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Utilization and degradation of an ester-based synthetic lubricant by *Acinetobacter lwoffi*

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Abstract

An oil-degrading bacterium, *Acinetobacter lwoffi*, isolated by elective culture from the Medway estuary, utilized an ester-based synthetic lubricating oil EMKARATE DE 155 as sole carbon and energy source. Analysis of culture supernatants by gas chromatography showed the accumulation of a nondegradable metabolite 1,1,1 Tris (hydroxymethyl) propane in addition to two metabolizable fatty acids, octanoic and decanoic acids as products of the synthetic oil degradation. Esterase activities were subsequently demonstrated in oil and acetate-grown cells. The synthetic oil therefore appears to be partially biodegradable in the environment.

Introduction

Lubricating oils derived primarily from crude petroleum have found diverse uses ranging from bearing lubrication and cooling in motor vehicles to hydraulic functions in industrial machines. Petroleum oils have in the past provided satisfactory lubrication for almost all straight-forward applications. However, synthetic lubricants have received considerable attention in recent years due to the failure of petroleum oils to meet some special lubrication requirements. Ester-based synthetic lubricants used in outboard engines have offered some advantages over mineral oil-based ones with respect to their low pour point, high viscosity index combined with low volatility, high temperature resistance and reduced toxicity.

Although the biodegradable nature of esters has made several manufacturers to use them in the formulation of two-stroke engine oils for outboard motors, the environmental friendliness and ultimate biodegradability of each formulation has assumed tremendous significance in view of stringent environmental standards expected of industries. This paper therefore reports the biodegradability of an ester-based synthetic oil, EMKARATE using an oil-degrading strain originally identified as *Acinetobacter lwoffi* (Amund 1982).

Materials and methods

Sources of microbial strain and oil samples

The oil sample EMKARATE DE 155 (Fig. 4) used in this study was provided by ICI Chemicals and Polymers Ltd., Middlesborough, Cleveland, U.K. The oil sample was sterilized by autoclaving at 121° C. The organism used for the biodegradability test was a strain of *Acinetobacter lwoffi* isolated by crude oil enrichment from a water sample collected from the Medway estuary, Kent, U.K. (Amund 1982). A freeze-dried culture of the organism was resuscitated in nutrient broth and purified on nutrient agar plates, on which the cultures were subsequently maintained.

Biodegradation and growth studies

Growth and degradation studies over a time course were carried out using the mineral salts medium of Mulkins-Phillips & Stewart (1974) supplemented with the trace elements stock of Bauchop & Elsden (1969) while the synthetic lubricant (EMKARATE DE 155) was used as the sole carbon source at 0.5% (v/v). Cultures (500 ml) were incubated with shaking (120 rev/min) at 25° C. The optical density (OD) at 600 nm, total viable counts (tvc), pH of culture medi-

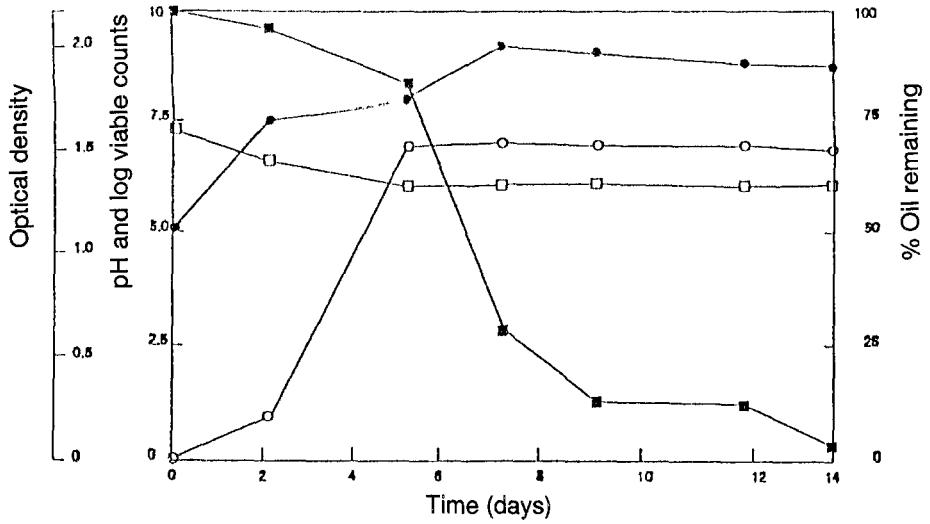

Fig. 1. Time course of growth of *Acinetobacter lwoffi* on the synthetic lubricant (EMKARATE DE 155). o Optical density; • log of viable counts; □ pH; ■ % oil remaining.

um and the percent oil remaining were measured at time intervals as previously described (Amund & Igiri 1990).

Gas chromatography of oils and degradation products

Fresh and degraded oils were analysed by gas liquid chromatography using a Pye Unicam series 304 gas chromatograph equipped with dual flame ionization detectors and fitted with a data-capture computing integrator (Drew Scientific Model 1040). A glass column of 2 m × 4 mm internal diameter containing a liquid phase of 3% OV-1 on supelcoport 100/200 was used. A temperature programme of 120–135° C increasing at 10° C/min and held isothermally at 350° C for 17 min was employed. Nitrogen (flow rate, 25 ml/min) was used as carrier gas while the flow rates for hydrogen and air were 14 ml/min and 7 ml/min, respectively. The oil extracts of culture supernatants were dissolved in carbon tetrachloride while a sample volume of 10 microliters was injected.

Hydroxylated products extracted from culture supernatants with diethyl ether were analysed by gas chromatography using the SP2340 column. The operating conditions were as follows: carrier gas (N_2) flow rate, 25 ml/min; column temperature, 180° C; detector temperature, 250° C.

Fatty acid products were first converted to methyl esters by treatment of culture supernatant with boron trifluoride-methanol and refluxing for 20 min. The hexane extracts containing the esters were analysed using a pre-packed column containing 3% (w/v) methylsilicone gum (SE 30) on chromosorb W (100–120 mesh). The operating conditions were similar to those used for hydroxylated products.

Measurement of oxygen uptake of cell suspensions

Oxygen consumption was measured using a magnetically-stirred oxygen electrode assembly (Rank Bros., Bottisham, Cambridge) linked to a chart recorder and with temperature maintained at 30° C by means of a circulating water bath. Reaction mixtures contained sodium phosphate buffer (0.01 M, 1.0 ml), cell suspension (1.0 ml, approx. 10 mg dry wt) and the substrate (20 μl, 1.0 mM). Cells of *Acinetobacter lwoffi* grown on the synthetic oil, n-hexadecane and sodium acetate were used for the measurements.

Enzyme assays

Esterase activities in lubricant-grown and acetate-grown cells were assayed using two model substrates, p-nitrophenyloctanoate and p-nitrophenylacetate from which the action of esterases released p-nitrophenol, which was determined spectrophotometrically at a wavelength of 400 nm (Wyatt 1983). Washed cell suspensions of the test organism were dispensed into flasks containing sodium phosphate buffer (0.01 M, pH 7.0)

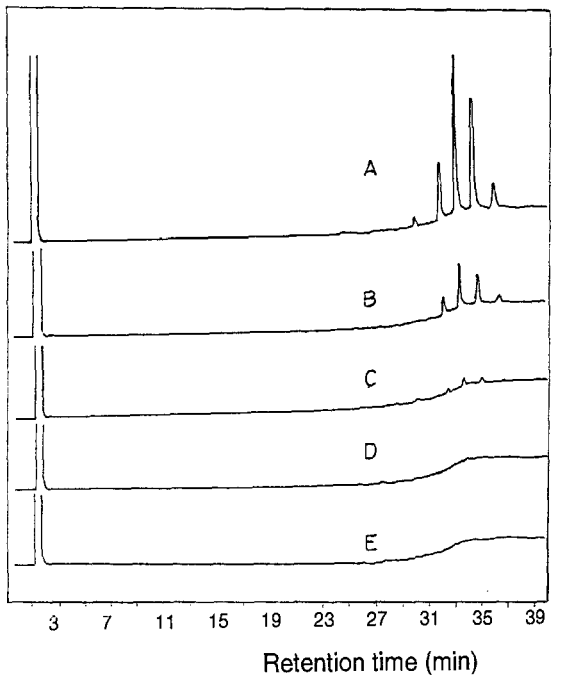

Fig. 2. Gas chromatographic profiles of the synthetic lubricant (EMKARATE DE 155) during a 14-day aerated batch culture of *Acinetobacter lwoffi*. A represents the undergraded oil while B, C, D and E represent the degraded oils after 2, 5, 7 and 8 days of incubation.

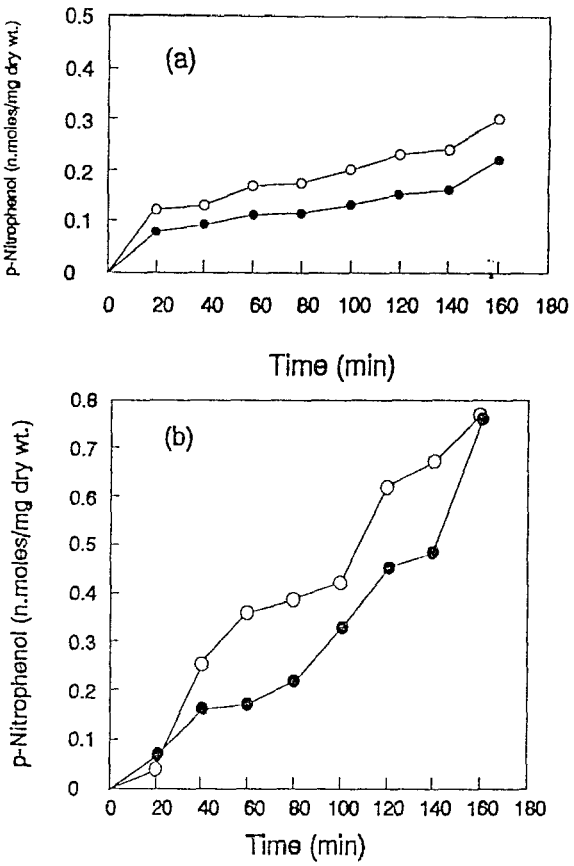

Fig. 3. Esterase activities of *Acinetobacter lwoffi* grown on two-stroke synthetic oil (EMKARATE DE 155) and sodium acetate as sole carbon sources. (*a*) represents cells grown on synthetic oil. (*b*) represents cells grown on sodium acetate. o Release of p-nitrophenol from p-nitrophenyl octanoate. • Release of p-nitrophenol from p-nitrophenyl acetate.

to give a total volume of 24 ml. The experiment was started by the addition of 1.0 ml of the model substrate (10 mM) and the flasks were incubated on a rotary shaker (100 rev/min) at 30° C. Samples (1.0 ml) were collected every 20 min and centrifuged to remove cell biomass after which 200 μl of supernatant was diluted to 1.0 ml and measured spectrophotometrically at 400 nm to detect p-nitrophenol. The optical density was related to a standard calibration curve (0–100 n moles/μl). Controls to which no cells were added were included in the assays to correct for the slow spontaneous hydrolysis of the model substrates.

Results and discussion

The microbial strain used in this study, *Acinetobacter lwoffi* S2 was a long-chain n-alkane utilizer which demonstrated the ability to grow on the synthetic lubricating oil (EMKARATE DE 155) as sole carbon source. Growth of the organism on the oil was accompanied by an increase in viable cell counts and optical density of the culture medium (Fig. 1), decrease in pH of the medium and a gradual depletion of the oil as monitored by a combination of gas chromatography (Fig. 2) and infrared spectrotrophotometry. A residual oil concentration of about 3.55% was left in the medium after a 14 day aerated batch culture. A gas chromatographic analysis of undegraded oil showed the presence of five peaks (Fig. 2A) indicating that the synthetic oil is probably a complex mixture of esters although the peaks were not identified. Analysis of the carbon tetrachloride extracts of culture supernatants during the growth studies showed that the esters were consumed within 7 days of incubation (Fig. 2B, C, D and E).

Analysis of ethereal extract of culture supernatants using the SP2340 column showed the accumulation of a compound (retention time 3.63 min) which was identified using an internal standard as 1,1,1 Tris (hydroxymethyl) propane, the alcohol base used in the synthesis of the lubricant EMKARATE DE 155. Analysis

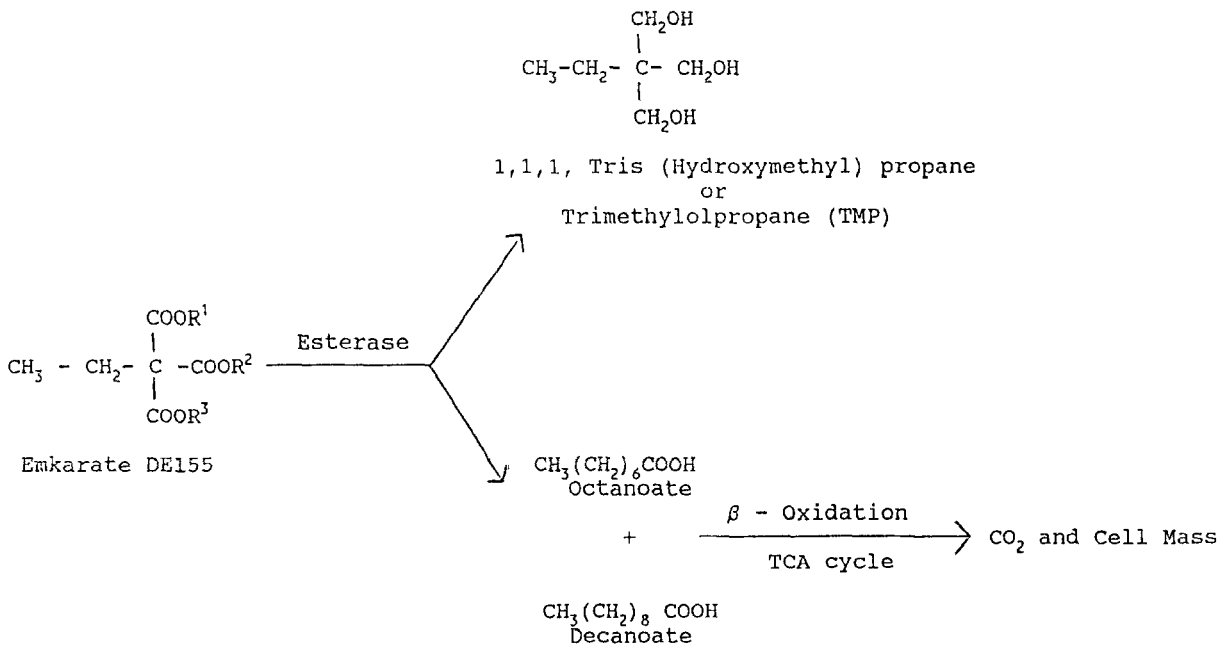

Fig. 4. Proposed scheme for the degradation of EMKARATE DE 155 by *Acinetobacter lwoffi*. The groups R^1, R^2, and R^3 are either octanoate or decanoate on a 50:50 ratio (Wright et al. 1993).

Table 1. Oxygen consumption of resting cell suspensions of *Acinetobacter lwoffi* during catabolism of the synthetic oil, hydrocarbons and possible metabolic intermediates. Oxygen uptake values were corrected for the endogenous rates.

Substrate oxidized	Oxygen uptake [nmol O_2/min/mg dry weight)		
	Growth substrate		
	Lubricant	n-Hexadecane	Acetate
Octanoate	12.43	17.39	0
Decanoate	11.74	7.86	0
Hexanoate	13.81	12.34	0
Acetate	7.60	11.78	2.78
Butyrate	2.07	10.66	0
Succinate	13.12	11.78	0.56
Laurate	7.60	13.46	0
1,1,1 Tris (Hydroxymethyl) propane	0	0	0
Synthetic lubricant (EMKARATE DE 155)	2.07	0.56	0
n-Dodecane	3.45	0.56	0
n-Hexadecane	0	5.61	0

of hexane extracts of methylated degradation products using internal standards also showed chromatographic peaks corresponding to methyl esters of octanoic and decanoic acids (retention time, 1.83 and 5.92 min, respectively). These two acids are the fatty acid components of the synthetic oil. The random positioning of these fatty acids molecules on the hydroxyl groups in 1,1,1 Tris (hydroxymethyl) propane could have result-

ed in a mixture of esters indicated by the five chromatographic peaks obtained from the fresh synthetic oil (Fig. 2A).

The results of polarographic oxygen uptake measurements comparing the ability of intact cells to oxidize possible intermediates of synthetic oil degradation after growth on the oil, n-hexadecane and sodium acetate are shown in Table 1. While the oil and hexadecane-grown cells could oxidize fatty acid substrates, acetate-grown cells could only oxidize acetate and succinate since growth on acetate did not elicit the synthesis of β-oxidation enzymes. However, cells grown on either of the three substrates could not oxidize the hydroxylated compound, 1,1,1 Tris (hydroxymethyl) propane. Substrate specificity tests also showed that the test organism could not utilize this hydroxylated degradation product as carbon source in the mineral salts medium. The recalcitrant nature of the compound could be attributed to the presence of a tertiary carbon in the structure which is unreactive to enzymes involved in the metabolism of alkyl chains. Experiments by Catelani et al. (1977) and McKenna (1972) have all indicated that tertiary groups in alkanes were not attacked and considerably reduced the biodegradabilities of such compounds.

The appearance of fatty acids and the hydroxylated compound in the culture supernatant during the growth of *Acinetobacter lwoffi* on the synthetic oil would suggest the presence of an esterase in this organism. Esterases of broad specificities have been reported in a wide spectrum of microbes especially those capable of degrading hydrocarbons (Brevil et al. 1978, Kosaric et al. 1979, Mattey & Morgan 1978). The results of esterase assays showed that the ester bond in p-nitrophenyloctanoate was more readily cleaved than the one in p-nitrophenylacetate (Fig. 3). The esterase in the test organism may therefore possess a higher affinity for long chain alphatic esters than short-chain ones. Higher esterase activities were also elicited in acetate-grown cells than in lubricant-grown cells probably due to the constitutive nature of these enzymes in *Acinetobacter lwoffi*.

The results of this study have therefore shown that the synthetic lubricating oil EMKARATE DE 155 was biodegradable and the proposed biodegradation scheme based on product identification and oxygen uptake measurements is shown in Fig. 4. These results seem to corroborate those of Wright et al. (1993) who tested the biodegradability of the synthetic oil with *Micrococcus roseus*. However, its degradation gave rise to a non-biodegradable metabolite 1,1,1 Tris (hydroxymethyl) propane which could accumulate in the environment. The sourcing of a new hydroxylated compound for use in the synthesis of ester-based oils may be necessary so as to produce oils whose residues would be completely biodegradable in the environment.

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