

## Biocatalytic Ring Cleavage of Aromatic Hydrocarbons and Heterocycles Commonly Present in Petroleum Distillates.

Q. Wu\*, P.M. Fedorak, M.A. Pickard, M.R. Gray, and J.M. Foght  
Univ. of Alberta, Edmonton, Alberta CANADA.

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### ABSTRACT

A potential alternative to conventional fuel upgrading techniques is to use bacteria under near-ambient conditions to catalyze aromatic ring cleavage in the first step of a two-stage upgrading process. The resulting biocatalytic products would be chemically hydrogenated under mild conditions in the second stage to produce the desired alkylbenzenes. Experiments testing biocatalytic ring cleavage of aromatic hydrocarbons are described in this study.

*Pseudomonas fluorescens* LP6a was chosen as the biocatalyst because of its broad-specificity aromatic hydrocarbon-degrading enzymes which confer the ability to oxidize a wide range of polycyclic aromatic hydrocarbons and heterocycles commonly present in petroleum middle distillates. Pre-grown, induced whole cell biocatalysts in aqueous suspension produced the predicted polar intermediates from aromatic substrates without altering the aliphatic carrier and without carbon loss from the aromatic substrate. Quantitative studies with  $^{14}\text{C}$ -labeled aromatic substrates incorporated into synthetic and authentic distillates were carried out to reveal potential process-limiting factors. Parallel studies were performed using gas chromatography with a flame ionization detector to quantify aromatic ring cleavage in authentic distillates.

### INTRODUCTION

Chemical hydrogenation processes are currently used to upgrade middle distillates produced by thermochemical cracking of heavy oils and bitumen. These distillates have a high content of low fuel value di- and tricyclic aromatics, which are reduced by expensive high temperature-high pressure hydrogenation to compounds such as alkylbenzenes. The catalysts involved are frequently deactivated by sulfur- and nitrogen-containing compounds in the feedstocks. These expenses make it desirable to explore an alternative to conventional upgrading techniques. An attractive alternative is to use bacteria to enzymatically cleave the fused-ring aromatics under near-ambient conditions, followed by mild chemical hydrogenation to produce the desired alkyl aromatics.

Biocatalytic technology has been used widely in crude oil biodegradation and is being assessed for biodesulfurization. It can be cost effective, competitive or compatible with current technology. It is well known that one ring of the di- and tricyclic aromatics can be opened selectively by bacterial enzymes. Although the oxygenated ring cleavage products are themselves undesirable, they may be converted to alkylaromatics under milder hydrogenation conditions than the parent feedstocks, resulting in cost savings.

Besides yielding the desired ring fission products, two primary requirements of the biological process are essential. First, the biocatalytic activity must be restricted to aromatic compounds and must be effective over the wide range of di- and tricyclic aromatic hydrocarbons, heterocycles and alkyl-substituted homologues common to middle distillates. Second, there should be no carbon loss from the aromatic substrates as a consequence of microbial oxidation; that is, the process has to be blocked at certain stage to prevent complete oxidation. Other advantages such as the ability of the cells to catalyze ring cleavage in a resting state and to be pre-grown quickly to high density in an active state would greatly benefit the process.

The research project described here deals with the biocatalytic stage for ring cleavage of aromatics in the middle distillates. *Pseudomonas fluorescens* LP6a was previously reported to possess several of the properties listed above and thus was chosen for this study. For example, LP6a mutant 21-41 has been shown to accumulate the expected ring cleavage products from a variety of pure aromatic compounds commonly present in petroleum distillates (Foght et al. 1997). In the experiments reported here,  $^{14}\text{C}$ -labeled phenanthrene was incorporated into synthetic and authentic distillates to detect biotransformation of the substrate into water-soluble ring cleavage products. Parallel studies were performed using gas chromatography (GC) with a flame

ionization detector (FID) to quantify aromatic ring cleavage in authentic distillates. These results are leading towards optimization of biocatalytic ring cleavage for bio-upgrading of petroleum distillates.

## METHODS AND MATERIALS

### Preparation of biocatalyst

Transposon mutants of *P. fluorescens* LP6a were generated by conjugation using a suitable Tn5 donor plasmid with subsequent screening for desired phenotypic changes (Foght and Westlake, 1996). Cultures of *P. fluorescens* LP6a mutants were incubated to high density in Tryptic Soy Broth (Difco; typically 200 mL) containing kanamycin (to maintain the transposon) at 30°C with agitation for 24 h. Enzymatic activity was specifically induced with salicylic acid (Yen and Serdar, 1988), and the cells were harvested by centrifugation and resuspension in 3 mM potassium phosphate buffer (pH 7). The protein content of biocatalyst suspensions was determined using the biocinchoninic acid assay (Pierce, Rockford IL).

### Substrates and middle distillates

Seventeen aromatic compounds including naphthalene and its methyl-, ethyl- and dimethyl-substituted homologues, phenanthrene and dibenzothiophene (DBT) have been tested for ring cleavage with the chosen mutant 21-41 (Foght et al. 1997). Selected aromatic model compounds were dissolved in the aliphatic carriers *n*-hexadecane or heptamethylnonane (HMN; Efroymson and Alexander 1991) to provide a two-phase system referred to as a "synthetic distillate," or presented directly in authentic distillates. A brief description of the distillates used in this study is summarized in Table 1. The substrate solution was then added to the suspension of induced biocatalyst and incubated for 24 h to effect biotransformation of the substrates. Parallel sterile controls were also prepared.

Table 1. Brief description of authentic distillates used in this study

SOURCE	TYPE	Boiling point range	S content * (mol% of cut)
3HPP0015	Middle distillate	177-343°C	0.118
3HPP0016	Middle distillate	177-343°C	0.319
3HPP0017	Middle distillate	177-343°C	0.407
3HPP0018	Middle distillate	177-343°C	0.359
E #1	Distillate cut	177-343°C	NA
LCGO	Gas oil	NA	NA

\* the 3HPP series was subjected to various hydrogenation conditions; elemental analyses were performed, with only the sulfur content reported here.

NA = not available

### Radioactive and chemical analysis

To determine the biotransformation of the aromatic compounds quantitatively, <sup>14</sup>C-labeled phenanthrene (Amersham) was incorporated into synthetic and authentic distillates and incubated with the biocatalyst as described above. A two-step extraction procedure was used to extract and recover the radioactivity. First, unaltered non-polar substrates and distillates were recovered by extraction with pentane in the presence of the cells at neutral pH. Second, after the cells had been removed by centrifugation and the supernatant acidified to pH < 2, ethyl acetate was used to recover the polar ring fission products. Radioactivity was measured by liquid scintillation (Beckman model LS 3801) and the ratio of radiolabel recovered from the polar phase to that of total added was calculated as the percent converted to polar metabolites ("biotransformation percentage").

Similar extraction procedures were applied to the incubated cultures in quantitative GC experiments. Phenanthrene was usually used as the model substrate and biphenyl and benzothiophene were added as the internal standards to determine the relative residual mass of the model substrate in the pentane extracts. The biotransformation percentage was determined by comparing the relative residual mass of the model substrate incubated with the biocatalyst with that of the model compound in a parallel sterile control.

A silica gel column fractionation technique (Fedorak and Westlake 1981) was used to characterize the composition of the authentic distillates. The saturates and aromatics fractions were studied in detail to determine their susceptibility to microbial transformation by the biocatalyst compared with the whole distillate. Routine GC analysis of fractions and distillates was performed on a Hewlett-Packard model 5890 GC system equipped with a FID and a sulfur-selective flame photometric ionization detector. Chromatography conditions have been described previously (Foght and Westlake 1996).

## RESULTS AND DISCUSSION

### Radiometric determination of substrate biotransformation

Radiolabeled phenanthrene was incorporated into an aliphatic carrier or an authentic distillate to serve as a surrogate substrate for biotransformation. The radioactivity of the corresponding extracts was measured by liquid scintillation and the total recovery of the radiolabel and the conversion percentage were calculated. In this way, conversion of a specific compound can be monitored in the context of a complex mixture of substrates. Results are shown in Table 2.

**Table 2.** Transformation of [9-<sup>14</sup>C]-phenanthrene presented in a hydrocarbon carrier: synthetic or authentic distillates or fractions of authentic distillates. Percent biotransformation is the fraction of added radiolabel recovered from the polar phase. Total recovery is expressed as a percentage of the radiolabel added.

Presentation of <sup>14</sup> C-phenanthrene in:	Radioactivity	
	% biotransformed	% recovery
Heptamethylnonane (HMN)	80	100
Mixture of HMN and the aromatics fraction of 3HPP0015 (2:1 V/V)	76	90
Aromatics fraction of 3HPP0015	68	91
Saturates fraction of 3HPP0015	72	91
Whole authentic distillate 3HPP0015	16	99
Whole authentic distillate 3HPP0016	58	102

The total recovery of the radiolabel was  $\geq 90\%$  for all experiments. Biotransformation of phenanthrene in HMN and the individual distillate fractions was relatively high, but biotransformation was substantially lower for whole 3HPP0015 than for whole 3HPP0016; the reason for this observation is unknown at present.

### Gas chromatographic quantitation of biotransformation

In general, biotransformation levels of  $>80\%$  can be obtained either for pure phenanthrene or phenanthrene presented in a synthetic distillate, and quantitation by GC is relatively easy because of the simplicity of the samples. However, quantitative GC analysis in the presence of the authentic distillates is difficult, with the results being semi-quantitative due to the complexity of the substrates.

A silica gel column fractionation technique was used to gravimetrically determine the composition of selected distillates (Table 3). Some fractions then were used as carriers for presentation of radiolabeled or unlabeled phenanthrene to investigate whether specific fractions or the whole distillate were inhibitory to biocatalysis. Biotransformation was analyzed radiometrically or by GC as reported in Table 4.

It can be seen from Table 4 that distillate saturates fractions did not inhibit biotransformation of phenanthrene. The saturates fractions were not oxidized by the biocatalyst, as determined by GC analysis (data not shown). However, certain aromatics fractions, especially the aromatics fraction of LCGO, had an inhibitory effect on phenanthrene bioconversion. The aromatics in the authentic distillates may compete with the analyte substrate (phenanthrene) for enzyme activity, or may inhibit the conversion of phenanthrene through toxicity to the biocatalyst directly or via their metabolites. Alternatively, inhibition may be a reflection of poor mass transfer during the biotransformation process.

**Table 3.** Fraction composition of selected distillates, prepared by silica gel column fractionation; mean  $\pm$  1 standard deviation, n=2

Distillate	Distillate composition (weight % of fraction)			
	Saturates	Aromatics	Polar	Asphaltenes
3HPP0015	55.4 $\pm$ 4.7	26.2 $\pm$ 2.2	0.80 $\pm$ 0	0.5 $\pm$ 0.4
3HPP0016	41.2 $\pm$ 2.6	41.2 $\pm$ 4.8	2.2 $\pm$ 0.6	ND
3HPP0018	59.7 $\pm$ 0.4	33.3 $\pm$ 0.1	1.2 $\pm$ 0.3	ND
E #1	59.0 $\pm$ 2.9	27.2 $\pm$ 1.6	6.2 $\pm$ 0.6	ND
LCGO	36.4 $\pm$ 3.7	55.5 $\pm$ 3.5	5.4 $\pm$ 0.2	ND

ND = not determined

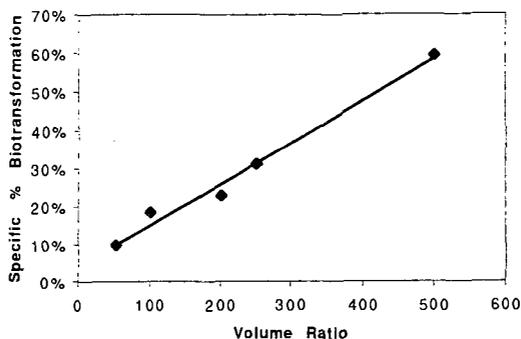
**Table 4.** Summary of biotransformation of phenanthrene presented in different carriers and incubated under standard conditions. Fractions were obtained by silica gel column chromatography of the authentic distillates listed in Table 3. The biotransformation percentage was determined either radiometrically or by quantitative GC, compared with parallel sterile controls.

Distillate	Presentation of phenanthrene in:	% Biotransformation	Analytical method
3HPP0015	Saturates fraction	72	Radiometric
	Aromatics fraction	76	Radiometric
	Whole distillate	36	GC-FID
3HPP0016	Whole distillate	60	GC-FID
E #1	Saturates fraction	74	GC-FID
	Aromatics fraction	64	GC-FID
	Whole distillate	62	GC-FID
LCGO	Saturates fraction	94	GC-FID
	Aromatics fraction	43	Radiometric
	Whole distillate	53	GC-FID

To distinguish among these possibilities, experiments were designed to elucidate limiting factor(s), using phenanthrene presented in the aromatics fraction of LCGO, an inhibitory carrier. In the first experiment, replicate samples of biocatalyst were suspended in different volumes of phosphate buffer to yield cell suspensions covering a 10-fold range of biomass density (measured as mg protein per ml suspension). To these suspensions, a constant volume of carrier containing 8.9 mg of phenanthrene was added, so that the only variable among tests was the biomass density. Biotransformation of the phenanthrene was calculated in either absolute units (percentage of phenanthrene biotransformed) or specific units, obtained by dividing the absolute units by the biomass density. Results are shown in Figure 1.

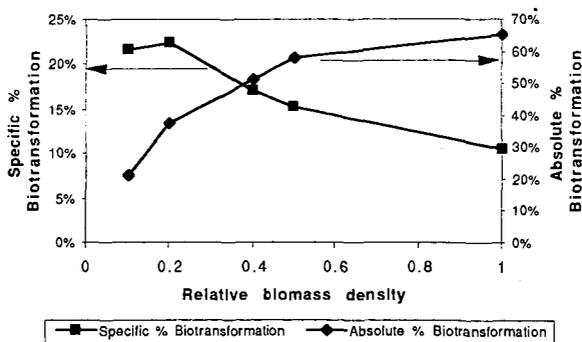
If the inhibitory effect of the carrier was due to competition for enzyme activity between phenanthrene and alternate substrates in the carrier, a constant specific % biotransformation would have resulted (i.e. a horizontal line) because the ratio of phenanthrene to the carrier was constant in the flasks and the same total biomass was present in all flasks. The results in Figure 1 are best explained if toxicity is the predominant inhibitory factor, because increasing biotransformation was observed as the volume ratio of cell suspension to carrier increased. That is, with increasing biocatalyst suspension volumes, the concentration of toxic compounds would decrease regardless of whether these toxic compounds were the parent aromatics or their metabolites.

**Figure 1.** Specific biotransformation (% biotransformation of phenanthrene per mg biomass protein per ml biocatalyst suspension) as a function of the volume ratio of biocatalyst suspension to carrier. Phenanthrene was presented in the aromatics fraction of LCGO and measured by quantitative GC-FID.



In the second set of experiments, the biomass density was varied over a 10-fold range by resuspending different amounts of biocatalyst in a constant volume of phosphate buffer, keeping the phenanthrene concentration and carrier volume constant. That is, the volume ratio of cell suspension to carrier was kept constant with only the biomass density varying. Phenanthrene bioconversion is presented in Figure 2 both as the absolute % biotransformation in a flask and as the specific % biotransformation, calculated per mg biomass protein. These values were plotted against the relative biomass density, with 1.0 being the cell density of an overnight culture and 0.1 being one-tenth this cell density.

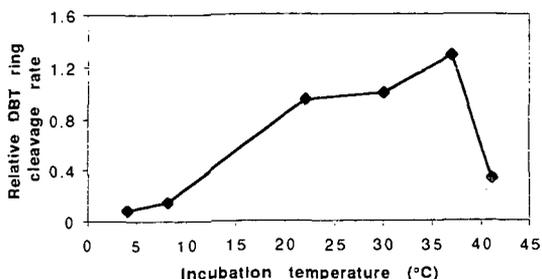
**Figure 2.** Absolute % biotransformation of phenanthrene and specific % biotransformation of phenanthrene as a function of the relative biomass density, with 1.0 being the biomass density of an overnight culture. Phenanthrene was presented in the aromatics fraction of LCGO and measured by quantitative GC-FID.



The absolute % biotransformation increased as the biomass density increased. This is as predicted, because the total amount of phenanthrene converted to polar metabolites should be greater if more cells are present. This biotransformation begins to plateau at about 60% when the relative biomass is approximately 1.0. In contrast, the "efficiency" of biocatalyst activity, calculated as specific % biotransformation, decreased above a relative biomass density of 0.2. That is, as the cell density increased, the biotransformation activity per mg biomass protein decreased. This may be due to mass transfer effects as the substrate has less access to individual cells due to "crowding" of the biocatalyst in suspension, or due to limitation of oxygen diffusion in the suspension, or it may be due to increased accumulation of toxic metabolites by the increased mass of cells. We cannot discriminate among these possibilities at present, although

mass transfer seems unlikely to be a significant factor at the relatively low cell densities used in these experiments, and it seems unlikely that oxygen is limited under the fairly vigorous aeration conditions provided in the flasks (200 rpm agitation).

**Figure 3.** Relative rates of DBT ring cleavage at increasing incubation temperatures, normalized to enzyme activity at 30°C.



The effect of incubation temperature on biocatalysis was determined at six temperatures by incubating replicate batches of biocatalyst with DBT, a heterocyclic aromatic substrate. Rates of DBT ring cleavage were calculated spectrophotometrically (Foght et al. 1997) as Absorbance Units at 475 nm (the maximum absorption wavelength for the DBT ring cleavage product) produced per min incubation. Rates were normalized to the rate at 30°C as the indicator of relative enzyme activity, as shown in Figure 3. The ring cleavage rate increased with temperature, reaching maximum activity around 37°C, typical for a mesophilic bacterium like *P. fluorescens* LP6a.

### CONCLUSIONS

- *P. fluorescens* LP6a biocatalyzes ring cleavage of the substrate phenanthrene when presented in several water-immiscible carriers including heptamethylnonane, various whole authentic distillates, and the saturates and aromatics fractions of those authentic distillates.
- Greater than 80% of the substrate can be biotransformed by the biocatalyst into polar ring cleavage products within a 24-h contact period.
- Ring cleavage activity is influenced by toxicity of some component(s) of the authentic distillates or their metabolites, particularly in the aromatics fraction. This knowledge may lead to general rules of distillate susceptibility to ring cleavage biocatalysis.
- Ring cleavage activity is influenced by temperature, with an optimum of ca. 37°C

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