Evaluation of biomass production in unleaded gasoline and BTEX-fed batch reactors


*** Laboratorio de Biorremediacion Ambiental, Facultad de Medicina, Universidad Autonoma de Nuevo Leon, Apdo. Postal 1563, Monterrey, Nuevo Leon, Mexico
** Department of Environmental Health Sciences, Tulane University Medical Center, School of Public Health and Tropical Medicine, 1430 Tulane Avenue, New Orleans, LA., 70112-2699, USA
*** Instituto Mexicano del Petroleo, Eje Central Lazaro Cardenas 152, Delegacion Gustavo A. Madero, 07730 México, D.F.

Abstract BTEX removal under aerobic conditions by unleaded gasoline acclimated biomass and BTEX acclimated biomass, and the effect of surfactant on BTEX biodegradation were evaluated. The effect of BTEX concentration as the sole source of carbon for biomass acclimation and the effect of yeast extract on cell growth in unleaded gasoline-fed reactors were also evaluated. For the unleaded gasoline acclimated biomass, benzene was shown the most recalcitrant among all BTEX, followed by o-xylene and toluene with 16–23%, 35–41% and 57–69% biodegradation, respectively. Ethylbenzene was consistently the fastest BTEX chemical removed with 99% biodegradation for the four bioreactor acclimated biomasses tested. For the 1,200 ppm BTEX acclimated biomass, benzene showed the highest removal efficiency (99%) among the four biomass environmental conditions tested, along with 99% toluene and 99% ethylbenzene biodegradation. O-xylene showed 92–94% removal. In all bioassays tested Tergitol NP-10 was fully removed, and did not have a substantial effect on BTEX biodegradation at the end of a 10-day evaluation.

Keywords Batch reactors; biodegradation; biomass acclimation; BTEX; unleaded gasoline

Introduction
Unleaded gasoline is a complex mixture containing more than 200 chemicals that may pose an environmental threat when released to air, soil and eventually to shallow and deep groundwater. Leaking underground storage tanks, production, transport and other storage facilities are frequently found as sources of environmental release of gasoline. The physicochemical properties allow some chemicals to evaporate while others may adsorb on to the soil or infiltrate to reach waterbodies. The major chemicals of environmental concern from unleaded gasoline are methyl tertiary-butyl ether (MTBE) and benzene, toluene, ethylbenzene and total xylenes (BTEX) due to their carcinogenic potential and other toxicity and their impact on property value (Hartley et al., 1999; Acuna-Askar et al., 2000; Chang et al., 2001; Wilson et al., 2001). BTEX are included in the current United States Environmental Protection Agency (USEPA) drinking water standards list under the National Primary Drinking Water Regulations (NPDWRs). The maximum drinking water levels for BTEX are 0.005, 1.0, 0.7 and 10 mg/L, respectively (USEPA, 2001). Environmental health water regulations applicable to public and private water supply systems in Mexico for potable water have set the maximum contaminant levels (MCL) for BTEX as 0.01, 0.3, 0.7, 0.5 mg/L, respectively (DOF, 2000). The MCL for benzene in unleaded gasoline distributed in the metropolitan area of the Mexico City Valley (MAMV) must not exceed 2.0% (v/v) (DOF, 1994). No maximum levels, however, are established for toluene, ethylbenzene and total xylenes in unleaded gasoline. Stringent environmental regulations require new and more sophisticated technologies to treat surface water as a result of potential
contamination from xenobiotic release. In those cases where groundwater is more available as a source of drinking water, prevention measures are to be undertaken to protect this valuable resource.

The purpose of this research is to evaluate predominant environmental conditions for biomass production in both unleaded gasoline and BTEX-fed batch bioreactors. Parameters including pH, oxidation reduction potential (ORP), temperature, cell mass as volatile suspended solids, (VSS), heterotrophic plate count, acid production, dissolved oxygen, BOD5, and COD were evaluated. BTEX removal capabilities of both unleaded gasoline acclimated biomass and BTEX acclimated biomass, and the effect of surfactant on BTEX biodegradation are also evaluated. The effect of BTEX concentration as the sole carbon source on cell growth in mixed reactors and the effect of yeast extract on cell growth in gasoline-fed reactors is also investigated. Predominant bacteria and fungi grown in the four mixed bioreactors are also characterized.

Materials and method

Chemicals
Benzene, toluene, ethylbenzene, mixed xylenes, o-, m-, p-xylene and Tergitol NP-10 (non-ionic surfactant) were purchased from Sigma-Aldrich Quimica S.A. de C.V. (Mexico) and were above 98% purity. Nutrient agar (Difco Laboratories, cat. No. DF 0001-17, Detroit, MI), and bacteriological agar (BIOXON, cat 150-1, Becton-Dickinson, Mexico) were purchased from Casa Rocas-Fisher Scientific (Mexico). Other chemicals used in this study were reagent grade and purchased from either Sigma-Aldrich Quimica, S.A. de C.V. or Casa Rocas-Fisher Scientific.

Biomass acclimation batch reactors
The biomass was grown using four separate 4-L dark glass bottles and aerated at a flowrate of 50 cc/s. Single daily manual additions of 100 ppm unleaded gasoline were made to two bioreactors for 18 months, one of which had unleaded gasoline as the sole source of carbon and the other had 0.005% yeast extract as an alternate source of carbon. BTEX bioreactors were fed with single daily manual additions of total concentration of 400 ppm BTEX (100 ppm benzene, 100 ppm toluene, 100 ppm ethylbenzene and 100 ppm mixed xylenes) and 1,200 ppm BTEX (200 ppm benzene, 200 ppm toluene, 200 ppm ethylbenzene and 600 ppm mixed xylenes) as the sole source of carbon for a period of 12 and 4 months, respectively. A maximum working volume of 2 L was maintained for all four bioreactors and culture medium was reconstituted once a week throughout the feeding time for all bioreactors.

Culture conditions
The following mineral concentration was prepared in deionized water and maintained in the seed biomass acclimation bioreactor (in mg/L) (Salanitro et al., 1994): KH2PO4, 17; K2HPO4, 44; Na2HPO4·2H2O, 67; MgSO4·7H2O, 23; NH4Cl, 3.4; (NH4)2SO4, 40; FeCl3·6H2O, 1. The four bioreactors were maintained at room temperature (27–32°C) and at pH 7.0–7.5. 1 N NaOH was added daily to keep the pH within this range. Separate bacteriological agar Petri plates spiked with unleaded gasoline and BTEX at concentrations exceeding the unleaded gasoline and BTEX water solubility were inoculated from the seed acclimation bioreactors for microbial isolation. The mineral concentration prepared for mixed culture batch reactor bioassays had the following composition (in g/L): Na2HPO4, 6; KH2PO4, 3; NaCl, 1; NH4Cl 1, MgSO4·7 H2O 0.5; CaCl2, 0.011; FeCl3·6H2O, 0.001.

Critical micelle concentration
The concentration of surfactant where a change in both culture medium density and surface
tension occurred was chosen as the critical micelle concentration (CMC). Several nonionic surfactant concentrations in the culture medium within the interval of 0.1 ppm to 1,000 ppm were evaluated.

**Experimental bioassays**

Bioassays were performed using 50 ppm as the initial concentration of each BTEX chemical to test the removal capabilities of unleaded gasoline and BTEX acclimated biomass of all four bioreactors. To evaluate xylene biodegradation, only o-xylene was added to all experimental bioassays. The initial surfactant concentration was set at 20 ppm for all bioassays. Samples were tested with and without surfactant and BTEX and surfactant concentrations were monitored for 10 days every 48 hours.

**Isolation of acclimated seed**

The unleaded gasoline and BTEX acclimated microorganisms were grown in unleaded gasoline and BTEX agar plates incubated at 28–30°C for 48–72 hours. Acid production in unleaded gasoline and BTEX agar plates was identified by change of color from blue to green, and in some cases from blue to yellow using bromothymol blue as indicator.

**Heterotrophic plate count**

The number of bacteria was counted for all four seed acclimation bioreactors once a week and for batch kinetic studies by using a Scienceware mini light box colony counter (Bel Art products Pequannock, N.J.) purchased from Sigma-Aldrich Quimica, S.A. de C.V. (Mexico), and reported as colony-forming units per milliliter of original solution (CFU/mL). Standard Methods 9215 A and 9215 B (*Standard Methods*, 1998) were followed for sample preparation and for estimating the number of heterotrophic bacteria.

**Volatile suspended solids**

Total cell mass was monitored as volatile suspended solids (VSS) following Standard Method 2540 E (*Standard Methods*, 1998) for all four bioreactors and for batch kinetic studies. Linear relationship of VSS concentration to heterotrophic plate count was followed by plotting the number of cells in the culture medium (CFU/mL) at different kinetic times with their equivalent VSS in mg/L. Correlation coefficients were evaluated to ensure appropriate fit of data.

**Incubation**

Microorganisms were incubated in a gravity flow Isotemp incubator (Fisher Scientific, USA), model 537D, purchased from Casa Rocas-Fisher Scientific (Mexico). Nutrient agar plates and unleaded gasoline and BTEX agar plates were incubated at 28–30°C for 48–72 hours.

**Batch study bioreactors**

Batch-scale kinetic studies were conducted using 40 mL Wheaton borosilicate glass EPA vials with Teflon™ fluorocarbon resin-lined top screw caps of GPI thread finish (Wheaton Science Products, Millville, NJ), leaving a headspace available for respiration.

**Mechanical shakers**

The vial kinetic batch bioreactors used for biotransformation studies were shaken using a Lab-line oscillating incubator shaker (Barnstead International, Dubuque, IA) model Orbit. Uniform shaking was maintained at 250 revolutions per minute at 30°C.
**Biomass centrifugation**

A fixed 320 mL culture medium volume containing acclimated biomass was taken from each of the four acclimation seed bioreactors and centrifuged in a Beckman Allegra™ centrifuge (Beckman-Coulter, Inc., Fullerton, CA) model 21R at 9,000 rpm at 28–30°C for 10 minutes. The biomass was resuspended in the mineral medium used for batch study bioreactors.

**Sample and control sterilization**

Samples and controls were autoclaved in a 21-L Presto autoclave (Industrias Steele, Mexico) following the time, pressure and temperature established (*Standard Methods*, 1998).

**Sonication extraction**

A Sonics and Materials, Inc., (Danbury, Connecticut) sonicator was used and programmed for each glass bottle at two cycles of two minutes each at a 80% duty cycle. USEPA method 3550 was used to release potential unleaded gasoline and BTEX trapped in cell membranes, and in any other cell compartment.

**Dissolved oxygen, BOD₅ and COD determination**

An Orion (Orion Research Inc., Boston, MA) model 97-08 Oxygen probe connected to an Orion EA 940 ion analyzer was used to measure dissolved oxygen (D.O.) in the acclimation bioreactor culture medium. D.O. calibration was effected on a daily basis and followed Standard Method 4500-O G (*Standard Methods*, 1998). BOD₅ and COD were determined for all four bioreactors following Standard Procedures 5210 B and 5220 A, respectively (*Standard Methods*, 1998).

**Determination of oxidation reduction potential (ORP) and pH**

A Corning redox combination model 476080 connected to an Orion EA 940 ion analyzer was used to measure the redox potential in samples and controls. For pH measurements an Orion combination pH electrode BNC model H-05711-41 connected to the Orion EA 940 ion analyzer was used. The reference electrode used was Ag/AgCl filled with a saturated KCl electrolyte solution. The redox and pH probes and the ion analyzer were checked for true readings on a daily basis.

**Gas chromatography**

The concentration of BTEX chemicals was determined by using a Varian 3400 GC/FID chromatograph. GC/FID determinations followed standard procedures (USEPA, 1986, 1995a, 1995b) with some modifications. A Petrochol™ (Supelco, Bellefonte, PA) 100 m × 0.25 mm ID × 0.5 µm film DH fused silica GC capillary column was used. The initial oven temperature was set up at 60°C and held for 30 minutes, after which the first temperature rate varied 10°C/min from 60°C up to 90°C, at which point the temperature was held for 20 minutes. A second temperature rate followed and varied 30°C/min from 90°C up to 150°C, at which point the temperature was held for 2 minutes. The injector was set up on a split/splitless mode (1:20) and its temperature was set at 250°C. The detector temperature was set at 300°C.

**Purge and trap concentrator**

The purge and trap concentrator was a Tekmar® (Cincinnati, OH) model LSC 2000. 5 mL samples were placed in the purge vessel. The purge and trap conditions followed Standard Method 6200 (*Standard Methods*, 1998) with some modifications. Samples were purged...
with nitrogen at 25°C for 10 minutes and desorbed at 225°C for 4 minutes. A baking time of 6 minutes at 230°C followed sample desorption.

**Results and discussion**

**Environmental conditions of acclimation bioreactors**

The environmental conditions were similar to all four bioreactors with the exception of the exposure to the type and amounts of the carbon source. Bacterial cell counts remained within the same order of magnitude in all four 2-L bioreactors. Increased biomass production was obtained by the addition of yeast extract to the unleaded gasoline bioreactor as indicated in Table 1. This increase in cell mass may have accounted for enhanced fungi growth. For the BTEX-fed bioreactors, cell mass was higher in the lower BTEX-fed reactor, suggesting potential inhibition of fungi growth for the higher BTEX concentration with no substantial change in bacterial cell counts (Table 2). Feeding time did not influence bacterial growth, but may have contributed to increased total cell mass, particularly for the BTEX-fed bioreactors. Among the bacteria isolated from experimental bioassays were *Pseudomonas aeruginosa*, *Pseudomonas* sp., *Bacillus* sp. and *Rhodococcus* sp. Fungi isolated included *Acremonium* sp., *Alternaria* sp., *Aspergillus* sp., *Curvularia* sp., *Humicola* sp. and *Penicillium* sp. Microorganisms isolated from all reactors tested positive on acid production as confirmed by color change of pH indicators (bromothymol blue and phenol red) in unleaded gasoline and BTEX agar plates.

**Acclimated seed from reactors 1 and 2**

BTEX removal by unleaded gasoline acclimated seed was evaluated to test the biomass capabilities to biodegrade each BTEX chemical under exposure to various environmental acclimation conditions. The biomass acclimated under 100 ppm unleaded gasoline as the sole source of carbon (reactor 1) was able to remove slightly higher BTEX concentrations than the biomass acclimated under unleaded gasoline using 50 ppm yeast extract as an alternate source of biomass (reactor 2). This suggests that yeast extract may inhibit the accli-

<table>
<thead>
<tr>
<th>Unleaded gasoline (ppm) (reactor)</th>
<th>Yeast extract (%)</th>
<th>Feeding time (months)</th>
<th>ORP range (mv)</th>
<th>T (°C)</th>
<th>Total cell mass (VSS) (mean) (mg/L) (std. dev.)</th>
<th>Bacterial cell counts (CFU/mL) (mean) (std. dev.)</th>
<th>pH mean (std. dev.)</th>
<th>D.O. mean (mg/L) (std. dev.)</th>
<th>BOD₅ (mg/L)</th>
<th>COD (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 (R1) 0</td>
<td>18</td>
<td>203–211</td>
<td>27–32</td>
<td>1,373</td>
<td>3.4 × 10⁹ (195) (4.6 × 10⁹)</td>
<td>7.18 (0.15)</td>
<td>9.03 (0.35)</td>
<td>406.3 (195)</td>
<td>1,975</td>
<td></td>
</tr>
<tr>
<td>100 (R2) 0.005</td>
<td>18</td>
<td>193–200</td>
<td>27–32</td>
<td>2,149</td>
<td>3.1 × 10⁹ (82) (1.8 × 10⁹)</td>
<td>7.32 (0.28)</td>
<td>8.9 (0.24)</td>
<td>440.2 (82)</td>
<td>1,472</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BTEX (ppm) (reactor)</th>
<th>Yeast extract (%)</th>
<th>Feeding time (months)</th>
<th>ORP range (mv)</th>
<th>Temp (°C)</th>
<th>Total cell mass (VSS) (mean) (mg/L) (std. dev.)</th>
<th>Bacterial cell counts (CFU/mL) (mean) (std. dev.)</th>
<th>pH mean (std. dev.)</th>
<th>D.O. mean (mg/L) (std. dev.)</th>
<th>BOD₅ (mg/L)</th>
<th>COD (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400 (R3) 0</td>
<td>12</td>
<td>214–225</td>
<td>27–32</td>
<td>2,307</td>
<td>2.4 × 10⁹ (463) (5.2 × 10⁹)</td>
<td>7.32 (0.28)</td>
<td>9.98 (0.27)</td>
<td>790.5 (463)</td>
<td>3298.1</td>
<td></td>
</tr>
<tr>
<td>1,200 (R4) 0</td>
<td>4</td>
<td>212–227</td>
<td>27–32</td>
<td>1,788</td>
<td>2.3 × 10⁹ (348) (1.6 × 10⁹)</td>
<td>7.27 (0.15)</td>
<td>9.0 (0.27)</td>
<td>1142.4 (348)</td>
<td>2422.4</td>
<td></td>
</tr>
</tbody>
</table>
mation of the seed for BTEX biodegradation as it constitutes an easily assimilated source of carbon. The two unleaded gasoline acclimated seed of reactors 1 and 2, were able to remove 99% ethylbenzene as indicated in Table 3. Benzene was the most recalcitrant to biodegradation (16–23%), followed by o-xylene (35–41%) and toluene (57–69%) for the unleaded gasoline acclimated biomass. This recalcitrance may be due to the low acclimation potential of the seed to benzene, since benzene concentration in unleaded gasoline is not expected to be higher than 2.0% v/v (DOF, 1994). In addition, benzene has been reported to be more recalcitrant than toluene (Chang et al., 2001). The nonionic surfactant Tergitol NP-10, was 96% removed and did not have a substantial effect on BTEX biodegradation for reactors 1 and 2 unleaded gasoline acclimated biomass at the end of the 10-day evaluation.

**Acclimated seed from reactors 3 and 4**

The biomass acclimated under BTEX as the sole source of carbon in reactor 3 at 400 ppm, showed higher BTEX removal efficiencies as compared to the unleaded gasoline acclimated biomass as indicated in Table 3. Toluene and ethylbenzene were 99% removed and the nonionic surfactant did not influence either toluene or ethylbenzene biodegradation at the end of the 10-day evaluation. The biomass acclimated under BTEX as the sole source of carbon at 1,200 ppm (reactor 4) showed the highest removal efficiency for benzene (99%). Although o-xylene was the slowest to remove, it showed little recalcitrance. Neither benzene or o-xylene removals were substantially influenced by Tergitol NP-10 at the end of the 10-day evaluation. Results from this research indicate that highest BTEX removal was obtained by the biomass acclimated with 1,200 ppm BTEX, particularly with respect to benzene, a known human carcinogen, suggesting that the biomass may need to be exposed to an amount as high as 200 ppm benzene under the conditions described in this study, to be able to remove 50 ppm benzene.

**Conclusion**

Biomass from all four acclimation bioreactors showed distinctive BTEX removal capabilities. For the unleaded gasoline acclimated biomass, benzene was the most recalcitrant of all BTEX chemicals followed by o-xylene. Ethylbenzene was the easiest biodegraded of all BTEX chemicals followed by toluene for all four bioreactor acclimated seed. Higher removal efficiencies were shown by the 400 ppm BTEX acclimated biomass with toluene, ethylbenzene and o-xylene being fully removed and benzene showing some recalcitrance. Highest BTEX removal efficiencies occurred with the 1,200 ppm BTEX acclimated biomass, suggesting that for 50 ppm benzene removal to occur, the biomass may need to be exposed to a benzene concentration higher than that under the environmental conditions described in this study. The nonionic surfactant Tergitol NP-10, was fully biodegraded in

<table>
<thead>
<tr>
<th>Bioreactor acclimated biomass</th>
<th>Benzone With surfactant</th>
<th>Toluene With surfactant</th>
<th>Ethylbenzene With surfactant</th>
<th>o-Xylene With surfactant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactor 1 UG*</td>
<td>23%</td>
<td>62%</td>
<td>99%</td>
<td>38%</td>
</tr>
<tr>
<td>Reactor 2 UG + yeast</td>
<td>19%</td>
<td>57%</td>
<td>99%</td>
<td>36%</td>
</tr>
<tr>
<td>Reactor 3 BTEX (400 ppm)**</td>
<td>83%</td>
<td>99%</td>
<td>99%</td>
<td>99%</td>
</tr>
<tr>
<td>Reactor 4 BTEX (1,200 ppm)</td>
<td>97%</td>
<td>98%</td>
<td>98%</td>
<td>92%</td>
</tr>
</tbody>
</table>

* Unleaded gasoline acclimation
** BTEX acclimation
all the experimental bioassays tested and did not have a substantial effect on either BTEX chemical at the end of the 10-day removal evaluation.

Acknowledgements
The authors thank Dr. Hugo A. Barrera-Saldana for providing special laboratory equipment, Dr. Jose G. Marmolejo, Dr. Guadalupe V. Nevarez-Moorillon, Dr. Humberto Rodriguez-Fuentes, Jorge J. Cervera-Maltos and Othonyel Gonzalez-Olazaran for their assistance in isolating and identifying fungal and bacterial strains. This research was funded by project No. FIES96-F-48VI under the auspices of the Instituto Mexicano del Petroleo; Start-up project No. I32968-B and Repatriation Fellowship No. C000/C300/1270 by CONACYT, and PAICYT projects No. SA241-99 and SA591-01 by UANL.

References


