Utilization of \( n \)-Alkanes by \textit{Pullularia pullulans}

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Received for publication 16 July 1970

\textit{Pullularia pullulans} was tested for its ability to utilize a series of \( n \)-alkanes for growth. It utilized hydrocarbons containing higher C-numbers (13, 14, 16, and 18) to a greater degree than those containing lower numbers; in addition, an induction phenomenon was noted. Gas-liquid and thin-layer chromatography of ether extracts of the growth media revealed that oleic and palmitic acids were formed from tridecane, tetradecane, hexadecane, and octadecane.

\textit{Pullularia pullulans} strain NRRL YB-4515, which was shown previously to grow on a variety of media (1, 2), was tested for its ability to utilize various hydrocarbons as the sole source of carbon. It was maintained on Nutrient Agar (Difco) slants and grown in Yeast Nitrogen Base (Difco). All hydrocarbons studied (hexane, heptane, octane, nonane, decane, undecane, tridecane, tetradecane, hexadecane, and octadecane) were obtained from Sinclair Oil Corp., Harvey, Ill., and ranged in purity from 95 to 98 moles \%. The medium and the individual hydrocarbons were sterilized by filtration through membrane filters (25-mm diameter; 0.45- \( \mu \text{m} \) pore size; Millipore Corp., Bedford, Mass.).

The depletion medium in which the organism was grown was prepared in three stages at 24-hr intervals. A loopful of the organism was transferred aseptically from a 48-hr-old stock culture into a 125-ml Erlenmeyer flask containing 30 ml of basal medium with 0.5\% glucose and incubated on a reciprocal shaker for 24 hr. A 0.1-ml amount of this culture was transferred into a flask containing 30 ml of the basal medium supplemented with 0.1\% glucose, and the flask was shaken for another 24-hr period. The third stage consisted of transferring 0.1 ml of the second stage culture into a 125-ml flask containing 30 ml of the yeast nitrogen base. After 24 hr of shaking, when the medium was entirely depleted of glucose, it was used as inoculum to test the organism for the ability to assimilate hydrocarbons.

Each of the 250-ml Erlenmeyer flasks containing 98 ml of basal medium was equipped with rubber-capped glass tubing which entered each flask and allowed aseptic delivery of inoculum as well as withdrawal of samples by needle and syringe. To each vessel, 1.0 ml of the third stage inoculum was introduced. Incrementally, 1.0 ml of one hydrocarbon was then added to each flask daily over a period of 20 days (one droplet per day). With the exception of the flasks containing hexane, heptane, and octane, all flasks were shaken for 21 days. Controls contained no substrate. All procedures were carried out at room temperature (28 \( ^\circ \)C), and the cultures were examined periodically for contamination. After 21 days of incubation, 5 ml was removed from each flask and added to the corresponding flask of the new series containing 94 ml of fresh sterile basal medium. Again, 1 ml of the respective hydrocarbons was added incrementally (two droplets per day) over a period of ten days with the proper controls (without substrate).

The cells of the known volume from each of the 21-day and the subsequent 10-day incubation series were collected by centrifugation, lyophilized, and weighed. Subsequently, they were transferred into Erlenmeyer flasks and extracted with ether for 24 hr (agitated by a magnetic stirrer). The ether layers were collected, the solvent was removed, and the residues were analyzed by thin-layer chromatography on plates coated with Adsorbisil-I. After a preliminary treatment with hexane to move any residual hydrocarbon present in the ether extract to the top of the plate, the chromatograms were developed with hexane-diethyl ether-acetic acid (40:10:1, v/v). The spots, made visible by spraying the plates with 0.2\% ethanolic 2',7'-dichloro-fluorescein, were located under ultraviolet light and compared to authentic standards.

For the separation, methylation, and identification of the fatty acids by gas chromatography, the procedure described by White and Cox (5) was employed. The methyl esters of the fatty acids were identified on the basis of their retention times as compared with standards of high purity. Although some authors have observed that static incubation enhances utilization of hydrocarbons by microorganisms (3, 4), we found that the shaking of our cultures resulted in a more
abundant growth. It also allowed more accurate daily growth measurements by colony counts. Without shaking, *P. pullulans* forms clusters which cannot be dispersed homogeneously. The amounts of growth of *P. pullulans* in individual alkanes are shown in Table 1.

The pattern of growth of *P. pullulans* during the subsequent 10-day experiment (Table 1) indicated adaptation of the organism to utilize hydrocarbons as a sole source of carbon. The pattern confirmed that the alkanes with a higher number of carbon atoms were better assimilated than those containing a lower number of carbon atoms. Undecane appears to fall into the former group, and nonane and decane into the latter. The data in Table 1 also indicate that the highest yield of cells was obtained with hexadecane and somewhat less with octadecane. The total yield of cells grown in the presence of the various hydrocarbons was 2.9 (n-hexane) and 5.7 (n-hexadecane) times greater than that of the controls in the 21-day experiment and 5.3 and 13.4 times greater, respectively, in the subsequent 10-day experiment than the control.

Thin-layer chromatography of the ether extracts from *P. pullulans* grown in tridecane, tetradecane, hexadecane, and octadecane for 21 days and the subsequent 10 days revealed spots corresponding to those of palmitic and oleic acids. The methyl esters of this material confirmed the presence of palmitic and oleic acids when analyzed by gas-liquid chromatography since they had the same retention times as authentic standards.

Several additional spots were also detected on the plates, but these have not been identified.

This investigation was supported by a grant from Abbott Laboratories, North Chicago, Ill.

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