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Microbial Upgrading of Shale Oils

Removal of Nitriles

Scientific Note

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ABSTRACT

After prolonged incubation on shale oil as the sole source of nitrogen, microbial cultures were obtained that degraded nitriles. These cultures degraded aliphatic nitriles, but did not degrade hydrocarbons or heterocyclic nitrogen compounds found in this oil. Lower-molecular-weight nitriles were biodegraded more rapidly than were higher-molecular-weight nitriles. A *P. aeruginosa* strain, isolated from one of the enrichments, was able to utilize the pure nitriles, undecyl cyanide, undecanenitrile, and stearonitrile as sole sources of carbon and nitrogen, and was able to remove the series of nitriles from the oil in pure culture.

Index Entries: Nitrile; biodegradation; shale oil; microbial upgrading; biotechnology.

INTRODUCTION

The possible application of microbial processes to the upgrading of oils (*2–6*) is a potential means of producing alternative fuels at competitive prices. There is increasing awareness that alternative fuels, such as coal derived liquids and shale oils, are needed, but it is necessary to reduce the nitrogen and sulfur contents of these alternative fuels without greatly increasing their costs if these fuels are to be widely used. If microorganisms could selectively remove the nitrogen and/or sulfur containing

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compounds without attacking the hydrocarbon components of the oil, a biotechnological process could reduce the cost of upgrading shale oil to a useful fuel.

In this report, we are concerned with the ability of microbes to remove nitrogen from shale. We are specifically interested in removal of nitrogen, since this is the element that is difficult to remove from shale oil by the conventional hydrotreatment process (*7*). The nitrogen content of shale oil needs to be reduced, since nitrogen acts as a poison for refinery catalysts, and, together with oxygen, causes poor storage stability of the oil. In the raw shale oil produced by pyrolysis of shale from the Stuart deposit of Queensland Australia, 10% of the nitrogen occurs as aliphatic nitriles, 40% as aliphatic amines, and 50% as nitrogen heterocyclics, such as quinoline. This report focuses on the removal of the nitriles.

METHODS

Enrichment Cultures

Soils that had been contaminated with petroleum were used as sources of inocula for enrichment cultures. For establishing enrichment cultures, 10 g of each soil were added to 100 mL of a nitrogen-free medium with the following composition: $MgSO_4$ (0.2 g/L), $CaCl_2$ (0.02 g/L), KH_2PO_4 (1 g/L), K_2HPO_4 (1 g/L), $FeCl_3$ (0.05 g/L). The pH was adjusted to 7.5 and the medium was then autoclaved. The medium was amended with a sterile glucose solution to give a final concentration of 0.1% (w/v) and shale oil was added to give a concentration of 1% (w/v). The shale oil was the sole source of nitrogen, but not the sole source of carbon for these enrichments. The shale oil used in these enrichment cultures was the middle cut of a raw shale oil (250–400°C boiling range) produced at the Taciuk Processor Pilot Plant by pyrolysis of shale from the Stuart Deposit, Queensland, Australia (*8*). This shale oil contains 0.87% Kjeldahl nitrogen (analysis provided by Galbraith Laboratories, Knoxville, TN). The enrichments were incubated at 28°C on a rotary shaker at 200 rpm with monthly transfers into fresh medium.

Biodegradation of Shale Oil Components

After repeated subcultures to ensure maintenance of growth on media in which the shale oil was the sole source of nitrogen but not carbon, a culture showing emulsification and visible changes was selected for further study. This culture came from a subsurface Alberta tar sands drill core. Aliquots (5 mL) from the enrichment were added to replicate flasks containing 100 mL of the nitrogen free medium described previously supplemented with glucose (0.1% w/v) and shale oil (1 mL), and these cultures were incubated at 28°C on a rotary shaker at 200 rpm. Sterile controls

were included to account for any substrate loss caused by volatilization or other nonbiological loss. Periodically, during a 14-d incubation period, the residual oil from duplicate control and inoculated flasks was extracted and analyzed for nitrile biodegradation, as described below.

Biodegradation of Aliphatic Nitriles and Aliphatic Amines by a Pure Culture

A bacterial strain, identified as a strain of *pseudomonas aeruginosa*, was isolated directly from the original enrichment by plating onto 0.1% strength Tryptic Soy Broth (BBL) solidified with purified agar (Difco). This strain was tested for growth on the nitriles; undecanenitrile, undecyl cyanide, and stearonitrile (Aldrich Chemical Co.) and the amines; decylamine, dodecylamine, and tetradecylamine (PolySciences Cor.) as the sole source of nitrogen and carbon. Nitrogen free medium was amended with the nitriles to give a final concentration of 0.1% (w/v) and the amines to give 0.05% (w/v). The ability of the organism to utilize the individual nitriles as sole nitrogen and carbon source was determined by gas liquid chromatography using a flame ionization detector and the chromatographic conditions described below. The ability of the organism to utilize the aliphatic amines was monitored by measuring bacterial growth; to detect growth of the organism on the aliphatic amines the numbers of bacteria were monitored by plating at regular intervals onto 0.1% Tryptic Soy Broth solidifed with purified agar. The *P. aeruginosa* strain also was inoculated into nitrogen free medium with glucose (0.1%) as a carbon source and shale oil (1%) as the sole source of nitrogen. After 14 d incubation at 28°C with shaking at 200 rpm, the remaining oil was recovered. A sterile control was included for determination of abiotic changes in the oil.

Analyses of Oil Fractions

The residual oil from each culture flask was extracted with methylene chloride (Burdick and Jackson) at neutral pH and the oil concentrated by using a rotary evaporator. The oil was fractionated by using a modification of the column chromatographic method described by Fedorak and Westlake (2), in which the alumina chromatography column was developed sequentially with 50 mL of pentane (MCB), 50 mL of benzene (Burdick and Jackson), and 70 mL of chloroform (Fisher Scientific), to give, respectively, the saturate, aromatic, and nitrile containing fractions. In some cases, the heterocyclic-nitrogen components were separated from the oil by acid extraction using the procedure of Bett et al. (9) prior to fractionation.

After the solvent volume had been reduced under vacuum, the nitrile containing fraction to which quinoline (Aldrich Chemical Co.) was added as an internal standard was analyzed by capillary gas liquid chromatography by using a Hewlett Packard (model 5840) GC with a nitrogen specific detector. A 25 m×0.2 mm ultra 2 coated fused silica column (Hewlett

Packard) was used for compound separation. The operational parameters were: injection port at 240°C; detector at 320°C, column temperature 100°C isothermal for 5 min, 5°C per min to 290°C, isothermal at 290°C for 20 min; helium as carrier and makeup gas.

Analyses of the oil for total nitrogen (percent Kjeldahl nitrogen) after extraction from the broth cultures was done by Galbraith Laboratories, Knoxville, TN.

RESULTS

Biodegradation of Shale Oil Components

The nitrile fraction of the Stuart shale oil used in these experiments was found to contain a series of *n*-nitriles from C_7 to C_{30} with a maxima at C_{12} and unsaturated nitriles as identified by gas chromatographic and mass spectrometric analyses. Some of these unsaturated nitriles are probably singly unsaturated alkylnitriles with the double bond at the opposite end to that of the nitrile group, as reported by Harvey et al. (*10*). The concentrations of the unsaturated nitriles were less than the saturated nitriles of comparable chain lengths, with the ratio of unsaturated to saturated nitrile getting progressively smaller with increasing chain length above C_{12}. The concentrations of these nitriles were essentially unchanged in the sterile controls after 14 d incubation compared to the nitrile fraction at the beginning of the experiment. In the enrichment cultures, where visible changes in the oil were detected, however, there was extensive removal of the saturated and unsaturated nitriles. The extent of removal varied among the various enrichment cultures.

In contrast to the nitrile fraction, there was no evidence of attack on the heterocyclic nitrogen compounds nor on the aliphatic and aromatic hydrocarbons found in this oil (*1*).

The enrichment culture chosen for further analysis showed a substantial decrease (45%) in the amount of nitrogen present in the oil after extraction, when compared to a sterile control. Subsequent gas chromatographic analyses of the oil fractions recovered from the broth enrichment cultures in which the shale oil was the sole nitrogen source and glucose was an alternate carbon source showed selective microbial attack on the aliphatic nitrile compounds from the shale oil (Fig. 1). Both saturated and unsaturated nitriles were rapidly degraded by the microbial populations in the mixed enrichment culture. There was a sequential utilization of nitriles, with lower molecular weight nitriles being degraded prior to higher molecular weight nitriles. As an example of this point, after 3 d 100% of the C_7-C_{12} nitriles had been degraded, but there was limited attack on C_{13}-C_{20} nitriles. However, after 14 d incubation, all C_7-C_{14} nitriles and more than 90% of the C_{15}-C_{20} nitriles were removed from the oil. After 14 d incubation, more than 90% of the total nitriles had been removed from the oil. The lower

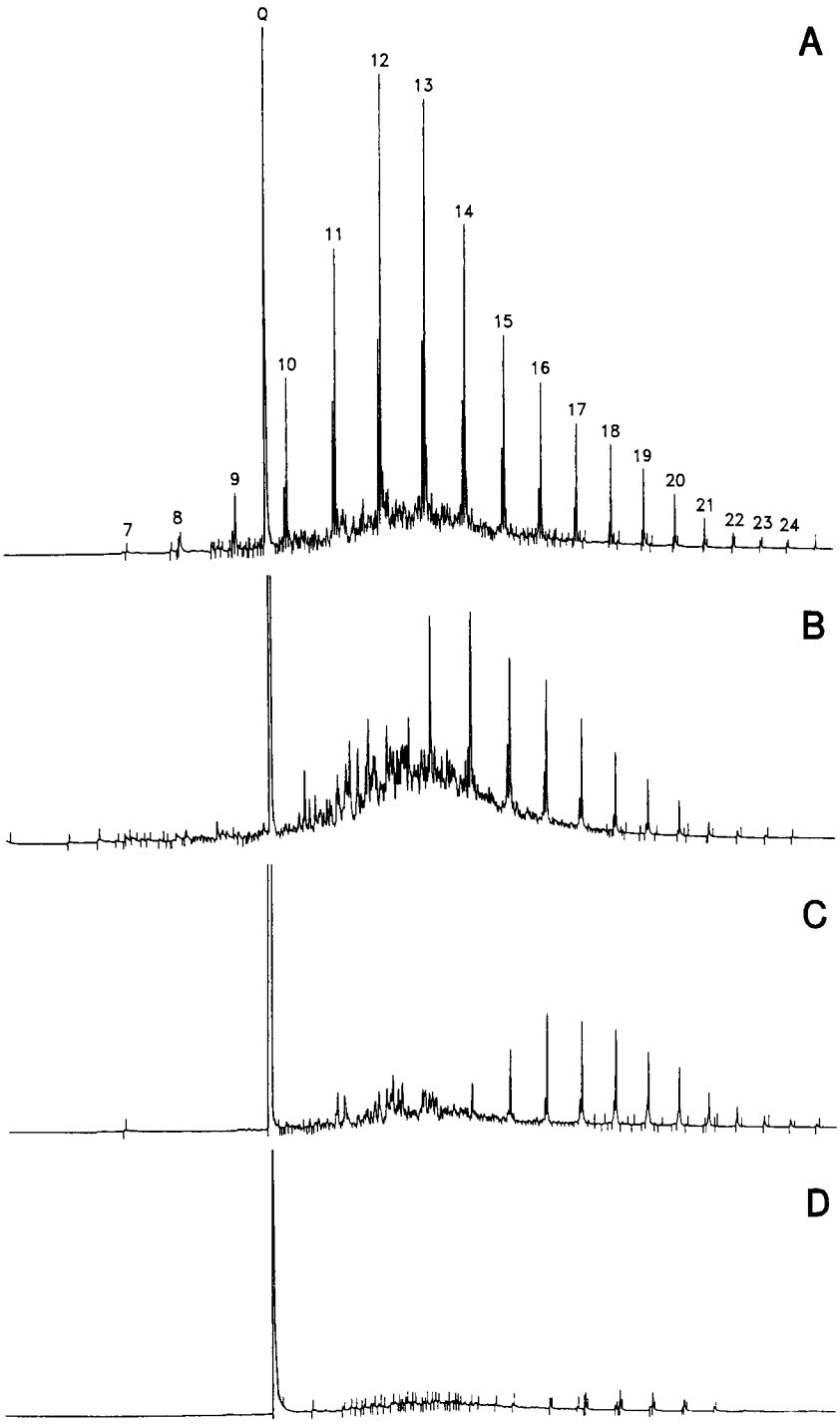

Fig. 1. Gas chromatographic tracings of nitrile fractions recovered from a sterile control after 14 d incubation (A), an enrichment culture after 3 d (B), 7 d (C), 14 d incubation (D). The profile of the nitriles in the sterile control after this incubation period is identical with that for the initial oil, indicating no abiotic degradation of these nitriles. The internal standard is quinoline (Q). The chain lengths of the *n*-nitriles are indicated. The unsaturated nitriles occur just preceding the *n*-nitriles of corresponding chain lengths.

molecular weight nitriles, thus, were degraded preferentially compared to the higher molecular weight nitriles, and at a faster rate. The results clearly indicate that the unsaturated nitriles were degraded at least as rapidly as the saturated nitriles of identical chain lengths.

Biodegradation of Nitriles by *P. aeruginosa*

After one week incubation of the strain of *P. aeruginosa* with either undecanenitrile, undecyl cyanide, or stearonitrile as the sole source of carbon and nitrogen, the undecanenitrile and undecyl cyanide could no longer be detected (100% degradation), and the stearonitrile had been reduced by 50%. This preferential degradation of the lower molecular weight nitriles is consistent with the observations in the mixed enrichment cultures. Further, when inoculated into a medium with shale oil as the sole source of nitrogen the nitriles were again selectively removed from the oil, and no microbial attack was detected on the hydrocarbon portion of the oil. As long as there is an alternate carbon source (glucose or succinate) this organism does not appear to attack aliphatic or aromatic hydrocarbons.

Biodegradation of Amines by *P. aeruginosa*

Because the decrease in the total nitrogen content of the oil indicates that more nitrogen than that contained in the nitriles is able to be removed from the oil by microbes in the enrichment and because there was no attack on the nitrogen heterocycles under the cultural conditions employed in this study, the ability of the pure culture of *P. aeruginosa* strain to biodegrade aliphatic amines was investigated. Surprisingly the numbers decreased 2-3 orders of magnitude, indicating that lower molecular weight aliphatic amines are toxic to this organism at a concentration of 0.05%.

DISCUSSION

Although various investigators have examined the abilities of microorganisms to degrade nitriles (*11–17*), with the finding that the degradation of aliphatic nitriles proceeds via the corresponding amide to the fatty acid plus ammonia by the action a nitrilase and an amidase, respectively (*11, 12*), previous studies have not examined the abilities of microorganisms to degrade nitriles within complex mixtures, such as shale oil (*18–21*).

The present study clearly shows that the nitrogen content of Stuart Shale oil can be reduced by biotechnology. Microbial attack on shale oil resulted in a 45% decrease in total nitrogen of the oil. Gas chromatographic analysis of the oil after microbial attack showed that nitriles were removed

from the oil. Subsequently, a *P. aeruginosa* strain was isolated from the mixed culture which is able to remove the series of nitriles, both saturated and unsaturated, from the shale oil without attack on the hydrocarbon portion nor on the heterocyclic nitrogen compounds, when used in pure culture with the shale oil provided as the nitrogen source and with an alternative carbon source. These results for aliphatic nitrile degradation contrast with those of Fedorak and Westlake (2) who found that biodegradation in a crude oil of heterocyclic nitrogen-containing carbazoles occurred together with degradation of aliphatic and aromatic hydrocarbons. In our study, the enrichment cultures did not attack any hydrocarbons (alkanes, alkenes, or condensed aromatics) under conditions where nitriles were attacked.

Like the enrichment cultures, the isolated *P. aeruginosa* strain showed preferential attack on the lower molecular weight nitriles. This preferential attack on lower molecular weight nitriles may be a function of the bioavailability of these compounds. At 28°C, undecyl cyanide and undecanenitrile are liquid whereas, stearonitrile is solid. Similar preferential attack on lower molecular weight alkanes has been reported for some microbial strains (*22,23*).

The pure culture of *P. aeruginosa* isolated in this study can be used in a biotechnological shale oil upgrading process. Although the aliphatic nitriles account for only 10% of the total nitrogen in Stuart shale oil, the ability of this organism to remove these nitrogen containing compounds without decreasing the hydrocarbon content of the oil clearly demonstrates the potential of microbes for selective modification of the oil. This selective removal capacity is critical for upgrading of shale oil or other synthetic fuels because of the necessity of retaining the caloric value of the hydrocarbons within the fuel.

Although 50% of the nitrogen in shale oil is contained in the heterocyclic nitrogen compounds, these complex aromatic compounds were not attacked under the conditions employed in this study. Heterocyclic nitrogen-containing compounds found in this oil have been reported to be subject to biodegradation (*2,24*) and we have also observed degradation of these compounds when the acid-extractable fraction of the oil that contains these basic nitrogen heterocyclic compounds is used as a source of carbon for enrichment cultures (unpublished results).

Because microbial attack on the shale oil resulted in a 45% decrease in the total nitrogen content of the oil and no attack on the nitrogen heterocyclics was detected, it is proposed that the aliphatic amines present in this oil were also removed by microbial activity. Subsequently, the ability of the *P. aeruginosa* strain to biodegrade pure aliphatic amines was investigated, with the finding that this strain does not attack these compounds under the conditions employed in this study. However, the *P. aeruginosa* strain might be able to attack aliphatic amines contained within shale oil. Alternately, an organism or organisms within the initial enrichment, other

than *P. aeruginosa* strain isolated from the culture, may have been able to attack aliphatic amines. Additional studies will be needed to determine the fate of the aliphatic amines contained in shale oil.

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REFERENCES

1. Aislabie, J. and Atlas, R. M. (1988), *Appl. Environ. Microbiol.* **54,** 2197–2202.
2. Fedorak, P. M. and Westlake, D. W. S. (1984), *Appl. Environ. Microbiol.* **47,** 858–862.
3. Finnerty, W. R. (1982), *Energy technology: Proceedings of the ninth energy technology conference,* (Hill, R. F., ed.), Government Institutes, Washington, DC.
4. Finnerty, W. R., Shockley, K., and Attaway, H. (1982), Microbial enhanced oil recovery, (Zajic, J. E., Cooper, D. C., Jack, T. R., and Kosaric, N., (eds.), PennWell Books, Tulsa, OK.
5. Hartdegen, F. J., Coburn, J. M., and Roberts, R. L. (1984), *Chem. Eng. Prog.* **80,** 63–67.
6. Monticello, D. J. and Finnerty, W. R. (1985), *Ann. Rev. Microbiol.* **39,** 371–389.
7. Harvey, T. G., Matheson, T. W., Pratt, K. C., and Stanborough, M. S. (1984), Proceedings of the 2nd Australian Workshop on Oil Shale. CSIRO Division of Energy Chemistry, Sutherland, Australia.
8. Baker, L. A. and Wright, B. C. (1987), Proceedings of the 4th Australian workshop on oil shale, Brisbane. CSIRO Division of Energy Chemistry, Menai, Australia.
9. Bett, G., Harvey, T. G., Matheson, T. W., and Pratt, K. C. (1983), *Fuel* **62,** 1445–1454.
10. Harvey, T. G., Matheson, T. W., and Pratt, K. C. (1986), Proceedings of the 3rd Australian workshop on oil shale, Lucas Heights, CSIRO Division of Energy Chemistry, Menai, Australia.
11. Asano, Y., Fujishiro, K., Tani, Y., and Yamada, H. (1982), *Agric. Biol. Chem.* **46,** 1165–1174.
12. Asano, Y., Tachibana, M., Tani, Y., and Yamada, H. (1982), *Agric. Biol. Chem.* **46,** 1175–1181.
13. Collins, P. A. and Knowles, C. J. (1983), *J. Gen. Microbiol.* **129,** 711–718.
14. Kuwahara, M., Yanase, H., Ishida, Y., and Kikuchi, Y. (1980), *J. Ferment. Technol.* **58,** 573–578.
15. Linton, E. A. and Knowles, C. J. (1986), *J. Gen. Microbiol.* **132,** 1493–1501.

16. Thiery, A., Maestracci, M., Arnaud, A., and Galzy, P. (1986), *Zentralbl. Mikrobiol.* **141,** 575–582.
17. Yanase, H., Sakai, T., and Tonomura, K. (1985), *J. Ferment. Technol.* **63,** 193–198.
18. Evans, E. J., Batts, B. D., Cant, N. W., and Smith, J. W. (1985), *Org. Geochem.* **8,** 367–374.
19. Harvey, T. G., Matheson, T. W., and Pratt, K. C. (1984), *Anal. Chem.* **56,** 1277–1281.
20. Regtop, R. A., Crisp, P. T., and Ellis, J. (1982), *Fuel* **61,** 185–192.
21. Rovere, C. E., Crisp, P. T., Ellis, J., and Bolton, P. D. (1983), *Fuel* **62,** 1273–1281.
22. Haines, J. and Alexander, M. (1974), *Appl. Microbiol.* **28,** 1084–1085.
23. Singer, M. E. and Finnerty, W. R. (1984), *Petroleum Microbiology,* (Atlas, R. M., ed.), Macmillan, NY.
24. Shukla, O. P. (1986), *Appl. Environ. Microbiol.* **51,** 1332–1342.

Session 5
Biological Production of Materials