTEX degrading microorganisms present in soils are a major factor for the remediation technologies (Singleton 1994). By adding nutrients and oxygen the degradation processes are enhanced (biostimulation). The fate and transport mechanisms of BTEX hydrocarbons are affected by the contaminant characteristics, which vary with the different BTEX compounds. In general, the volatilization, dissolution and degradation determine the concentrations of BTEXs, and the sorption and dissolution determine the transport in soil and groundwater systems.

In this paper, we utilize the FAME technique to characterize the fatty acids produced by the isolated microorganisms related to surfactant activity as previously described (Youssef et al,

2005). Biosurfactant activity can influence other microorganisms as well as hydrocarbons of concern (Al-Tahhan et al, 2000).

The aim of the study was to evaluate the biodegradation of individual BTEX hydrocarbons and BTEX a mixture of two biosurfactant-producing bacteria.

Material and Methods

Site characterization

The 100-year-old Czechowice-Dziedzice Oil Refinery (CZOR) in Poland produced an estimated 120,000 tons of acidic, highly weathered, petroleum sludge deposited into three open waste lagoons, 3 meters deep and covering 3.8 hectares. One of the waste lagoons (0.3 hectare) was chosen for aerobic biopile bioremediation monitoring and physicochemical characterization. The waste from the lagoon was removed, and heavily petroleum contaminated soil was subjected to the bioremediation process. The biopile with active and passive aerated sections, referred to further as biopile 1 or engineered, was constructed in 1997 in the smallest lagoon at the Czechowice-Dziedzice Oil Refinery (Altman *et al*. 1997; Płaza *et al*. 2003). The purpose was to evaluate novel technologies and applications for environmental restoration of soils heavily contaminated with petroleum waste by comparing bioremediation processes under active *vs*. passive aeration and the removal rates of both, easily biodegradable and recalcitrant petroleum hydrocarbons. The project focused on the application of cost-effective amendments for biostimulation, including additions of mineral NPK fertilizers and the surfactant, Rokafenol N8, to enhance hydrocarbon biodegradation.

In 2001, a second biopile was constructed to cleanup soil mixed with petroleum hydrocarbon waste (Worsztynowicz *et al*. 2001). This biopile, referred to as biopile 2 or non-engineered, is situated within the second (middle) lagoon at the refinery. In this biopile contaminated soil was mixed with mineral fertilizers and wood chips, and covered with a dolomite layer. Subsequently, the dolomite layer was covered with unpolluted soil and grass seeds sown over the biopile area. A simple drainage system was built to allow natural soil aeration. The bacteria employed in this work were isolated from soil core taken from these two biopiles.

Isolation and identification of bacteria-utilizing hydrocarbons

For isolation of hydrocarbon degrading cultures 10 g of mixed soils from biopile 1 and biopile 2 were initially inoculated in 100 ml of the mineral medium (MM). The composition of the medium used was the following (g/l): NH_4NO_3 - 1; $MgSO_4$ x $7H_2O$ – 0,2; $CaCL_2$ x $2H_2O$ – 0,03; K_2HPO_4 – 1; KH_2PO_4 – 1. The medium was supplemented with 1 ml of the trace elements solution (Gerhardt 1981), and 1% (v/v) of crude oil as carbon and energy source. The incubation was performed at 30^{o}C for 24 – 48 h. Development of bacterial colonies was obtained by a serial dilution-agar plating technique on standard methods agar (SMA, Biomerieux). Cultures were then reisolated by streaking on noble agar plates utilizing naphthalene as a sole carbon and energy source. Then, the isolates were tested for their ability to grow on solid mineral medium with different hydrocarbons. The composition of the medium used was the following (g/l): NH_4NO_3 - 1; $MgSO_4$ x $7H_2O$ – 0.2; $CaCL_2$ x $2H_2O$ – 0.02; K_2HPO_4 – 1; KH_2PO_4 – 1 and 1 ml/l of the trace elements solution. Cyclohexane, hexadecane, xylene, benzene, toluene, heptane, decane, isooctane, hexane, mineral oil, pristane, and squalene were used as carbon and energy sources. All chemicals used were of analytical grade and purchased from Sigma-Aldrich Co. and Polish Chemical Reagents S.A., Gliwice. 200 µl of hydrocarbons were put on the filter paper and then overlaid with the mineral medium containing 20 g of agar (DIFCO). The incubation of Petri dishes was carried out at 30^{o}C by two weeks.

Isolates, which grew well in the presence of hydrocarbons were initially identified with PCR (Steffan & Atlas 1991). Isolates were grown on SMA plates, and single colony of each isolate was resuspended and washed three times in 100 µl of sterile distilled water. The whole cell washed cell suspension was added to the PCR reaction mixture as described by Furlong et al. (2002). Briefly, the PCR reaction was performed with puReTaq™ Ready-To-Go™ Polymerase Chain Reaction (PCR) beads (Amersham Biosciences). Beads were premixed and predispensed to enable complete reactions for performing PCR amplifications. With the exception of primers and template, the ambient temperature-stable beads provide all the necessary reagents to perform 25 µl polymerase chain reactions, e.g. stabilizers, BSA, dATP, dCTP, dGTP, dTTP, ~2.5 units of puReTaq DNA polymerase and reaction buffer. When a bead was reconstituted to a 25 µl final volume, the concentration of each dNTP was 200 mM in 10 mM Tris-HCl (pH 9.0), 50 mM KCl and 1.5 mM $MgCl_2$. Each PCR contained

< 1μg of template DNA and primers at a concentration of 0.2-1 μM. The samples were subjected to 30 cycles of 94°C for 1 min, 72°C for 2 min and 61°C for 1 min for denaturation, annealing and elongation steps, respectively. An initial denaturation step (95°C, 1 min) was used to ensure complete denaturation of the DNA. PCR amplification was performed in a Mastercycler® gradient machine (Eppendorf). The primers for the reactions were as follows: forward primer: 27f (5'-TTCCGGTTGATCCYGCCGGA-3') and reverse primer: 1492 universal (5'- ACGGGCGGTGTGTRC-3') (Furlong et al., 2002). Partial sequences of rRNA genes were obtained using an ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA) with an ABI PRISM BigDye terminator sequencing Lab kit at the Genome Analysis Facility in the Botany Department at the University of Georgia, Athens. Isolates were tentatively identified by similarity to sequences in the GenBank data base using the FastA algorithm (Pearson & Lipman 1988) of the GCG software package (Genetics Computer Group, Wisconsin).

Biosurfactant Production

Isolates were assayed for biosurfactant production utilizing a modified CTAB-methylene blue agar plate method described by Gunther et al., 2005. The method is for identifying rhamnolipids, glycolipids with biosurfactant activity. Bacteria were grown on agar plates containing 1% mineral oil for 48 h at 30ºC. A *Pseudomonas fluorescens* strain (ATCC 31483) was included as a control. Small shallow wells were cut into the indicator plates with the hot tip of a metal inoculating loop. Loopfuls of bacteria from overnight cultures on mineral oil plates were placed in the shallow wells. The plates were then incubated for 48-72 h. A positive reaction for rhamnolipids production is the appearance of dark blue colony pigmentation with clearly defined edges. The plates were then placed in a refrigerator at 4ºC which allows the positive wells to darken, distinguishing them from any potential false positives.

Representative fatty acid profiles of the bacteria were obtained by direct extraction as with a modified method described by Sasser, 1990. Pure culture suspensions were obtained from overnight cultures on TSA medium. The cultures were transferred to sterile 10 ml glass tubes with Teflon®-lined caps. One ml of methanolic sodium hydroxide solution (15% [wt/v] NaOH in 50% [v/v] methanol) was added to saponify the samples. Tubes were capped, vortexed for 10 s, incubated at 100^{o}C in a water bath for five min, vortexed for 10 s, and

incubated at 100°C for an additional 25 min. Tubes were cooled in a cold water bath, and two ml of 3.25 N HCl in 46% (v/v) methanol was added to the tubes to esterify the fatty acids. The samples were capped, vortexed for five s, placed in a water bath at 80°C for 10 min, and cooled. The fatty acids in the samples were extracted with 1.25 ml of methyl-tert-butyl ether:hexanes (1:1, v/v). The samples were capped, inverted continuously for 10 min, and the aqueous (lower) phase was removed using a glass Pasteur pipette. Three ml of 1.2% (wt/vol) NaOH were added to the tubes to wash the FAMEs; the tubes were inverted continuously for five min. Two-thirds of the organic (upper) phase was transferred with a glass Pasteur pipette into a two ml glass gas-chromatography vial, which was sealed with a Teflon-lined cap. The FAME in the organic layer were derivatized with BSTFA (Pierce, Rockford, IL) and analyzed by gas chromatography/mass spectrometry (GC/MS) (Agilent Technologies 6890N Network GC systems/ 5973 Network Mass Selective Detector, Wilmington, DE).

The controls consisted of polycarbonate membranes with and without 50-55 mg of either 24-hour cultures of TSA-grown *Stenotrophomonas maltophilia* (ATCC 13637)and reagent blanks.

BTEX biodegradation by Ralstonia picketti (BP-20) and Alcaligenes piechaudii (CZOR L-1B) under aerobic conditions

Incubation conditions

Experiment was performed using 125 ml serum bottles containing 25 ml medium as described by Abu-Ruwaida *et al*. (1991). The composition of the medium was the following (g/l): Na_2HPO_4 – 2.2, KH_2PO_4 – 1.4, $MgSO_4$ x $7H_2O$ – 0.6, $(NH_4)_2SO_4$ – 3, yeast extract – 1, NaCl – 0.05, $FeSO_4$ x $7H_2O$ – 0.01, 1 ml microelements solution (Gerhardt 1981). 500 µl of 24–hours bacteria culture (10^4 – 10^5 cells/ml) as inoculum were added. The vials were sealed with a Teflon-coated rubber and aluminum septum-cap. Headspaces of the bottles were flushed with oxygen. Two experiments were established. In the first experiment, BTEX mixture of 50 mg/l as initial hydrocarbons concentration was added to the cultures using a glass microsyringe (Hamilton Co, # 701). In the second experiment, all the BTEX compounds at a final concentration of 100 µg/l were added separately to each culture. All hydrocarbons, 99% analytical standards, were purchased from the Supelco Co. The incubation was carried out at 30°C for a total of 30 days. Measurements of BTEX were done after 1, 2, 5, 10, 20 and 30 days of the incubation time. Sterile controls were prepared to evaluate hydrocarbon evaporation. The experiments were done in triplicate.

Analytical procedure

BTEX hydrocarbons concentrations were determined according to the method described by Wypych & Mańko (2002). The Head-Space Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (HS-SPME-GC/MS) technique was used. The hydrocarbon analysis was carried out with Star 3400 Cx gas chromatograph (equipped with a ^{63}Ni Electron Capture Detector); it was coupled to Saturn 3 mass spectrometer and Varian 8200 Cx Autosampler with 10 ml autosampler vials. The chromatographic column with the phase DB624 and length 30 m x 0.32 mm ID (1.8 μm film thickness) was used. As the carrier gas helium was used: purity 99.999%, flowing capacity of 1.0 ml/min (in the temperature of 35°C). The gas chromatographic conditions were as follows: the oven temperature was held isothermally at 40°C for 10 minutes, then increased to 250°C by 10°C/min, and finally increasing by 5^{o}C/min to 270^{o}C. The total analysis time was 45 minutes. The injector temperature was 250°C. The MS operating conditions were the following: the mass range scanned was 30-250 amu at 1 sec/scan, temperature of ion trap was 170°C, multiplier voltage was 2700 V, ionization energy was 70 eV (electron impact mode EI). Transfer line temperature was 250°C. The temperature of the ECD detector (^{63}Ni) was 300°C.

Results and discussion

BTEX biodegradation in mixture

The time course concentration of each BTEX component in the mixture is presented in Figure 1. Both bacteria had the ability to degrade BTEX hydrocarbons but *Alcaligenes piechaudii* was found to degrade more hydrocarbons over shorter time periods. Table 1 summarizes the removal of BTEX hydrocarbons by both bacteria by % removal after 30 days of incubation. *Alcaligenes piechaudii* removed 96% and 97% of toluene and m+p xylenes respectively. Depletion of m+p-xylenes was evident during the first stage of growth, see Figure 1 Both strains were found to efficiently degrade BTEX in the culture medium at 30^{o}C and pH 7.0. The results showed the order of degradation to be: m+p-xylene > toluene > o-xylene > ethylbenzene > benzene. Benzene biodegradation was 52% and 84% for *Ralstonia picketti* and *Alcaligenes piechaudii*, respectively. Under the experimental conditions *Alcaligenes*

piechaudii was a better BTEX degrader than *Ralstonia picketti*. The bacteria species were able to grow on BTEX mixture as sole carbon and energy sources. Behavior of BTEX mixture involves complex interaction among all these hydrocarbons similar to those conditions that may be observed in subsurface environments (McInerney et al., 2005). In mixture experiment the rate of consumption of one substrate was found to be affected by the presence of the other, although the degree of influence varied widely. The occurrence of hydrocarbons in the mixture is an important problem because the degradation of one substrate can be inhibited by the other compounds in the mixture, and because different conditions are required to treat different compounds within the mixture (Reardon *et al*. 2000). Again discussion – I would compare rates of degradation along with total removal. That data could be added to Table 1.

BTEX biodegradation individually

The removal of BTEX hydrocarbons by *Ralstonia picketti* and *Alcaligenes piechaudii* is presented in Table 1. Both bacteria had the ability to degrade BTEX compounds individually. Figure 2 presents BTEX biodegradation pathways which were different than in BTEX's mixture. Generally, *Alcaligenes piechaudii* had higher ability to degrade BTEX than *Ralstonia picketti*. The removal of toluene, m- xylene and p- xylene reached almost 100% after 2 days of the incubation. The pathways of biodegradation for ethylbenzene and o- xylene were similar for two bacteria – how do you know this? This sounds like a literature citation not a result. They were degraded simultaneously and in parallel way? Not sure what this means. Benzene degradation was very different for each bacteria. *R. picketti* degraded benzene very well, after 5 days of the incubation degradation of benzene reached almost 100%. *Alcaligenes piechaudii* degraded only 59% of the benzene after 30 days. However, during the next days of the incubation the benzene biodegradation reached 95% (data not shown).If the data is not shown then don't talk about it or talk about and add the data *A. piechaudii* degraded toluene and m+p- xylenes faster than the other hydrocarbons. The results showed the order of degradation to be: m+p-xylene > toluene > o-xylene > ethylbenzene > benzene. The order is similar which was obtained in the experiment in which BTEXs were degraded in the mixture. The degradation order depended on structure of hydrocarbons and their substitutes. The concurrent presence of multiple BTEX compounds reveal a range of substrate interactions including no interaction, stimulation, competitive inhibition, non-competitive inhibition and cometabolism.

Biosurfactant Production.

Alcaligenes piechaudii colonies on CTAB medium were observed to turn dark blue in 48 hours. The *Ralstonia picketti* colonies took 72 hours to turn a lighter blue on the CTAB medium. The agar plates were place in a refrigerator overnight for 24 h and darkened further (Figure 3A and 3B). The *Alcaligenes piechaudii* colonies remained lighter than the *Ralstonia picketti* colonies on the CTAB medium. The fact both cultures showed this type of pigmentation o\n CTAB medium indicate rhamnolipid production (Gunther et al., 2005). Growth was observed for the *P. fluorescens* control culture with no darker pigment production for the same time period.

FAME identified the *Alcaligenes piechaudii* fatty acid profile and it was shown to produce 8:O 3OH (2.4%), 10:0 3OH (2.8%), and 12:3OH (3.5%) fatty acids. FAME identified the *Ralstonia picketti* fatty acid profile and it was shown to produce 14:0 3OH/16:1 *iso* 1 (3.3%), 10:0 3OH, and 12:3OH fatty acids. This is significant since the percentage of 3-hydroxy even numbered fatty acids has been correlated with biosurfactant activity (Youssef et al., 2005).

Discussion

In our experiments all BTEX compounds were degraded more individually than in the combined mixture. According to Bielefeldt & Stensel (1999) the slower degradation rates of the BTEX compounds in the mixture versus alone could be due to three potential interactive effects: toxicity, non-competitive inhibition, and competitive inhibition. BTEX compounds incubated individually can be transformed by specific enzyme pathways which have been very well characterized. Most of the enzymes involved in the degradation of all BTEX hydrocarbons pathways are the same, and competitive inhibition effects during degradation of the BTEXs in the mixture are the most probable interactions. All substrates are metabolized by the same enzymes. The competitive inhibition reduced the biodegradation of individual BTEX compounds in the mixture in comparison to the biodegradation of each compound alone. Chang *et al*. (1993) measured the Monod biokinetic parameters for BTEX degradation by two pure *Pseudomonas* cultures. The authors developed equations to predict competitive

inhibition effects only for two compounds. However, Bielefeldt & Stensel (1990) evaluated the biodegradation rates of BTEX compounds in the mixture. The biodegradation rates in the mixture were predicted using a basic on competitive inhibition model. The results obtained here for hydrocarbon degradation were similar those described by Reardon *et al*. (2000). The results showed that degradation rates of each of the compounds in the mixture were significantly slower than the individual compounds degradation rates. They concluded that competitive inhibition appeared to be the predominant factor of the BTEX degradation in the mixture.

In this work two bacterial strains *Ralstonia picketti* and *Alcaligenes piechaudii* that have the ability to produce biosurfactant and degrade BTEX were isolated. The identified species were responsible for BTEX compounds degradation and played a key role in production of biosurfactants, which are recognized to enhance degradation of aliphatic and aromatic hydrocarbons. Biosurfactant characteristics and biochemical activities were identified. How biosurfactants influence hydrocarbon degradation in different sites can depend on the type of microorganisms present (Al-Tahhan, et al., 2000). Biosurfactant production has been demonstrated as a microbial mechanism to increase petroleum and other hydrocarbon biodegradation by increasing the bioavailability of the hydrocarbons (McInerney et al., 2005).

FAME was employed here to characterize fatty acids in two hydrocarbon degrading environmental isolates. Other reports (e.g., Cavigelli *et al*. 1995 & Glucksman et al. 2000) have used the FAME approach to study microbial communities from environmental samples. Future studies could examine environmental samples for key signature fatty acids relevant to biosurfactant applications including bioremediation or biorecovery of hydrocarbons.

The BTEX degradation experiments demonstrated that isolated bacteria are useful to assess the potential for natural attenuation of hydrocarbon-contaminated environments. Concerning the limitation in degradative capacities observed for benzene, the study allowed a fine discrimination of the specific capacities for the degradation of toluene and xylene isomers. In a hydrocarbon-contaminated site, it is necessary to understand what the microbial population is present and associated activities (ie biosurfactant production) as well as what factors influence their metabolic activities to take advantage of their remedial capacity (McInerney et al., 2005). Perturbations to microbial populations could explain different rates of BTEX biodegradation often observed in heterogeneous environments (Fischer et al., 2006)). From an

applied point of view, the results indicate that, in remediating contaminated environments the site-specific microbiological activities should be taken into account.

Conclusions

In this study revealed that two bacteria strains have the special properties for petroleum hydrocarbons remediation, e.g. they produce biosurfactant and degrade aromatic hydrocarbons. There is no data on biosurfactant production, concentrations, or mode of action

Of particular interest is that the hydrocarbon biodegrading bacteria mechanism found here is combined with their ability to produce biosurfactants and increase BTEX uptake, probably by the changes of the surface active properties. The beneficial effect of biosurfactants on BTEX biodegradation has been observed. Evidence of biosurfactant production by these two strains was demonstrated by both microbial and biochemical characterization and further characterization in planned. Biosurfactants increase hydrocarbons availability and dependent on their growth conditions and substrate. Biosurfactant production changes the medium surface active properties which are responsible for increasing the cell/hydrocarbon interactions and ultimately increase BTEX biodegradation rate. The properties, biosurfactant production and modulation of the cell surface hydrophobicity play an important role in efficient hydrocarbon assimilation/uptake. Based on this experimental evidence these natural environmental isolates show potential for hydrocarbon bioremediation and biorecovery applications.

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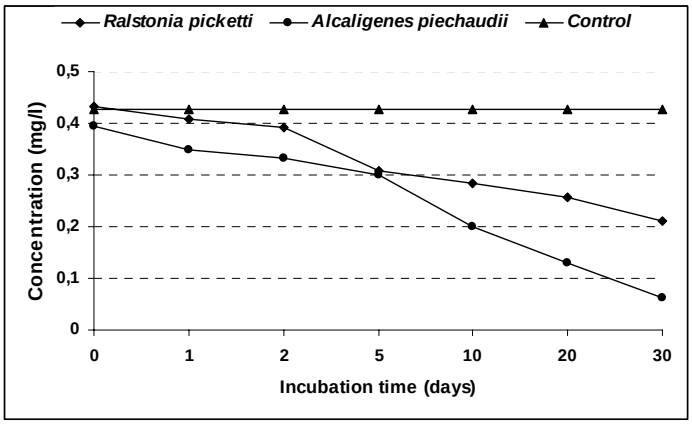
Table 1

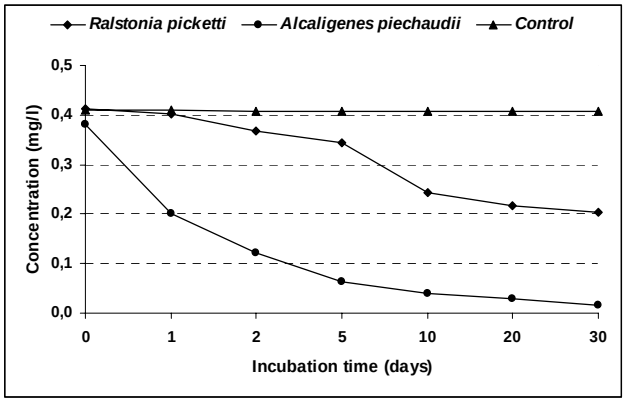
Strains	Biodegradation Percentage (%)*				
	Benzene	Toluene	Ethylbenzene	m+p-Xylene	o-Xylene
	IN THE MIXTURE				
Ralstonia picketti	52	53	63	65	65
Alcaligenes piechaudii	84	96	85	97	85
	INDIVIDUALLY				
Ralstonia picketti	100	89	82	98[1]; 84[2]	66
Alcaligenes piechaudii	59	100	77	100[1]; 100[2]	77

Standard deviation (SD) values were $\leq 2\%$;
* % of biodegradation after 30 days of the incubation time
[1] – m-xylene
[2] – p-xylene

BENZENE



TOLUENE



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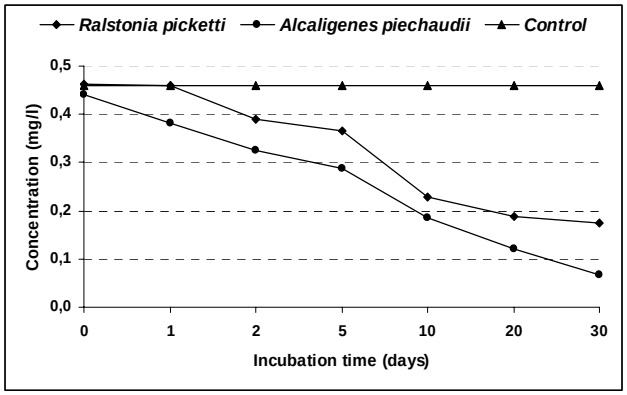
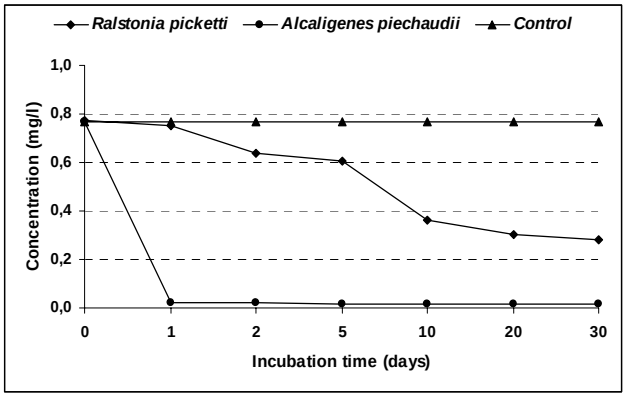


m+p- XYLENE



o- XYLENE

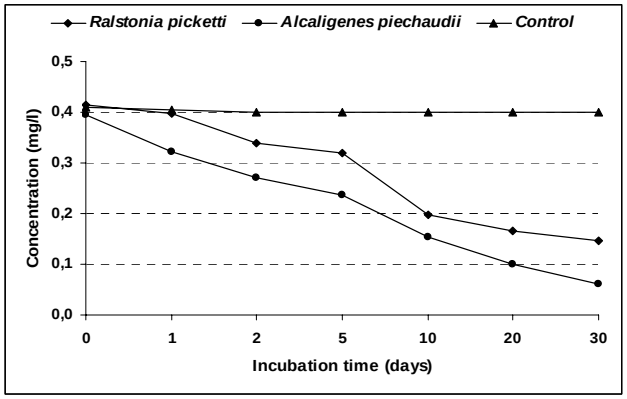
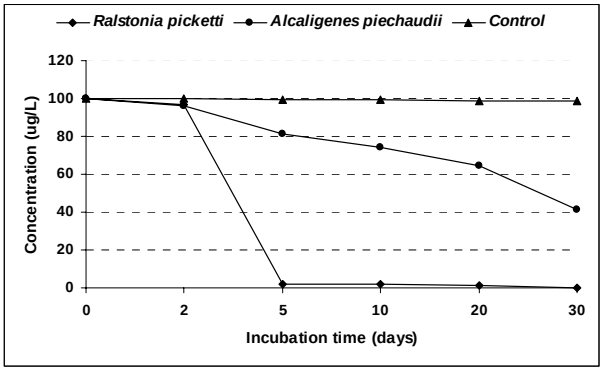


Figure 1.

BENZENE



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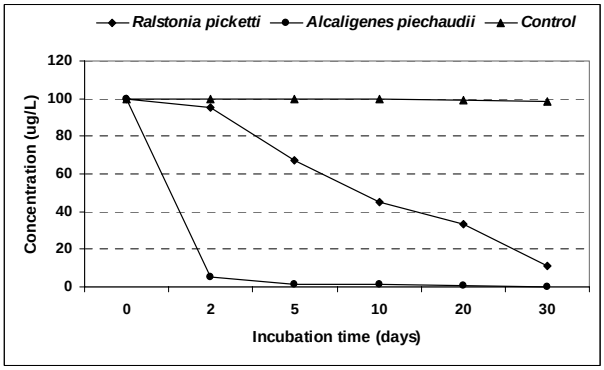

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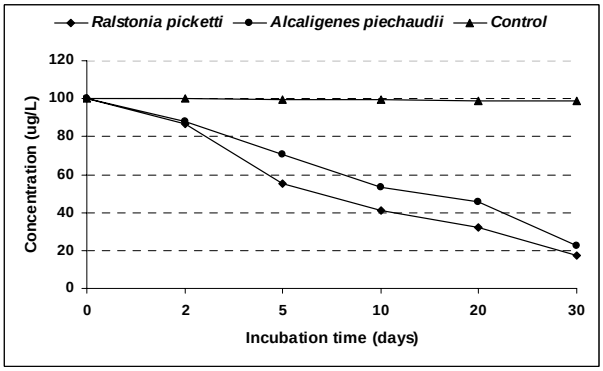

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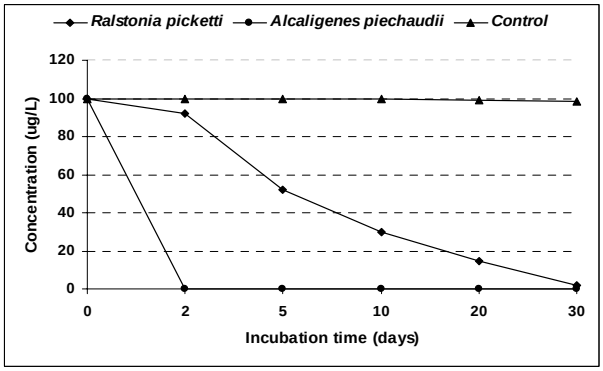

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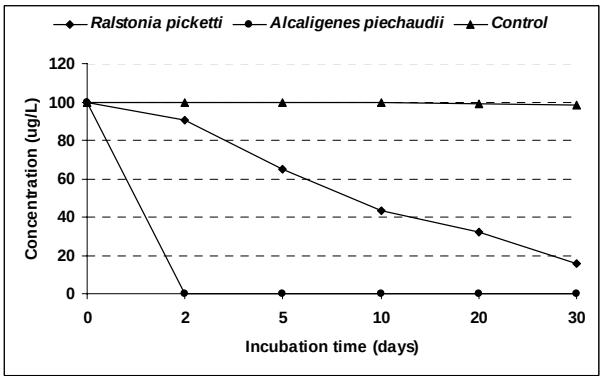

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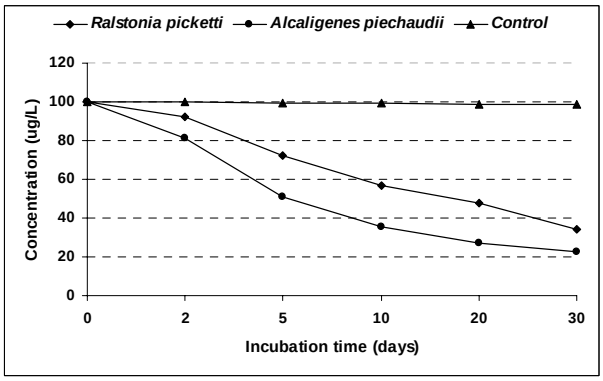

Figure 2.

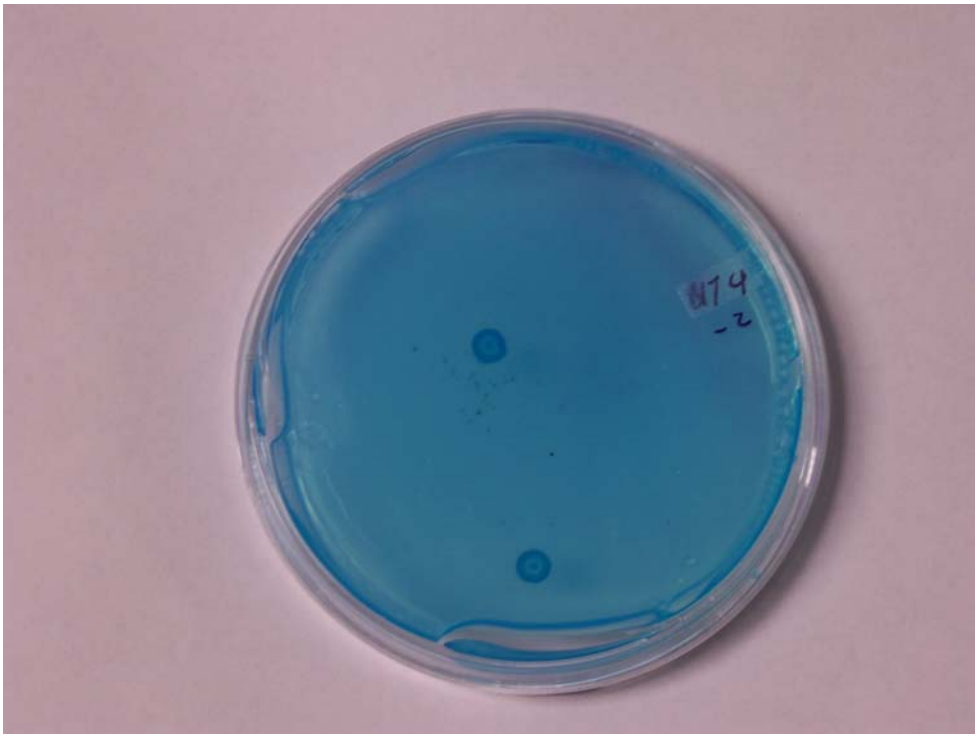

Figure 3A Dark Blue pigmented *Alcaligenes piechaudii* colonies characteristic of rhamnolipid production on selective medium

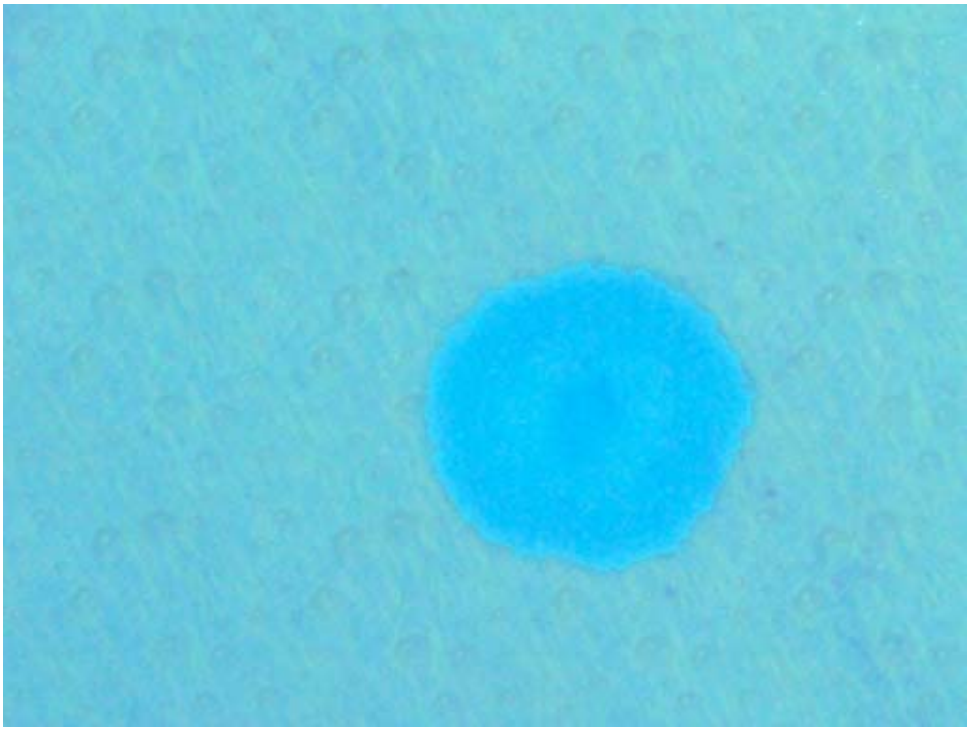

Figure 3B Close up of dark Blue pigmented *Ralstonia picketti* colonies characteristic of rhamnolipid production on selective medium

Figure legends

Figure 1 BTEX biodegradation by *Ralstonia picketti* and *Alcaligenes piechaudii* in the mixture during the course of the experiment (mean values: SD ≤ 0.1 mg/l)

Figure 2 BTEX biodegradation by *Ralstonia picketti* and *Alcaligenes piechaudii* individually

Figure 3A Dark Blue pigmented *Ralstonia picketti* colonies characteristic of rhamnolipid production on selective medium

Figure 3B Close up of dark Blue pigmented *Ralstonia picketti* colonies characteristic of rhamnolipid production on selective medium