Appl Microbiol Biotechnol (1986) 23:384–388

Applied and Microbiology Biotechnology

The uptake of *n*-alkanes from alkane mixtures during growth of the hydrocarbon-utilizing fungus *Cladosporium resinae*

N. D. Lindley* and M. T. Heydeman

Department of Microbiology, University of Reading, Reading RG1 5AQ, UK

Summary. *Cladosporium resinae* growing on alkane mixtures removed *n*-alkanes sequentially in order of increasing molecular weight, each at about the same rate as during growth on it as single alkane. This sequence is not in order of the ability of each alkane to support growth. No alkane-specific extracellular solubilizing agent able to affect the order o metabolism could be detected during the growth of *C. resinae* on mixed *n*-alkanes, but supplementing the medium with phospholipids like those produced during growth on specific alkanes increased the rate or removal of the respective alkanes. Kinetic analysis indicated that the uptake of dodecane, hexadecane and octadecane from a mixture could be by a common mechanism, the order being determined, through competition, by the affinity of the system for each alkane.

Introduction

Blocking of fuel-line filters due to microbial contamination, and the resulting restriction of fuel supply causes operational problems in ships powered by gas-turbines (Neihof et al. 1981). To maintain ballast such ships displace the diesel fuel with seawater. An elaborate coalescer-filtration system is needed to remove traces of corrosive salt water from the fuel before it reaches the engines. Hydrocarbon-utilizing organisms proliferate in the resultant biphasic mixtures of fuel + seawater in fuel tanks and on filters. Biomass dispersed in the fuel becomes entrapped in the filters thus shortening their effective life. Biocide development has not yet found a suitable compound. Evidently much more information is needed about the growth on hydrocarbons of major fuel-contaminating fungi.

Marine diesel fuel is a complex mixture of hydrocarbons whose exact composition depends on the origin of the parent crude oil. However, it is generally considered to be the saturated aliphatic fraction, particularly *n*-alkanes (C_{10-20}), which support microbial growth (Cofone et al. 1973; Lindley and Heydeman 1985a). Although the growth of micro-organisms on single hydrocarbons has been much studied, little is known about their growth on mixed hydrocarbon substrates. Alkane mixtures, being a relatively cheap and plentiful product of 'cracking', have been investigated as potential substrates for protein production, but most of this work relates to the effect of dispersion and droplet size on growth rates (Einsele et al. 1975; Miura et al. 1977; Käppeli and Fiechter 1980). The physiology of the cultures with respect to preferred utilization of substrates has been neglected although clearly of importance for a number of biotechnological applications. Goma et al. (1973a) reported that the yeast *Yarrowia lipolytica* initiated removal of alkanes from a mixture in order of increasing molecular weight, attributing this to the sequential production of extracellular solubilizing agents (Goma et al. 1973b) essential for alkane uptake. However, this finding has not been substantiated with other species, many of which rely upon direct contact with hydrocarbons rather than solubilization into the aqueous phase. For this reason the growth in the presence of multiple hydrocarbon substrates of

* *Present address:* Departement genie biochimique, INSA, 31077 Toulouse, France

Offprint requests to: N. D. Lindley, Departement genie biochimique, INSA, Avenue de Rangueil, F-31077 Toulouse cedex, France

Cladosporium resinae, a fungus isolated from fuel tanks, was examined; the results are presented here.

Materials and methods

Organism and cultivation. The strain of *C. resinae* (ATC 52833) used in this study was isolated from a contaminated fuel tank sample and identified by conventional taxonomic methods. Growth was in shaken or static flasks at 30° C using the mineral medium of Turner et al. (1980). Hydrocarbons (2% w/v) either as single substrates or as mixtures (final total concentration 2%) were used as sole source of carbon. Homogeneous cell suspensions were grown for whole cell physiological experiments using the same medium in a vortex-mixed fermenter which prevented mycelial 'clumping', a common occurrence in flask cultures.

Growth rate determinations. Growth rates were assessed by a spectrophotometric method the validity of which was checked by periodic dry weight determinations.

Hydrocarbon analysis. Uniform sampling of single shaken or static flasks was not reliable so multiple flasks were prepared in an identical manner and whole flasks taken for each data point. Culture fluid was filtered through membranes of 0.45 μm pore size. Alkanes adhering to the biomass were washed off in situ with 10 ml acetone per 25 ml culture. The combined filtrates plus 0.05 ml Tween 80 were converted to an emulsion by ultrasonic disruption and analysed by vapour phase chromatography. Amounts of alkanes within the range C_{12-18} were assessed using a Porapak PS column (1 m long, 0.4 cm internal diameter) with an oven temperature of 220° C and flame ionization detector. Peak height measurements were a sufficiently accurate method of estimating the concentration of each alkane.

Identification of extracellular products. The lipid fraction of the culture filtrate was extracted with chloroform + methanol (2 : 1 v/v) and concentrated by rotary evaporation. Products were separated by two dimensional TLC (Minnikin et al. 1979) and identified by co-chromatography with known standards.

Alkane uptake assay. Alkane emulsions were prepared by ultrasonic treatment of [^{14}C]dodecane, [^{14}C]hexadecane or [^{14}C]octadecane in citrate-phosphate buffer (100 mM, pH 5.5). Cells were harvested by centrifugation, washed twice and resuspended in the same buffer to a final concentration of approximately 1 mg protein ml^{-1}. This cell suspension (2.5 ml) was pre-incubated at 30° C for 15 min before addition of the alkane emulsion (2.5 ml). The mixture was agitated by magnetic stirring bar at 500 rpm. Samples (0.5 ml) were removed at intervals, vacuum-filtered through glassfibre discs and washed three times in situ with ice-cold hexane to remove all superficial alkane adhering to the cells. The radioactivity remaining within the cells was then assessed by scintillation counting as previously described (Lindley and Heydeman 1983). Specific uptake rates were related to the protein concentration estimated by the Lowry technique using bovine plasma albumin as standard.

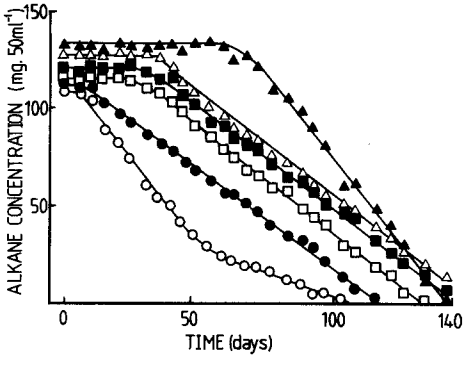

Fig. 1. Removal of *n*-alkanes from a mixture during growth of *Cladosporium resinae* ○, dodecane; ●, tridecane; □, tetradecane; ■, pentadecane; △, hexadecane; ▲, octadecane

Results and discussion

C. resinae growing on alkane mixtures (C_{12-18}) started to remove alkanes in a sequence similar to that reported for *Y. lipolytica* (Goma et al. 1973a), i.e. in order of increasing molecular size (Fig. 1). This sequence was common to all fungal species isolated from fuel tanks (results not shown, see Lindley 1984) and appears to contradict Walker et al. (1975) who reported no characteristic order of metabolism common to fungi. In no instance were the smaller alkanes, whose removal began first, capable of supporting either the most rapid growth, or the highest biomass yields, so the observed sequence cannot be viewed as analogous to classical diauxie. The sequence correlated with extended lag phases of *C. resinae* on larger alkanes (Lindley and Heydeman 1985a) suggesting that some modification of the cell structure, biochemistry or environment was essential before successful exploitation of specific alkanes could occur.

Exponential increases in biomass were not facilitated by increased removal of a specific alkane, rather by initiation of uptake of other alkanes. The rates of removal of individual alkanes were proportional to but lower than those observed during growth on single alkanes (Fig. 2).

During growth the aqueous phase became brown and more readily formed emulsions with alkanes, but this surfactant activity appeared to be non-specific throughout growth with, for example, no enhancement of emulsion stability for any alkane during the period of its utilization. No evidence was found for such specific alkane solubilization as was reported for *Y. lipolytica* (Goma et al. 1973b).

During growth on both glucose and alkanes, *C. resinae* produced extracellular fatty acids and phospholipids. Our studies showed similar prod-

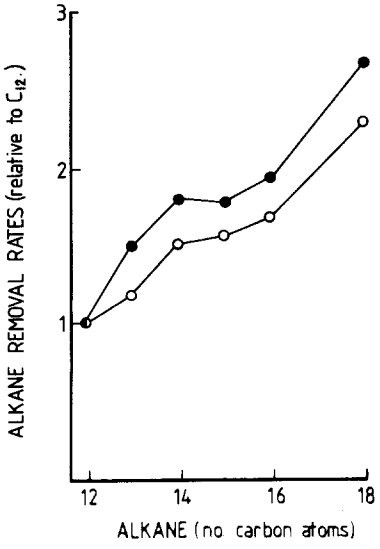

Fig. 2. Relative removal rates of alkanes by *Cladosporium resinae* grown on individual alkanes (○) and alkane mixtures (●). The rates quoted are relative rates and are compared to the rate at which dodecane was removed

ucts to those described before (Siporin and Cooney 1975), phosphatidylcholine and dodecanoic acid being the major products. The hydrocarbon components of such extracellular products differ from those of cellular lipids (Kan and Cooney, 1975), reflecting the size of the alkane substrate (Siporin and Cooney 1975). Two phosphatidylcholines (*bis*-dodecanoyl and *bis*-hexadecanoyl) did not affect the sequence of alkane removal from mixtures, although the rates at which specific alkanes were removed was affected (Fig. 3). Removal of dodecane was stimulated by *bis*-dodecanoyl-substituted lecithin whilst addition of *bis*-hexadecanoyl-substituted lecithin produced faster rates of removal of larger alkanes, especially hexadecane. Addition of dodecanoic acid to cultures did not effect either the sequence or the rate at which alkanes were removed.

Changes in the chemical nature of the culture filtrate which take place during growth of *C. resinae* on hydrocarbons thus increase the efficiency with which this fungus can utilise each alkane, but such changes are not a sufficient explanation of the observed sequence of alkane removal.

Pregrowth of the inoculum on hexadecane did not alter the removal sequence, although growth on the longer alkanes began sooner than in control flasks inoculated with glucose-grown cells. Any necessary modifications to the physiology of the cells allowing uptake and utilization of the longer alkanes thus does not prevent preferential removal of the shorter alkanes. As neither changes in the cell nor changes in extracellular surfactant production can satisfactorily explain the order of metabolism, the possibility that the preference resulted from the characteristics of the uptake system was investigated.

Uptake has been characterized as a rapid saturation of the outer cell surface (Käppeli and Fiechter 1976; Miura et al. 1977; Käppeli and Fiechter 1981) followed by a non-passive movement of alkane into the cell (Käppeli and Fiechter, 1981; Lindley and Heydeman 1985b). Kinetic constants were assigned to the uptake of three *n*-alkanes by *C. resinae* using [^{14}C]-labelled uptake techniques (Fig. 4): dodecane ($K_m = 1.0$ mM, $V_{max} = 12.2$ nmol min^{-1} mg protein^{-1}), hexadecane ($K_m = 4.6$ mM, $V_{max} = 13.6$ nmol min^{-1} mg protein^{-1}) and octadecane ($K_m = 9.9$ mM, $V_{max} = 13.7$ nmol min^{-1} mg protein^{-1}). These figures obtained by double reciprocal plots of uptake rates against substrate concentration were confirmed by statistical methods of analysis (Eisenthal and Cornish-Bowden 1974; Wilkinson 1961). The similarity of the theoretical maximum rates suggests a common mechanism of alkane uptake, the affinity of which favours uptake of shorter alkanes. The change in affinity constants was in close agreement with the culture time be-

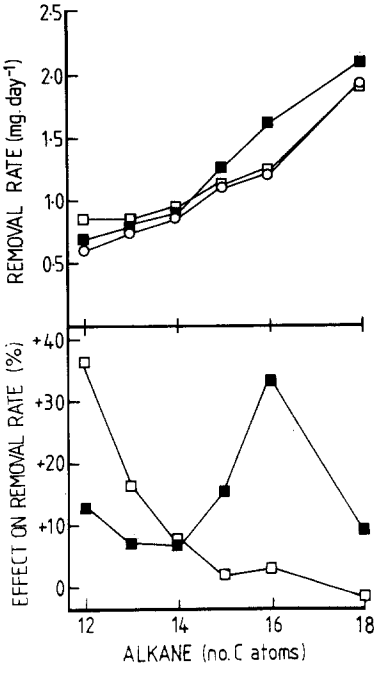

Fig. 3. Effect of phosphatidylcholines on the removal of alkanes from a mixture by *C. resinae*. ○, no addition to medium; □, bis-dodecanoyl-phosphatidylcholine (3 mg in 50 ml), ■, bis-hexadecanoyl-phosphatidylcholine (3 mg in 50 ml)

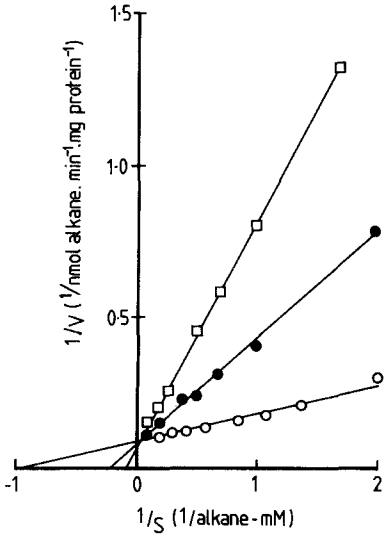

Fig. 4. Double reciprocal plots of alkane uptake rates as a function of substrate concentration for *C. resinae*. ○, dodecane; ●, hexadecane; □, octadecane. Rates of uptake were calculated as nmol min^{-1} mg $protein^{-1}$ and substrate concentrations were in mM

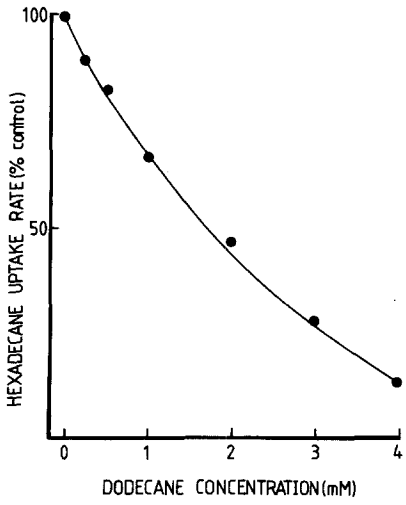

Fig. 5. Effect of various concentrations of dodecane on hexadecane uptake by *Cladosporium resinae*. In all assays the concentration of hexadecane was 2 mM

fore initiation of each alkane's removal from mixtures. This kinetic analysis suggestive of competition between alkanes at a common site provides a likely explanation for sequential removal of alkanes from mixtures.

The effect of dodecane upon the uptake of hexadecane was further investigated to characterize the interrelationship between potentially competing substrates. Less hexadecane was taken up by cells in the presence of dodecane (Fig. 5). As the concentration of hexadecane was insufficient to saturate the assay system, the effect of dodecane was not one of dilution, but rather of favoured uptake. When a fixed quantity of dodecane was added to various concentrations of hexadecane, the V_{max} value remained unaltered whilst the affinity constant (K_m) changed (4.6 mM for hexadecane; 9.1 mM for hexadecane + 2 mM dodecane) (Fig. 6). Thus a second alkane influences uptake of an alkane as if it were a competitive inhibitor.

Such findings clarify the explanation of sequential initiation of alkane removal from mixtures by *C. resinae* since the uptake of shorter alkanes is competitively favoured by the uptake mechanism. Since the K_m values approximately double for each additional C_2H_4 extension, it might be predicted that alkanes of larger molecular weight than those found in diesel fuel (e.g. $C_{22}H_{46}$) would be unlikely, because of transport problems, to support significant growth. The biochemical basis of this affinity phenomenon remains obscure but cannot be explained by specific chemical modification of either the cell surface or the aqueous phase environment. A more likely explanation would be the chemical nature of the substrate, whose molecular fluidity decreases with increasing chain length making penetration of lipid membranes subject to physical limitations.

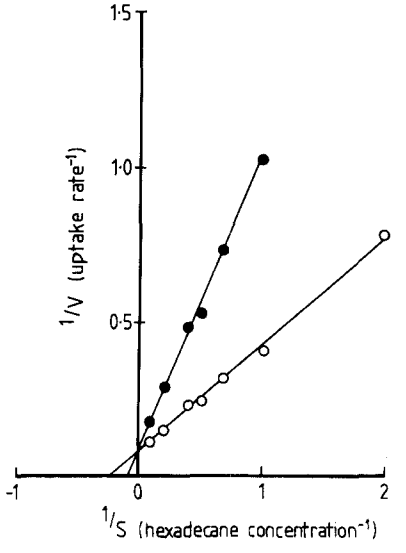

Fig. 6. Double reciprocal plot of hexadecane uptake by *C. resinae* and the competitive influence of dodecane. ○, hexadecane; ●, hexadecane + 2 mM dodecane

Acknowledgement. We thank the Procurement Executive of the Ministry of Defence for financial support.

References

Cofone L, Walker JD, Cooney JJ (1973) Utilization of Hydrocarbons by *Cladosporium resinae*. J gen Microbiol 76:243—246

Einsele A, Schneider H, Fiechter A (1975) Characterisation of microemulsions in a hydrocarbon fermentation by electron microscopy. J Ferment Technol 53:241—243

Eisenthal R, Cornish-Bowden A (1974) The direct linear plot: a new graphical method for estimating enzyme kinetic parameters. Biochem J 139:715—720

Goma G, Pareilleux A, Durand G (1973a) Cinetique de degradation des hydrocarbures par *Candida lipolytica*. Arch Mikrobiol 88:97—109

Goma G, Pareilleux A, Durand G (1973b) Specific hydrocarbon solubilization during growth of *Candida lipolytica*. J Ferment Technol 51:616—618

Kan CM, Cooney JJ (1975) Phospholipids of *Cladosporium resinae* cultured on glucose and on *n*-alkanes. Can J Microbiol 21:1205—1210

Käppeli O, Fiechter A (1976) The mode of interaction between the substrate and the cell surface of the hydrocarbon-utilizing yeast *Candida tropicalis*. Biotechnol Bioeng 18:967—974

Käppeli O, Fiechter A (1980) Partition of hexadecane to the cell surface of *Candida tropicalis:* mechanism for the transport of water-insoluble substrates. Biotechnol Bioeng 22:1829—1841

Käppeli O, Fiechter A (1981) Properties of hexadecane uptake by *Candida tropicalis*. Curr Microbiol 6:21—26

Lindley ND (1984) PhD Thesis, University of Reading, UK

Lindley ND, Heydeman MT (1983) Uptake of vapour phase [^{14}C]dodecane by whole mycelia of *Cladosporium resinae*. J Gen Microbiol 129:2301—2305

Lindley ND, Heydeman MT (1985a) Alkane utilization by *Cladosporium resinae:* the importance of extended lag phases when assessing substrate optima. FEMS Microbiol Ecol 31:307—310

Lindley ND, Heydeman MT (1985b) The mechanism of dodecane uptake by whole cells of *Cladosporium resinae*. J Gen Microbiol (in press)

Minnikin DE, Collins MD, Goodfellow M (1979) Fatty acid and polar lipid composition in the classification of *Cellulomonas, Oerskovia,* and related taxa. J appl Bacteriol 47:87—96

Miura Y, Okazaki M, Hamata S, Murakawa S, Yugen R (1977) Assimilation of liquid hydrocarbons by micro-organisms. Biotechnol Bioeng 19:701—714

Neihof RA, Klemme DE, Patouillet CE, Hannan PJ (1981) Microbial contamination of ships fuels. Distillate Fuel Stability and Cleanliness ASTM STP 751 (Stavinoha LL, Henry CP (eds) American Society for Testing and Materials, pp 126—135

Siporin C, Cooney JJ (1975) Extracellular lipids of *Cladosporium (Amorphotheca) resinae* grown on glucose or on *n*-alkanes. Appl Microbiol 29:604—609

Turner APF, Higgins IJ, Gull K (1980) Microbodies in *Cladosporium resinae* grown on glucose and on *n*-alkanes. FEMS Microbiol Lett 9:115—119

Walker JD, Austin HF, Colwell RR (1975) Utilization of mixed hydrocarbon substrates by petroleum-degrading micro-organisms. J Gen Appl Microbiol 21:27—39

Wilkinson GN (1961) Statistical estimations in enzyme kinetics. Biochem J 80:324—332

Received July 4, 1985/Revised August 24, 1985