Utilization of \( n \)-Alkanes by \textit{Cladosporium resinae}

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Four different isolates of \textit{Cladosporium resinae} from Australian soils were tested for their ability to utilize liquid \( n \)-alkanes ranging from \( n \)-hexane to \( n \)-octadecane under standard conditions. The isolates were unable to make use of \( n \)-hexane, \( n \)-heptane, and \( n \)-octane for growth. In fact, these hydrocarbons, particularly \( n \)-hexane, exerted an inhibitory effect on spore germination and mycelial growth. All higher \( n \)-alkanes from \( n \)-nonane to \( n \)-octadecane were assimilated by the fungus, although only limited growth occurred on \( n \)-nonane and \( n \)-decane. The long chain \( n \)-alkanes \((C_{14} \text{ to } C_{18})\) supported good growth of all isolates, but there was no obvious correlation between cell yields and chain lengths of these \( n \)-alkanes. Variation in growth responses to individual \( n \)-alkane among the different isolates was also observed. The cause of this variation is unknown.

For the past decade the growth of microorganisms in jet fuel, resulting in the formation of biological sludge and the corrosion of fuel tanks, caused serious concern in aviation industry. To date, no completely satisfactory control of microbial contamination has been found (6, 7, 16). It is now generally believed that a filamentous fungus, \textit{Cladosporium resinae}, plays the major role in most cases of fuel system fouling (5, 8, 16). The organism has also been found to grow in hydraulic fluid, diesel fuels, lubricating oil, and other kerosene-type fuels (16).

During its growth on jet fuel, \textit{C. resinae} preferentially utilized the component \( C_{4} \text{ to } C_{8} \) \( n \)-alkanes (16). On the other hand, gasoline which contains mostly short-chain alkanes is usually not susceptible to attack by the fungus (10). Recently, Cooney and Proby (3) reported that the growth of \textit{C. resinae} on all individual \( n \)-alkanes, with the exception of \( n \)-dodecane, decreased in shake cultures as the chain length increased from \( n \)-decane to \( n \)-tetradecane. These reports suggest that \textit{C. resinae} may exhibit a certain degree of substrate specificity with regard to the \( n \)-alkanes. At this time, studies on \( n \)-alkane utilization by the fungus, which were usually carried out with a single isolate, are either incomplete or qualitative in nature (3, 10, 16, 19). The natural variability of \textit{C. resinae} (16) is such that results obtained from work done with only one isolate could not be considered representative. Furthermore, differences in cultivation methods and in the amount of hydrocarbon substrate actually available to the organism, which depends partly on the physical state of the hydrocarbon, would undoubtedly complicate the interpretation of observations obtained by different investigators.

This communication reports quantitative data on the comparative utilization of individual liquid \( n \)-alkanes, ranging from \( n \)-hexane to \( n \)-octadecane, by several isolates of \textit{C. resinae} under standard conditions. An attempt was also made to investigate the effect of various \( n \)-alkanes on the growth of the organism on glucose.

**MATERIALS AND METHODS**

**Organism.** Isolates 35A, 89A, 95B, and 102B, which were isolated from Australian soils and identified as \textit{C. resinae f. avellaneum} (14), were obtained from D. G. Parbery, University of Melbourne, Victoria, Australia. They were maintained on Bushnell-Haas glucose-agar slants.

**Media.** Bushnell and Haas (1) mineral salts solution, containing ammonium nitrate as nitrogen source, was used as the aqueous phase in all experiments. Bushnell and Haas agar contained additional 2% agar and 1% glucose. Normal alkanes (\( \geq 99\% \) purity) were obtained from Koch-Light Laboratories, Colnbrook, England, and Eastman Kodak Co., New York. Purity of the \( n \)-alkanes was checked by both gas-liquid chromatography and thin-layer chromatography; the latter showed that oxygenated impuri-
ties were absent in all of the n-alkanes used. Jet A-1 commercial fuel was kindly supplied by the Shell Co. of Singapore. Glucose, when used as a carbon source in liquid cultures, was autoclaved separately in concentrated solution and added aseptically to the medium to give a 2% final concentration.

Cultivation methods. All liquid culture experiments were carried out in duplicate in standard 250-ml Erlenmeyer flasks. For the preparation of liquid inoculum, the organism was grown on Bushnell-Haas agar slants for one to two weeks at room temperature. A spore suspension was obtained by washing the spores off the agar slants with 10 to 15 ml of sterile distilled water. A 0.3- to 0.5-ml sample of this suspension was continuously agitated during inoculation and then pipetted into each flask.

The effect of hydrocarbon substrate concentration on growth was determined by adding increasing quantities (1 to 40 ml) of Jet A-1 fuel to a constant volume (40 ml) of the aqueous salt medium. After inoculation, the flasks were incubated statically at 30 C for 35 days.

Utilization of individual n-alkanes by the fungus was determined under the following standard conditions. Each flask contained 40 ml of mineral salt medium and 5 ml of the n-alkane being tested; these were added as an overlay on the aqueous surface. After inoculation, all flasks were incubated statically at 30 C for 35 days. For reference, glucose and Jet A-1 fuel were included as substrates.

The effect of individual n-alkanes on spore germination in glucose solution was determined by adding 0.5 ml of the spore suspension to a mixture of 5 ml of the hydrocarbon being tested and 40 ml of the mineral salt medium containing 2% glucose. The flasks were incubated statically at 30 C for 21 days. To investigate the effect of n-alkanes on mycelial growth, flasks containing 2% glucose-mineral salt medium were inoculated with spores and incubated at 30 C for 24 h, by which time tiny white mycelial colonies were clearly visible. A 5-ml sample of the sterile n-alkane being tested was then added aseptically to the mixture, and incubation was continued thereafter for 20 days at 30 C.

Thin-layer chromatography. This was carried out on plates spread with Silica Gel G (Merck and Co., Inc.). The plates were developed with hexane-diethyl ether-acetic acid (95:15:2), sprayed with 0.2% ethanolic 2',7'-dichlorofluorescein, and examined under ultraviolet light (254 nm).

Gas-liquid chromatography. This was performed with a Varian Aerograph model 1200 gas chromatograph equipped with a flame ionization detector. A stainless steel column (6 ft by ½ inch ca. 81.28 by 0.05 cm in) packed with 80/100 mesh Chromosorb G and coated with 5% SE-30 was used. Temperature was programmed from 40 to 100 C at 2 C/min for the lower n-alkanes and from 100 to 200 C at 4 C/min for the higher boiling n-alkanes. The carrier gas was nitrogen at a flow rate of 25 ml/min. Temperatures of the injection port and the detector were 250 C. Only a single peak was obtained for all of the n-alkanes tested.

Dry weight determination. At the end of the incubation period, the cells from each flask were collected by vacuum filtration. They were washed successively with small portions of distilled water and petroleum ether (bp 40 to 60 C) to remove salts and residual hydrocarbon. Finally, the cells were transferred quantitatively to a preweighed dry aluminium boat and dried to constant weight in an oven at 105 C.

RESULTS AND DISCUSSION

In view of the influence of flask shape and size on the growth of C. resinae (15), uniform batches of 250-ml Erlenmeyer flasks were used in all experiments. Since shaking generally led to lower cell yields, the flasks were incubated statically in agreement with a previous finding (15). The optimum temperature for growth for the four isolates appeared to be at or near 30 C. Hydrocarbon substrate was added as an overlay, since C. resinae is known not to utilize emulsified jet fuel for growth in liquid cultures (2). The organism grew at the hydrocarbon-water interface, forming a partial or complete mycelial mat. The effect of increasing hydrocarbon concentration on the growth of isolate 35A is shown in Fig. 1. It can be readily seen that growth of the organism in the static two-phase liquid medium was dependent upon the concentration of hydrocarbon and that a high cell yield could only be attained when the hydrocarbon concentration exceeded 10% (vol/vol). Similar phenomenon has been reported previously for a hydrocarbon-utilizing Candida species (17). A likely explanation seems to be the higher oxygen availability to the aqueous medium, with the imposition of a discrete layer of hydrocarbon (4, 12).

Results of the utilization of individual n-alkanes for growth by C. resinae are shown in Table 1. Although the fungus grew rapidly in 2% glucose and all four isolates gave almost identical large cell mass, the amount of growth on n-alkanes or jet fuel was relatively small. This is, perhaps, a common feature of hydrocarbon-utilizing filamentous fungi, since there have been very few reports of any mold being able to grow rapidly on hydrocarbons (11, 18). The three short-chain n-alkanes (n-hexane, n-heptane, and n-octane) did not support any visible growth of the isolates examined. Tanaka et al. (19) had previously reported that n-hexane and n-heptane could not be utilized by a C. resinae isolate, although Leonard & Klemme (10) claimed that their Hormodendrum (later revised as Cladosporium) isolate from jet fuel showed limited growth in n-octane. All of the higher liquid n-alkanes from n-nonane to n-octadecane were assimilated by the fungus, although only limited growth occurred on n-nonane and n-decane. The considerable natural variability of C. resinae (16) was reflected in its utilization of long- and intermediate-chain n-alkanes. For example, n-un-
decane supported good growth of isolates 35A and 89A; however, it did not support growth of the other two isolates. On the other hand, n-tridecane elicited relatively high growth response in all isolates except isolate 95B. What caused these differences is unknown. In general, the series of n-alkanes, ranging from n-tetradeccane to n-octadecane, appeared to be well utilized, but there was no obvious correlation between cell weights and chain length of the n-alkanes. For a given isolate, one particular n-alkane might give a higher growth response than others; the particular n-alkane eliciting that response could be any member of the n-alkane series that supported good growth. Similar behaviour has been described for other filamentous fungi growing on the C₁₀ to C₁₄ n-alkanes (13). Although Cooney & Proby (3) found that the cell yield of *C. resinae* decreased with increasing chain length of the n-alkanes from n-decane to n-tetradeccane, such was not observed in our investigation. The difference could be due to the different cultural conditions used or the fact that only one isolate was tested on a limited number of n-alkanes by these investigators.

Both Lowery et al. (11) and Nyns et al. (13) have observed that n-alkanes shorter than n-decane generally supported little or no growth of a large number of hydrocarbon-utilizing filamentous fungi. Some workers have attributed this to the toxicity of the short-chain n-alkanes which, being generally good lipid solvents, could extract certain essential cell lipids from the cell membrane (9, 13). In our present study, the inability of the *C. resinae* isolates to grow on n-hexane, n-heptane, and n-octane suggests either a lack of specific enzymes for transporting and oxidizing these n-alkanes or toxicity due to the short chain n-alkanes themselves, or both. The possible toxicity of n-alkanes was, therefore, tested experimentally by growing *C. resinae* on glucose together with various n-alkanes added in excess. Results are shown in Table 2.

Both n-hexane and n-heptane completely inhibited spore germination in 2% glucose solution, even after prolonged incubation. Only partial inhibition was brought about by n-octane, n-Nonane and all higher n-alkanes tested had no inhibitory effect on spore germination.

### Table 1. Growth of four isolates of *Cladosporium resinae* on various liquid n-alkanes, Jet A-1 fuel, and glucose

| Carbon source | Total dry-cell weight (mg) |
|---------------|--------------------------|
|               | Isolate 35A | Isolate 89A | Isolate 95B | Isolate 102B |
| n-Hexane      | 0           | 0           | 0           | 0           |
| n-Heptane     | 0           | 0           | 0           | 0           |
| n-Octane      | 0           | 0           | 0           | 0           |
| n-Nonane      | <1          | <1          | <1          | 1           |
| n-Decane      | 2           | 1           | 2           | 1           |
| n-Undecane    | 19          | <1          | 2           | 34          |
| n-Dodecane    | 21          | 6           | 1           | 9           |
| n-Tridecane   | 53          | 27          | 2           | 28          |
| n-Tetradecane | 46          | 44          | 29          | 17          |
| n-Pentadecane | 52          | 28          | 36          | 19          |
| n-Hexadecane  | 54          | 29          | 26          | 21          |
| n-Heptadecane | 32          | 26          | 34          | 21          |
| n-Octadecane  | 44          | 34          | 26          | 32          |
| Jet A-1 Fuel  | 37          | 34          | 26          | 22          |
| Glucose       | 133         | 135         | 138         | 126         |

### Table 2. Effect of various n-alkanes on the growth of *Cladosporium resinae* in 2% glucose

| n-Alkane added | Total dry-cell weight (mg) |
|---------------|--------------------------|
|               | Isolate 35A | Isolate 89A | Isolate 102B |
| n-Hexane      | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> |
| n-Hexane      | <1           | <1           | <1           |
| n-Heptane     | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> |
| n-Heptane     | 15           | 18           | 22           |
| n-Octane      | 15           | 34           | 10           |
| n-Nonane      | 78           | 87           | 88           |
| n-Nonane      | 86           | 90           | 91           |
| n-Decane      | 91           | 96           | 94           |
| n-Decane      | 82           | 89           | 101          |
| n-Dodecane    | 87           | 93           | 96           |
| n-Tetradecane | 87           | 98           | 87           |
| n-Hexadecane  | 92           | 91           | 103          |
| Jet A-1*      | 90           | 91           | 105          |
| Jet A-1<sup>a</sup> | 85       | 90           | 100          |
| Control<sup>c</sup> | 86 | 93           | 96           |

<sup>a</sup> Hydrocarbon (5 ml) added before inoculation.

<sup>b</sup> Hydrocarbon (5 ml) added 24 h after inoculation.

<sup>c</sup> Fungus grown in 2% glucose-mineral salt medium without alkanes.

<sup>d</sup> No visible growth after a further 21-day incubation period at 30°C.
and subsequent growth, the final cell yields being similar to that of the control. The effect of short-chain n-alkanes on mycelial growth appeared to be less marked. Only n-hexane stopped mycelial growth completely, whereas n-octane and higher n-alkanes had no significant inhibitory effect. On removal of the short-chain n-alkanes from the culture flasks under a gentle stream of sterile nitrogen gas, spor germination and resumption of mycelial growth were observed. The final cell yields were similar to those of the control experiments. Thus, the lower n-alkanes, particularly n-hexane, are fungistatic towards C. resinae growing glucose. Preliminary work also indicates that n-hexane and n-heptane inhibited spor germination and mycelial growth when Jet A-1 fuel was used as the sole carbon source. This may explain why C. resinae does not grow readily on gasoline, since the latter is rich in short-chain n-alkanes. At present, the inhibitory action of the short-chain n-alkanes on the growth of C. resinae is not well understood, although it is likely to be related to the cell membrane and the higher solubility of these n-alkanes. While this work was in progress, Walker and Cooney (20) reported in a communication that n-hexane apparently altered the cell membrane and decreased the endogenous respiration of C. resinae.

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