

MICROBIAL DENITROGENATION OF FOSSIL FUELS

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ABSTRACT

The microbial degradation of nitrogen compounds from fossil fuels is important because of the contribution these contaminants make to the formation of nitrogen oxides (NO_x) and hence to air pollution and acid rain. They also contribute to catalyst poisoning during the refining of crude oil, thus reducing process yields. In this study carbazole was used as the model nitrogen aromatic and 1-methylnaphthalene was used as the model fuel. Two-phase bioconversions were carried out with carbazole-degrading strains to investigate the feasibility of microbial upgrading of fossil fuels by the removal of nitrogen aromatic contaminants.

INTRODUCTION

Several heterocyclic aromatic nitrogen compounds are found in fossil fuels¹. Crude oil is a heterogeneous mixture of organic molecules including all-hydrocarbon alkanes and aromatics, as well as sulfur-, oxygen-, and nitrogen-containing heteroaromatic compounds. Many applications of crude oil are hindered by the presence of sulfur-, oxygen-, and nitrogen-containing aromatic compounds. The nitrogenous compounds found in crude oils fall into two classes. The 'nonbasic' molecules include pyrroles and indoles but are predominantly mixed alkyl derivatives of carbazole, while the 'basic' molecules are largely derivatives of pyridine and quinoline (Fig. 1). The total nitrogen content of crude oils averages around 0.3% of which the nonbasic compounds comprise approximately 70-75% (Petrolite). As existing supplies of high quality, low-boiling-point crude decrease, there is a trend towards the use of lower-volatility oils with higher nitrogen contents.

The removal of aromatic nitrogen contaminants from petroleum is important for many reasons. First, their combustion leads directly to the formation of nitrogen oxides (NO_x); emissions of NO_x, which contributes to acid rain, are under increasingly stringent control by environmental regulations^{2,3}. Second, the presence of aromatic nitrogen compounds can lead to significant poisoning of refining catalysts, resulting in a decrease in yield. Carbazole, the major nonbasic species, directly impacts the refining process in two ways: (1) it is converted during the cracking process into basic derivatives that can adsorb to the active sites of the cracking catalyst; and (2) it is unexpectedly potent as a direct inhibitor of hydrodesulfurization, which is commonly included in the refining process in order to meet sulfur content criteria^{4,6}. The practical consequence of this catalyst poisoning is that the removal of carbazole and other nitrogen species can significantly increase the extent of catalytic-cracking conversion and the yield of gasoline. With a 90% reduction in nitrogen content, an increase in gasoline yields of up to 20% may be achievable (Petrolite), which would represent a major economic improvement in low-margin, high volume refining processes. Finally, the presence of nitrogen compounds promotes the corrosion of refining equipment such as storage tanks and piping, which adds to the refining costs⁷.

Nitrogen heteroaromatics can be eliminated from petroleum using high pressure, high temperature hydrotreating, but such processes are expensive and hazardous, and also modify many other constituents of petroleum. Current research on microbial denitrogenation focuses on the degradation of the nonbasic nitrogen compounds and their alkyl derivatives, because they represent the majority of the total nitrogen and the basic nitrogen compounds can be readily extracted if desired. Although solvent extraction methods also exist for the nonbasic species, approximately 30% of the oil is retained in the extract phase. Such solvent treatments are thus ill-suited to the efficient removal of the nitrogen content.

We believe that microbial transformation of nitrogen heteroaromatics can be used to alleviate catalyst inhibition in several ways. Carbazole, for example, can be completely metabolized to CO₂ and biomass, or (using appropriate blocked mutant strains) converted to anthranilic acid or other intermediates. These appear likely to cause less catalyst inhibition than their parent compound, and many polar intermediates could be readily extracted from petroleum streams into water. It has been reported that carbazole enrichment cultures are capable of degrading a wide range of alkylcarbazoles present in crude oil, generally yielding water-soluble, nontoxic metabolites⁸.

In this study *Pseudomonas* LD2 received from Prof. Phil Fedorak's at the University of Alberta was used due to its extensive metabolic characterization⁹ and its high activity relative to other isolates obtained in our lab. Based on published reports of cloning of carbazole degrading activity in the literature^{10,11}, the carbazole genes were cloned and mobilized to several other strains of bacteria for two-phase experiments. Many *pseudomonads* were selected for known solvent resistance¹². In other model systems based on dibenzothiophene, hexadecane was used as the model fuel. However, given the low solubility of carbazole in hexadecane (~0.03 wt%), 1-methylnaphthalene proved to be a better choice due to higher solubility (~0.8 wt%), low freezing point (-22°C), and ease of emulsion separation in the two-phase system. While 1-methylnaphthalene proved to be a superior solvent, the toxicity of the oil phase to the microorganisms was problematic compared to hexadecane. Thus several organisms were tested in the model system to find a strain compatible with the 1-methylnaphthalene two-phase system.

MATERIALS AND METHODS

Microorganisms and media

A pure culture of *Pseudomonas* sp. LD2 was obtained from Phil Fedorak's lab at the University of Alberta. Other *Pseudomonas* strains were obtained from Dr. Jurtschuk at the University of Houston. The growth medium for the culturing of the microorganisms had the following composition (g/L) Tryptone 10, Yeast Extract 10, K_2HPO_4 5, and glycerol 10. Trace metals solution was added at 5 ml to 1 L and had the following composition (g/L) $MgSO_4$ 4.0, NaCl 0.2, $FeSO_4 \cdot 7H_2O$ 0.2, $MnSO_4 \cdot 4H_2O$ 0.2, D.I. H_2O 100ml. Solid media were prepared by adding 15 g/L agar to LB medium. LB medium had the following composition (g/L) Tryptone 10, Yeast Extract 5, NaCl 10.

Growth conditions

Seed cultures were started by inoculating 5 ml of media with a sterile loop dipped in -80°C frozen seed cultures with 25% glycerol. The seed cultures were incubated at 30°C in a water bath shaker for 6-8 hours or until approximately 1-2 OD was reached. One ml of this seed culture was added to 1L of media in a 2.8L Fernbach baffled shake flask and incubated at 30°C and 250rpm. After 12-16 hours of growth, the cells were harvested by centrifugation at 4,000 rpm in a Beckman J6B. The supernatant was discarded, and the cells were resuspended in 500 ml of LB for use in two-phase experiments.

Two-phase model system

Thirty ml of cell slurry was added to a 300 ml baffled shake flask. To this flask, 10 ml of 1-methylnaphthalene with 0.8 wt% carbazole was added. In hexadecane experiments, 10 ml of carbazole-saturated hexadecane were added. These flasks were incubated at 30°C and 250 rpm. Time samples were taken by removing a flask and emptying the contents into a 40 ml polypropylene centrifuge tube. The sample was then centrifuged at 15,000 rpm for 30 minutes. After centrifugation a disposable pipette was used to remove approximately 2 ml of the 1-methylnaphthalene layer. The 1-methylnaphthalene phase was added to an amber 1.5 ml GC sample vial for analysis.

GC analysis

The 1-methylnaphthalene phase was analyzed on a HP 6890 gas chromatograph. One μ l of sample was injected on a HP-5 column at an initial column temperature of 160°C. The method held the column at the initial temperature for 2 minutes then increased the temperature at a rate of 8°C/min until 250°C was attained. This final temperature was held for 2 minutes. The detector was a NPD, which is specific for nitrogen.

Results and Discussion

Several strains were tested, and the results are summarized in Table 1. All strains tested in the hexadecane model systems showed removal of a low level (~0.03 wt%) of soluble carbazole. However, only one strain showed any ability to remove carbazole in the 1-methylnaphthalene two-phase system. None of the tested strains were observed to grow on 1-methylnaphthalene as the sole source of carbon. Thus, the 1-methylnaphthalene did not supply a co-metabolic substrate for carbazole degradation. In the systems containing *P. fraggi*, the total observed removal of carbazole was 4.7% after 3 hours, 11.3% after 18 hours, and 13.3% after 28 hours relative to the stock solution. An additional flask at 45 hours did not show any further decrease in carbazole concentration relative to the stock solution. In the hexadecane experiments, no carbazole was detected after 2 hours of incubation. No intermediates of carbazole metabolism were detected in

the GC analysis in either of the model system runs. However, control samples analyzed by GC were able to detect anthranilic acid, which is a known intermediate in carbazole degradation¹³. The control flask not containing microorganism did not show any change in carbazole concentration relative to the stock solution. A specific activity was not calculated since growth occurred during the reaction, and the 1-methylnaphthalene prevented measurement of optical density or dry cell weight.

CONCLUSIONS

The investigation of biodenitrogenation of fossil fuels requires the selection of a model fuel for use in two-phase experiments. While systems utilizing hexadecane have been used for similar studies in desulfurization, the low solubility of carbazole in hexadecane eliminates this solvent from serious consideration. 1-methylnaphthalene is a good solvent for carbazole, is easily purchased, and forms an emulsion during testing that was easily broken by centrifugation. However, the observed toxicity of the solvent to many normally solvent-resistant strains of bacteria necessitated the search for a compatible biocatalyst for the model system. This initial screening indicates that at least one strain, *P. fraggi*, is capable of removing carbazole with 1-methylnaphthalene as the model fuel. This strain may also prove more resistant to the toxicity of petroleum refining streams

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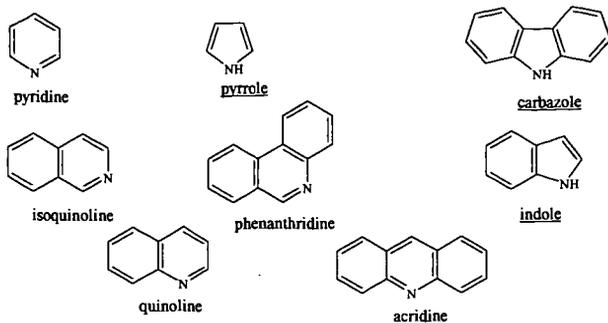


Figure 1: Examples of common nitrogen aromatic compounds found in fossil fuels. The nonbasic species are underlined.

Strain	Hexadecane system	1-methylnapthalene system
<i>E. coli</i>	+	-
<i>P. sp. LD2</i>	+	-
<i>P. fraggi</i>	+	+
<i>P. mendicino</i>	+	-
<i>P. idaho</i>	+	-
<i>P. putida</i>	+	-

Table 1. Tested strains harboring the carbazole degradation genes. Removal of carbazole from the model oil phase is denoted with a +.