

# BIOCATALYTIC RING CLEAVAGE OF DIBENZOTHIOPHENE AND PHENANTHRENE IN A BIPHASIC FERMENTER SYSTEM

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Keywords: Biotransformation, petroleum, fermenter

## ABSTRACT

Our previous research has demonstrated the ability of *Pseudomonas fluorescens* LP6a to cleave the fused-ring aromatic hydrocarbons and heterocycles commonly present in petroleum distillates. In this study, experimental conditions for biocatalytic ring cleavage of dibenzothiophene and phenanthrene in a biphasic 3 liter fermenter system were investigated.

Compared with batch flask experiments, a fermenter system provides the advantage of large scale, easy automation and versatile control of the experimental conditions. Growth curves of the biocatalyst and the relative oxidation rate of dibenzothiophene at different growth phases were determined. Incubation of dibenzothiophene and phenanthrene in various carriers was performed in the fermenter system. Rapid complete biotransformation of dibenzothiophene in light mineral oil and improved biotransformation of phenanthrene in diesel fuel were obtained in a fermenter system compared with batch flask conditions.

## INTRODUCTION

Petroleum middle distillates contain low fuel value di- and tricyclic aromatics. Currently, expensive and non-specific chemical hydrogenation processes involving high temperature and high pressure are used to reduce di- and tricyclic aromatics to alkylbenzenes to increase their fuel value. It is desirable therefore to explore other economical alternatives to conventional upgrading techniques.

Our previous research has demonstrated the ability of *Pseudomonas fluorescens* LP6a to cleave fused-ring aromatic hydrocarbons and heterocycles commonly present in petroleum distillates [Wu et al., 1998]. This provides an attractive potential alternative to conventional fuel upgrading techniques as a two-stage upgrading process: first bacteria would enzymatically cleave the fused-ring aromatics under near-ambient conditions, then mild chemical hydrogenation of the ring opening products would yield the desired alkyl aromatics.

We previously demonstrated ring cleavage of aromatics in middle distillates under batch flask conditions, typically using 200 mL of biocatalyst suspension and <1 mL of distillate [Foght et al., 1997; Wu et al. 1998]. To scale up the reaction volumes, experimental conditions for biocatalytic ring cleavage of dibenzothiophene and phenanthrene in a 3 L fermenter system were investigated in this study. A biphasic system was used, with the ring cleavage substrate dissolved in a water-immiscible carrier and the biocatalyst suspended in an aqueous buffer.

Compared with batch flask experiments, a fermenter system provides the advantage of large scale, automation and versatile control of the experimental conditions such as aeration and mixing. Growth curves of the biocatalyst and the specific rate of dibenzothiophene (DBT) ring cleavage at different growth phases were determined. Results are presented from biotransformation of the model ring cleavage substrates dibenzothiophene or phenanthrene dissolved in light mineral oil, diesel fuel or a petroleum middle.

## METHODS AND MATERIALS

### Preparation of biocatalyst

A transposon mutant of *P. fluorescens* LP6a was generated by conjugation with a suitable *Tn5* donor plasmid [Foght and Westlake, 1996] and subsequent screening for desired ring cleavage products. Cultures of *P. fluorescens* LP6a mutant #21-41 were incubated to high density at 30°C with agitation for 24 h in Tryptic Soy Broth (Difco; typically 200 mL) containing kanamycin to maintain the transposon. The 200 mL seed culture was then used to inoculate a fermenter (New Brunswick, NJ) containing 3 L Tryptic Soy Broth plus kanamycin. The culture was incubated at 30° C with aeration at 4 L min<sup>-1</sup> sterile air and stirring at 300 rpm.

To determine optimum biocatalyst preparation conditions, the culture optical density at 600 nm and protein concentration (BCA protein assay; Pierce, Rockford IL) were determined at intervals in the fermenter culture. As well, approximately 50 mL subsamples of the growing culture were assayed for ring cleavage activity, using crystalline dibenzothiophene (DBT) as the substrate. The

rate of DBT biotransformation was determined spectrophotometrically at 475 nm, the wavelength of maximum absorbance of the DBT ring cleavage product [Kodama et al., 1973]. Thereafter, for convenience, the fermenter cultures were grown to stationary phase overnight (ca. 18 h) under the same conditions.

Enzymatic ring cleavage activity was induced in the grown culture by adding salicylic acid [Yen and Serdar, 1988]. The induced cells were harvested with a high speed continuous centrifugation system (CEPA; New Brunswick, NJ) and resuspended in potassium phosphate buffer (pH 7) to the same density as the original grown culture. This suspension was used in the same fermenter system as the "active biocatalyst", and subsequently incubated with a model substrate dissolved in a carrier.

#### Model substrates and carriers

Quantitative analysis of specific changes in the composition of petroleum products is difficult due to the chemical complexity of the substrate. Therefore, we chose to "spike" petroleum products with individual model substrates and quantify their ring cleavage against the complex background. Phenanthrene and DBT were selected as model substrates for biocatalysis, representing tricyclic and heterocyclic aromatic substrates, respectively. DBT was dissolved in 20 mL of light mineral oil while phenanthrene was dissolved in 30 mL of authentic hydrotreated middle distillate HP16 and straight run diesel and added to the suspension of induced biocatalyst. This reaction mixture was incubated with aeration and agitation for 24 h to effect biotransformation of the substrates. Parallel cell-free controls were also prepared and compared with the biocatalytic samples.

#### Analytical methods

The rate of biotransformation of DBT dissolved in light mineral oil was determined spectrophotometrically at intervals during biocatalysis by clarifying ca. 1 mL of reaction mixture and measuring absorption at 475 nm. Additionally, 10.0 mL samples were removed and the internal standard was added for pentane extraction of residual DBT and quantitative gas chromatography (GC) [Wu et al., 1998]. The percentage of DBT biotransformed was then calculated by difference.

Similar extraction procedures were used during biocatalysis to recover residual phenanthrene dissolved in HP16 or straight run diesel. Quantitative GC was used to estimate the percentage of phenanthrene biotransformed, using biphenyl and benzothiophene as 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 recovered after incubation with the biocatalyst to that of the model compound in a parallel sterile (cell-free) control.

Routine GC analysis of fractions and distillates was performed on a Hewlett-Packard model 5890 GC system equipped with a flame ionization detector (FID) and a sulfur-selective flame photometric ionization detector (FPD). Chromatography conditions have been described previously [Foght and Westlake, 1996].

## RESULTS AND DISCUSSION

### Specific ring cleavage activity of the biocatalyst during culture growth

It was necessary to determine the point in the growth curve at which a growing culture should be harvested for use as a biocatalyst. We observed a short culture lag time in the inoculated fermenter, with stationary phase achieved after 6 to 8 h incubation (Figure 1). The absolute DBT ring cleavage rate of this growing culture increased during incubation (data not shown), but the specific DBT oxidation rate (i.e. the ring cleavage rate normalized to biomass protein) achieved a maximum after 6 hours (Figure 1), i.e. in stationary phase. For convenience, we chose thereafter to standardize culture growth to an overnight incubation (ca. 18 h).

### Biotransformation of DBT dissolved in light mineral oil by pre-grown, induced biocatalyst

Light mineral oil was chosen as the carrier for this experiment because it approximates the aliphatic fraction of petroleum products and does not interfere with quantitative GC analysis of the residual DBT.

A rapid decrease in the residual DBT was observed in the biphasic fermenter system. By 7 hours incubation with the biocatalyst, less than 10% of the DBT remained, and within 20 hours the DBT was completely removed (Figure 2). Concomitant accumulation of the DBT ring cleavage

product, monitored spectrophotometrically at 475 nm, reached a maximum at ca. 8 h. then decreased, most likely due to side-reactions of the ring cleavage product.

Biotransformation of phenanthrene dissolved in middle distillate HP16 or straight run diesel by pre-grown, induced biocatalyst

Biotransformation of the model substrate phenanthrene incubated with biocatalyst in a biphasic fermenter system is shown in Figure 3. Middle distillate HP16 or diesel were used as carriers. Unlike DBT biocatalysis (Figure 2), complete removal of phenanthrene was not achieved within 22 h. However, significant biotransformation was observed, with similar initial rates for both carriers. The middle distillate permitted slightly better biotransformation than the diesel (ca. 80% versus ca. 60% substrate removal). The fermenter system achieved better biotransformation than typical batch flasks, which achieved 60% removal of phenanthrene dissolved in middle distillate HP16 in 24 h [Wu et al., 1998].

## CONCLUSIONS

Preparation and use of the biocatalyst in fermenter system is rapid and easily controlled using aeration, agitation and temperature. In a single fermenter the biocatalyst can be grown, induced, then re-suspended in buffer (after harvesting) for incubation with the substrate. Biotransformation of model substrates in various carriers was achieved at rates surpassing those achieved in small volume batch flasks, possibly due to superior mixing in the fermenter. The biphasic fermenter system is promising and warrants further research for optimization of operating conditions and for scaling up to larger volumes.

## ACKNOWLEDGMENTS

Grateful acknowledgment is made of support from Texaco Group, Inc., the National Centre for Upgrading Technology (Canada), and the Natural Sciences and Engineering Research Council of Canada (Collaborative Research & Development Program).

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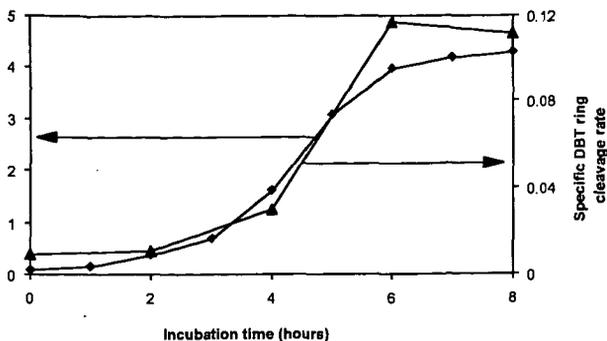


Figure 1. Correlation of culture growth and DBT ring cleavage activity. Growth curve of *P. fluorescens* #21-41 at 30°C measured as optical density at 600 nm (OD<sub>600</sub>), and specific DBT ring cleavage rate (absorption units at 475nm·min<sup>-1</sup>·mg biomass protein<sup>-1</sup>) during growth.

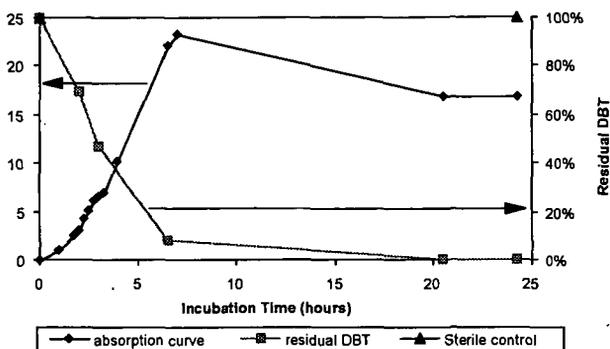


Figure 2. Biocatalysis of DBT and formation of ring cleavage product. Biotransformation of DBT dissolved in light mineral oil by *P. fluorescens* LP6a #21-41 and accumulation of its ring cleavage product, measured at 475 nm; residual DBT measured quantitatively by GC.

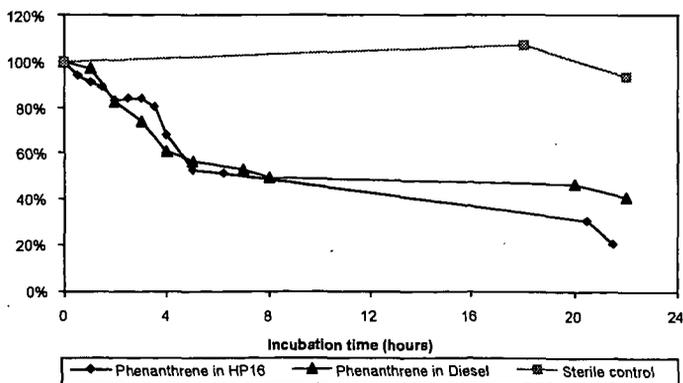


Figure 3. Biocatalysis of phenanthrene in biphasic fermenter system. Biotransformation of phenanthrene dissolved in middle distillate HP16 or diesel fuel by pre-grown, induced biocatalyst, measured by quantitative GC as removal of residual phenanthrene.