Aerobic and Anaerobic Biodegradation of the Methyl Esterified Fatty Acids of Soy Diesel in Freshwater and Soil Environments

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Abstract
Soy diesel is composed of a mixture of methyl esterified long chain fatty-acids (e.g., C₁₈-C₂₀). Its biodegradation was demonstrated in both freshwater and soil environments under oxic and anoxic conditions as determined by flask test analysis. Bacterial populations increased by 4 to 5 orders of magnitude using soy diesel as the sole source of carbon. Soy diesel was completely degraded by 7 days in aerobic flasks and by 14 days in anaerobic flasks, as determined by gas chromatography. An aerobic bacterium, strain GSOY, and a denitrifying bacterium, strain H₁, were isolated using soy diesel as the sole source of carbon with oxygen (GSOY) or nitrate (H₁) as the terminal electron acceptor. Phylogenetic analysis of 16s rRNA gene sequence revealed that the two strains were members of the β Proteobacteria and related to the common soil bacteria Burkholderia cepacia and Burholderia solanacearum.
respectively. In subsequent experiments, GSOY, *B. cepacia* (ATCC 25416) and *B. solanacearum* (strain AW1ØA) were shown to express an inducible esterase and produce methanol when grown on soy diesel. Preliminary evidence suggests that the fatty acid is then either degraded via β-oxidation or incorporated directly into phospholipids. These results demonstrate that soy diesel is readily biodegraded under both oxic and anoxic conditions by the natural flora of soil and freshwater.
INTRODUCTION

Biodiesel, derived from crop oils such as soybean, rapeseed, and sunflower, is composed of small number of methylated fatty acids. Current interest centers on its use as an alternative or additive to petroleum diesel and as a replacement for other petroleum based products (e.g., lubricants, cutting fluids). In trials where biodiesel was used by itself or in blends with petroleum diesel, CO, particulate, NOx, sulfur and aromatic emissions were reduced while not sacrificing engine performance(9,19,27,30).

Biodegradation of hydrocarbons by natural populations of microorganisms represents one of the primary mechanisms by which petroleum and diesel products are removed from the environment (1,2,14,23,34). Many of the components of petroleum diesel, however, are recalcitrant to microbial degradation or are simply nonbiodegradable(1,2). Petroleum diesel consists of a complex array of hydrocarbons including alkanes, branched alkanes, cycloalkanes, and aromatic compounds(3). While many species of microorganisms can degrade the simple alkane components, other classes of diesel hydrocarbons such as branched and aromatic hydrocarbons are less susceptible to microbial attack(32). This is particularly true when oxygen is not available as the terminal electron acceptor. Soy diesel, or methyl soyate, is a less complex mixture having a limited variety of structures as compared to petroleum diesel, and is comprised predominantly of 8 different C16-18 fatty acid methyl esters (FAME). These include methyl esters of oleate, palmitate, stearate, linoleate, myristate, iso-stearate, laurate, and linolenate(26). Preliminary studies on its toxicity and biodegradability have
suggested that it is less toxic than petroleum diesel and biodegradable (26-28). These studies, however, have provided little information on the range of environmental conditions under which biodiesel can be degraded, the by-products formed, the rates and pathway of degradation, and the organisms involved in the process.

We report here the results of our study on the biodegradation of soy diesel in freshwater and soil environments under oxic and anoxic conditions, and the identification of soy degrading bacteria including species which can grow on soy diesel anaerobically with nitrate as the terminal electron acceptor. We also discuss the proposed pathway of degradation based on the current results.

MATERIALS AND METHODS

Aerobic flask tests. Aerobic flask tests were set up in 250 ml Erlenmeyer flasks. Each flask contained 100 ml of sterile minimal salts media (MS&I, 0.2g MgSO₄·7H₂O, 0.02g CaCl₂, 1.0 g KH₂PO₄, 1.0g K₂HPO₄, 1.0g NH₄Cl, and 10 ml base mineral mix (24) per liter of distilled water) with a final pH of 7.0. Test flasks were amended with 0.1 ml (88mg) of filter-sterilized soy diesel (Interchem Environmental, Incorporated, Leawood, KS) and inoculated with 1ml of freshwater sample (Squaw Run Pond or Old Hickory Lake), 1gm of soil sample (top soil), or 200μl of a 7 day pure culture grown on soy diesel. Two different controls were used. The first set of controls (soy diesel controls) included MSM and soy diesel without inoculum in order to determine if there were any abiotic breakdown of the methyl esters. The second set of controls (bacterial controls) included MSM and the inoculum without soy diesel. This control was
used to determine the amount of growth which occurred as a result of nutrient carryover. Flasks were placed in a gyrotory water bath (Model G76, New Brunswick Scientific) or in a gyrotory shaker (Model G10, New Brunswick Scientific) at 22-23°C for 14 days. All tests were run in triplicate.

**Anaerobic flask tests.** Anaerobic flask tests were set up in 125ml Wheaton septum top bottles. Each bottle contained 100ml of the MSM with KNO₃ (5g/L) and was rendered anoxic by sparging with oxygen-free CO₂ and N₂ (20:80) (18). After autoclaving, the medium was amended with 0.1ml of filter sterilized soy diesel and inoculated with either 1g of soil, 1ml of degassed freshwater sample, or 200μl of a 7 day pure culture grown on soy diesel using anaerobic technique (18). Three controls were done. The first contained MSM with soy diesel but no inoculum (again to test the stability of the methyl esters), the second was inoculated MSM without soy diesel (the bacterial growth control), and the third was inoculated MSM with soy diesel but without nitrate (NO₃-minus medium). This last control was done to determine if any growth occurred without the terminal electron acceptor. In addition, the production of nitrite was monitored using the methods from Parsons et al. (29). Flasks were shaken on a gyrotory shaker (Model G10, New Brunswick Scientific) at 200 rpm at 22-23°C for 14 days. All tests were run in triplicate.

**Bacterial enumeration.** Direct cell counts were done by epifluorescence with acridine orange using the method from Parsons et al. (29) with a Nikon Microphot SA. Samples were taken in tandem
with samples for the GC analysis at 0, 7, and 14 days and all counts were done in triplicate.

Gas chromatography. Samples were extracted by the method of Douglas et al. (10) using methylene chloride. One microliter of diluted sample (1:10) was analyzed on a Hewlett Packard 5890 Series II gas chromatograph equipped with a high-resolution fused capillary column (J&W Scientific DB-5, 30 m, 0.25 ID mm, 0.25 mm film) and a split/splitless injection port. Helium was the carrier gas. The injection port and detector temperatures were 300°C and 320°C, respectively. The oven temperature was maintained at 150°C for 4 min followed by a programmed temperature rise of 2.0°C/min to 195°C. A three-point calibration curve was used to establish the linear range for analysis and for quantification of sample analytes. Sample analyte recovery was corrected for through the use of an internal standard, methyl myristate. Although methyl myristate can be a component of soy diesel, it was only present in trace amounts in the soy diesel used in these experiments.

Isolation and culture of the soy degrading bacteria. The aerobic soy degrading bacterium, strain GSOY, was isolated by streak plating using MSM with 1.5% agar and soy diesel as the sole source of carbon. The anaerobic soy degrading bacterium, H1, was isolated by serial dilution using MSM with nitrate and soy diesel as the sole carbon source. Pure cultures were grown in septum top tubes (20ml) containing 10ml of sparged MSM with nitrate amended with 10 ml of soy diesel.

Pure cultures of Burkholderia cepacia (ATCC 25416), B. solanacearum (strain AW10A, kindly provided by Dr. Mark Schell,
University of Georgia) and *Pseudomonas oleovorans* (ATCC 8062) were maintained on nutrient broth (DIFCO).

**Phylogenetic analysis.** The 16s rRNA gene sequences for GSOY, H1, and *P. oleovorans* were obtained using PCR amplification with forward and reverse sequencing primers (15, 21) and have been deposited in Genbank (accession numbers U16142, U16144, and UXXXXXXX respectively). Additional sequences were obtained directly from Genbank and the Ribosomal Database Project (22). Sequences were aligned using the Clustal W program (17) and phylogenetic analysis done using a version of PHYLIP (maximum parsimony, 12) and MEGA (neighbor joining, 20). Evolutionary distances were estimated with the correction of Jukes and Cantor from 1200 bases. Bootstrap numbers are based on 1,000 trees.

**Esterase activity.** Esterase activity was detected using the methods in Shum and Markovitz (33). Cells from flask tests or pure cultures grown on soy diesel were harvested by centrifugation (5,000 x g, 20 min), lysed by sonication (three 30 sec bursts with a Braunsonic Ultrasonicator), and fractionated into cytoplasmic (supernatant) and membrane (pellet) fractions by centrifugation at 200,000 x g for 1 hr. Membrane fractions were run on native polyacrylamide gels (8% resolving gel, 4% stacking gel, 1% CHAPS detergent) and then stained with a naphthyl acetate and fast blue RR. Control cultures were grown on nutrient broth or MSM with oleic acid (1g/L) and harvested as described above.

**Methanol detection.** The presumptive analysis of methanol was determined with Draeger tubes (Fisher Scientific). Flasks were capped with a polypropylene stopper and evacuated
through the Draeger tube at 20psi for 2min. Crimp top Wheaton bottles were measured in the same manner through the septum. The results were determined using the methanol Draeger scale using the relative values: -- =0 (none detected), + =<500, ++ =500-2000, and +++ >2000.

RESULTS

Environmental Samples. Soy diesel stimulated the growth of bacterial populations in freshwater and soil flask tests both under oxic and anoxic conditions. Initial studies on population growth done under oxic conditions over 21 days using a freshwater inoculum showed that after an initial lag phase of about 2 to 4 days, the populations grew exponentially up until 7 days and then maintained stationary phase (Figure 1). Subsequently, samples for bacterial counts and GC analysis were taken at 0, 7, and 14 days.

Flask test analyses using freshwater inoculum showed the concomitant growth of microbial populations with the disappearance of the fatty acid methyl esters (FAMEs) of soy diesel (Figures 2A and 3A). Bacterial populations increased from $10^4$ to $10^{10}$ cells/ml for Old Hickory Lake samples (Figure 2A) and from $10^5$ to $10^9$ cells/ml for Squaw Run Pond samples in the aerobic flask tests (Figure 3A). Control flask tests which contained only MSM and inoculum increased only two orders of magnitude. Bacterial populations increased from $10^5$ to $10^9$ cells/ml for Old Hickory Lake samples and from $10^5$ to $10^{10}$ cells/ml for Squaw Run Pond samples in the anaerobic flasks that had been amended with nitrate (Figures 2B and 3B respectively). Again, the population in control flasks not amended with soy diesel increased less than two orders of magnitude.
Anaerobic flask tests using MSM prepared without the addition of KNO3 had bacterial counts no greater than controls (2.8 x 10^5 cells/ml vs. 2.1 x 10^5 cells/ml for Old Hickory Lake, 2.7 x 10^5 cells/ml vs. 2.1 x 10^5 cells/ml for Squaw Run Pond).

FAMEs were reduced to undetectable levels by seven days in the aerobic freshwater (Figures 2 and 3) and soil flask tests (Figure 4). Appreciable degradation occurred by day 7 with complete disappearance by day 14 in anaerobic freshwater and soil flasks. There also was a noticeable loss of FAME after 14 days in the sterile controls (Figures 2, 3 and 4) but no increase in microbial population (Figures 2 and 3).

Soy degrading isolates. Nine isolates of aerobic soy degrading bacteria were obtained from Old Hickory Lake enrichments. All nine strains were identified as strains of Burkholderia cepacia based on physiological characteristics (Rapid ID). One strain, GSOY, was selected for 16s rRNA sequence analysis.

Four strains of soy degrading bacteria were isolated from Old Hickory Lake samples using anaerobic conditions with nitrate as the terminal electron acceptor. They were shown to also grow aerobically on soy diesel, but were not further characterized biochemically. One strain, H1, was chosen for 16s rRNA sequence analysis.

Phylogenetic analysis. Phylogenetic analysis with maximum parsimony and neighbor joining methods using the 16s rRNA sequence revealed that both GSOY and H1 were members of the β subclass of the Proteobacteria (Figure 5). GSOY was most closely related to B. cepacia and B. gladioli, while H1 was most closely related to the plant pathogen B. solanacearum and B. pickettii (Table
Our analyses have also shown that the original petroleum degrading bacterium, *Pseudomonas oleovorans*, is a γ Proteobacterium most closely related to *P. mendocina* (Figure 5).

Soy diesel degradation in pure cultures. Strains of *Burkholderia cepacia* (GSOY and ATCC 25416) and *B. solanacearum* (strain AW10A) were tested for their ability to grow on soy diesel. Populations of GSOY increased from $10^4$ cells/ml to $10^6$ cells/ml in 14 days with the concomitant disappearance of FAMES (Figure 6). Populations of *B. solanacearum* grown under anoxic conditions increased from $10^4$ cells/ml to $10^6$ cells/ml in 14 days also with the complete utilization of FAMES and production of nitrite (Figure 7).

Membrane fractions of all strains tested showed that when the organisms were grown on soy diesel, they expressed an inducible esterase (Figure 8). It was not expressed when the cells were grown on either nutrient broth (Figure 8) or MSM with oleate (data not shown). Furthermore, methanol was detected in only cultures fed soy diesel (Table 2).

**DISCUSSION**

The data presented here show that soy diesel can support the growth of bacteria with concomitant disappearance of FAMES. It can be degraded under both oxic and anoxic conditions by the microbial populations found in freshwater and soil environments. An extended lag phase was observed in all experiments using natural samples or pure cultures which had not previously been exposed to soy diesel (Figure 1). This lag phase was eliminated when samples were preadapted with soy diesel (e.g., 7 day enrichments or pure cultures)
suggesting that the organisms don't grow on the soy diesel until the esterase is expressed.

Phylogenetic analysis based on 16s rRNA sequence has revealed interesting relationships in the soy degrading bacteria. The anaerobic soy degrading bacterium strain H1 is closely related to the plant pathogen *B. solanacearum*, while GSOY is closely related to *B. cepacia* and *B. gladioli* also plant pathogens. These results initially suggested that the soy degrading bacteria might be a closely related phylogenetic group (e.g., confined to the β-Proteobacteria). However, examination of other species including *Acinetobacter lwoffii*, *Pseudomonas oleovorans*, and a marine isolate (*Pseudomonas* sp. strain Marsoy) show this is not the case. Rather, the presence of an inducible esterase is the defining characteristic of a FAME degrader.

Our results also have begun to elucidate the pathway of soy diesel degradation. The first step is the cleavage of the methyl group by an inducible esterase. An inducible esterase has been detected in all species we have examined which can grow on soy diesel (GSOY, *B. cepacia* ATCC 25416, *B. solanacearum* strain AW10A, *Acinetobacter lwoffii* strain RAG-1). This was not surprising given that *Pseudomonas* sp. and *B. cepacea* had previously been shown to degrade short chain FAMEs to the fatty acid and methanol (31,33) and esterase activity had been detected in *B. solanacearum* (7).

Once the free fatty acids are formed, they may enter intermediary metabolism to be oxidized to acetate via β-oxidation (32) or be directly incorporated into phospholipids. Labeling experiments using $^{14}$C-labeled oleic acid revealed the presence of labeled phospholipid in both GSOY and *B. cepacia* (M. Davis, personal
communication). Furthermore, the cells accumulate conspicuous intracellular inclusions suggestive of poly-β-hydroxyalkanoates or unmetabolized FAME (16,32). These inclusions could apparently provided substrate for growth as growth occurred in control flasks without soy diesel when washed cell suspensions of preadapted cultures were used (Figure 7).

The biodegradation of FAMEs under anoxic conditions also has precedent. *Eubacterium limosum* and *Acetobacterium woodii* have been shown to anaerobically metabolize methyl esters of acetate and propionate with CO₂ and H₂ (25). Our results show that *B. solanacearum* is able to grow anaerobically with soy diesel as the sole source of carbon with nitrate as the terminal electron acceptor. It expresses the inducible esterase under these conditions producing both methanol and nitrite. This result also suggests that other electron acceptors such as Fe(III) and sulfate may be suitable so long as the species has an inducible esterase.

It is estimated that 3.3 million barrels of petroleum diesel are consumed daily for transportation, residential, commercial, and industrial uses (24). Petroleum diesel is involved on average in 21% of all spill incidents (11). Although petroleum diesel is degradable under oxic conditions, it is recalcitrant to degradation under anaerobic conditions (4,5). Thus, the environmental impact of a biodiesel spill, as a result of transportation, use, and storage, should be far less than petroleum diesel especially under anoxic conditions. However, the ability of microbes to utilize soy diesel under anoxic conditions with alternative electron acceptors such as NO₃, may impact its long term stability in pipelines and storage tanks.
ACKNOWLEDGEMENTS
We acknowledge the Ribosomal Data Base Project and James Garey for assistance in sequence comparisons. The work was supported under a cooperative agreement with the USDA.

REFERENCES


Figure legends

**Figure 1.** Bacterial growth under oxic conditions using freshwater inocula. Old Hickory Lake (■) with soy diesel, (□) without soy diesel; Squaw Run Pond (●) with soy diesel, (○) without soy diesel; (△) soy diesel no inoculum.

**Figure 2.** Bacterial growth with the concurrent disappearance of fatty acid methyl esters with Old Hickory Lake samples. A) oxic conditions, B) anoxic conditions with nitrate as the terminal electron acceptor. (□) inoculum without soy diesel, (●) inoculum with soy diesel, (■) FAMES without inoculum, (●) FAMES with inoculum. Bacterial counts are in cells/ml, FAMES in mg.

**Figure 3.** Bacterial growth with the concurrent disappearance of fatty acid methyl esters with Squaw Run Pond samples. A) oxic conditions, B) anoxic conditions with nitrate as the terminal electron acceptor. (□) inoculum without soy diesel, (●) inoculum with soy diesel, (■) FAMES without inoculum, (●) FAMES with inoculum. Bacterial counts are in cells/ml, FAMES in mg.

**Figure 4.** Bacterial degradation of soy diesel in soil flask tests based on GC analysis (FAMES in mg). (□) oxic conditions without inoculum, (■) oxic conditions with inoculum, (□) anoxic conditions without inoculum, (□) anoxic conditions with inoculum.
Figure 5. Phylogenetic analysis of the soy degrading isolates based on 16s rRNA sequence. Although both maximum parsimony and neighbor joining methods were used only the PHYLIP generated tree is shown. Only bootstrap numbers greater than 50 are shown.

Figure 6. Soy diesel degradation by strain GSOY, (□) inoculum without soy diesel, (○) inoculum with soy diesel, (■) FAMEs without inoculum, (●) FAMEs with inoculum.

Figure 7. Soy diesel degradation by *Burkholderia solanacearum* grown under anoxic conditions with soy diesel and nitrate. A) cell growth and FAME disappearance, (□) inoculum without soy diesel, (○) inoculum with soy diesel, (■) FAMEs without inoculum, (●) FAMEs with inoculum. Bacterial counts are in cells/ml, FAMES in mg/ml. B) nitrite production (○) cells/ml, (■) mmol of nitrite.

Figure 8. Esterase activity in pure cultures. Lane 1, GSOY on soy diesel, lane 2, GSOY on nutrient broth, lane 3, *Burkholderia cepacia* on soy diesel, lane 4, *B. cepacia* on nutrient broth, lane 5, *B. solanacearum* aerobic on soy diesel, lane 6, *B. solanacearum* aerobic on nutrient broth, lane 7, *B. solanacearum* anaerobic with nitrate and soy diesel. Each lane was loaded with 75 µg of total protein. Arrow indicates esterase.
TABLE 1

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Organism (RDB #): Bacillus subtilis (449), Escherichia coli (2150), Pseudomonas oleovorans (this study), Pseudomonas mendocina (2007), Pseudomonas aeruginosa (2000), Pseudomonas flavescens (2005), Oceanospirillum multiglobuliferum (1985), Marinomonas vag a (1995), GSOY (this study), Burkholderia gladioli (1811), Burkholderia solanacearum (1786), H1 (this study), Burkholderia cepacia (1809), Burkholderia pickettii (1797), Burkholderia andropogonis (1806).
<table>
<thead>
<tr>
<th>Strain</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Burkholderia cepacia</em></td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>GSOY</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><em>B. solanacearum</em> (aerobic)</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><em>B. solanacearum</em> (anaerobic)</td>
<td>-</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>
Escherichia coli

Pseudomonas flavescens

Pseudomonas oleovorans

Pseudomonas mendocina

Flavobacterium lutescens

Pseudomonas aeruginosa

Burkholderia pickettii

Burkholderia solanacearum

Alcaligenes eutrophus

GSOY1

Burkholderia cepacia

Burkholderia gladioli

Burkholderia caryophylli

Burkholderia andropogonis

Bacillus subtilis