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Prototheca zopfii Krüger Strain UMK-13 Growth on Acetate or *n*-Alkanes

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A new strain of *Prototheca zopfii* Krüger was grown on acetate or on pure *n*-alkanes. A maximum acetate-supported exponential growth of 12 divisions day^{-1} occurred at pH 5 and 30°C. At 25°C, growth on *n*-alkanes was almost as fast, but no growth occurred at 30°C. After 4 days at 25°C, 34 to 45% of the *n*-alkanes had been removed, whereas at 21°C and slower growth, utilization was twofold greater after 15 days. Rates of growth and utilization increased markedly after a point of sudden emulsification.

Since the initial report by Kocková-Kratochívlová and Havelkova (9) on alkane utilization by an isolate designated *Prototheca hydrocarbonea*, others have described features of hydrocarbon heterotrophy in *P. zopfii*. Particular emphasis has been on crude oil degradation and utilization. Walker et al. (11, 12), using a *P. zopfii* strain isolated from Chesapeake Bay, reported growth on crude oil and mixed hydrocarbons. Moreover, Walker et al. (11) compared hydrocarbon heterotrophy of *P. zopfii* with that of crude oil-degrading bacteria and observed that *P. zopfii* was able to remove an amount of crude oil comparable to that biodegraded by some bacteria. Walker and Pore (13) tested 55 isolates of *Prototheca* and found that 75% of the *P. zopfii* isolates grew on hydrocarbons.

The ability of a microorganism to utilize hydrocarbons is influenced by the physical and chemical features of its environment. Whereas the literature is replete with reports on effects of environmental factors on hydrocarbon heterotrophy and growth of bacteria and fungi, there have been only limited references to growth responses of *P. zopfii* to physical and chemical variables (1, 4, 7, 8). A comprehensive review of microbial degradation with an environmental perspective has been presented by Atlas (2).

We first established optimum conditions of pH, temperature, and salinity for acetate-supported growth of a new isolate of *P. zopfii*. We then tested the organism for its ability to biodegrade selected *n*-alkanes. We report herein the results of these investigations and describe features of *n*-alkane emulsification by the new isolate.

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Initially, a culture of *P. zopfii* Krüger ATCC 30253 was tested for growth in enriched media. During our first trials, we isolated from the ATCC 30253 strain a variant which exhibited markedly increased growth rate with acetate supplied as a primary carbon source. We abandoned the ATCC 30253 parent strain in favor of the new isolate, designated strain UMK-13.

Stock cultures were maintained on Sabouraud Dextrose (Difco) 2% agar. Experimental cultures were grown aerobically in the dark in liquid Bristol medium (3) modified by substituting NH_4NO_3 (0.25 g/liter) for $NaNO_3$ and adding $FeCl_3$ (2.0 mg/liter), glycine (0.25 g/liter), yeast extract (0.25 g/liter), thiamine (0.001 g/liter), and 1.0 ml of H_5 microelement mix per liter (6). This formulation was used as a basal mineral medium. Thiamine was filter sterilized and added after autoclaving. Adjustments of pH with KOH or HCl were made before and after autoclaving and reevaluated at the termination of growth runs. Salinity, expressed as parts per thousand (‰) as read with an American Optical model 10400 refractometer, was adjusted by the addition of NaCl to the basal medium.

For studies on growth responses to environmental factors, we supplied sodium acetate (2.0 g/liter) as the primary carbon source (no growth occurred on basal medium alone) in 50 ml of basal medium contained in 300-ml Nephelo sidearm flasks (Bellco Glass, Inc., Vineland, N.J.). Each of three replicates was inoculated with 1.0 ml of 48-h-old starter culture and grown in a temperature-controlled chamber. Suspensions were stirred at 300 rpm by 5-cm Teflon-coated bars driven by magnetic stirrers supporting the culture flasks.

Specific growth constants, expressed as divisions day^{-1}, were calculated during exponential growth according to the method of Guillard (5).

Growth was measured as increase in optical density (O.D.) at 750 nm. Growth on pure *n*-alkanes was studied by adding 1% (vol/vol) of filter-sterilized *n*-alkane in place of acetate to 50 ml of basal medium, pH 5. All *n*-alkanes were purchased from MCB Manufacturing Chemists, Inc., Cincinnati, Ohio. Tridecane (lot B2L23), tetradecane (lot A4N16), hexadecane (lot 5N05), and a mixed alkane substrate (MAS) were tested separately at 25°C. The MAS was an equal mixture of tridecane, hexadecane, tetradecane, and octadecane (lot 11H28), each at 0.25% (vol/vol). We did not include octadecane in the individual tests because it exists as a solid at 25°C. Octadecane used alone liquified at 30°C or solubilized at 25°C when mixed with the other hydrocarbons. We performed our experiments at less than 30°C because the alga would not grow on any tested *n*-alkane at 30°C. Trials consisted of three replicates, each of which was inoculated with 1.0 ml of a 12-day-old preconditioned MAS starter culture derived from a 48-h-old acetate-enriched culture. Control flasks containing basal medium minus added *n*-alkane received a similar cell inoculum.

The degree of utilization of *n*-alkanes was determined by gas chromatographic analysis of whole-cell extracts. The time-course study consisted of MAS cultures grown at 21°C, harvested, and extracted at different points of the growth cycle. For each assay, replicate cell suspensions and cell-free controls were extracted with an equal volume of benzene according to the method of Walker and Colwell (10), after which the solvent layer was evaporated. The residue was dissolved in 5 ml of CS_2. A 1.0-μl sample was injected into a Hewlett-Packard 5840A gas chromatograph fitted with a glass column (3.67 m by 2 mm inside diameter) containing OV-17 on 80-100 mesh, 3% Chromosorb WHP (Altech Associates, Inc., Deerfield, Ill.). The instrument was programmed to increase column temperature from 100 to 190°C at 8°C min^{-1}. The flame ionization detector and injection port were set at 275°C and the nitrogen flow rate was 20 ml/min. Data were expressed as the percent decrease in peak areas relative to controls.

In an acetate-enriched medium, strain UMK-13 grew within a pH 3 to 9 range, with best growth occurring at pH 3 to 5. At pH 5, growth was best at 25 to 35°C, with maximal growth occurring at 30°C. At 40°C no growth occurred. As salinity increased (the basal medium was at 8.0‰), the growth rate of the alga decreased. At 22.7‰ salinity and above, the rate of growth for strain UMK-13 decreased markedly. No growth was observed at 35‰. Under optimum conditions (pH 5.0, 30°C) in an acetate-enriched medium, strain UMK-13 grew exponentially at approximately 12 divisions day^{-1}. We note, however, that the rapid exponential growth of strain UMK-13 occurred only during the first 20 h of culture, after which growth was near linear until beyond an O.D. of 1.0. Under similar conditions in our laboratory, the ATCC 30253 parent strain grew at approximately 3 divisions day^{-1} although the slower exponential growth

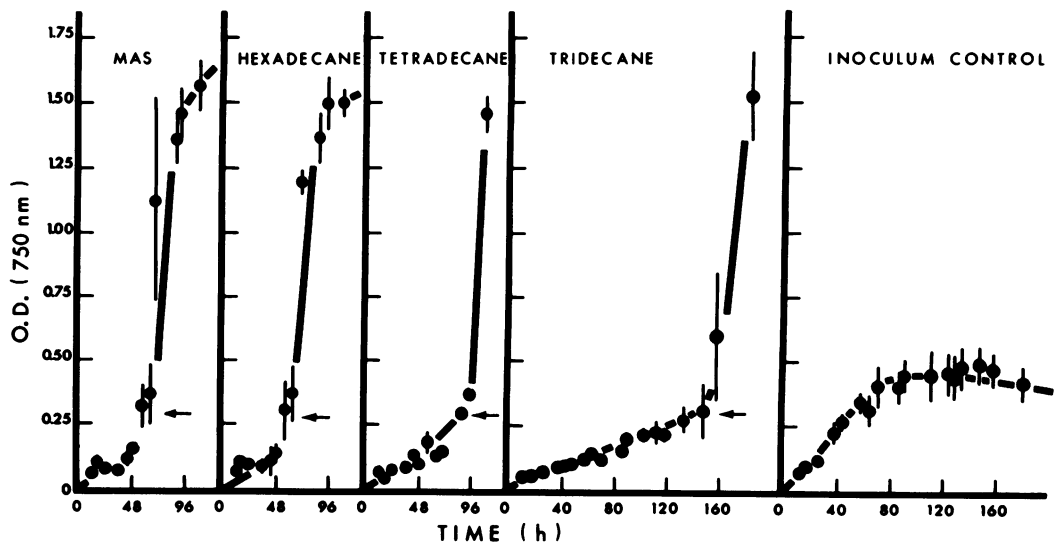

FIG. 1. Growth of UMK-13 on individual and MAS 1% (vol/vol) pure *n*-alkanes. The arrows designate the point of culture at which emulsification occurred. The exponential growth rate on MAS was 3.0 divisions day^{-1}. Vertical lines represent 1 standard deviation from the mean of three replicates.

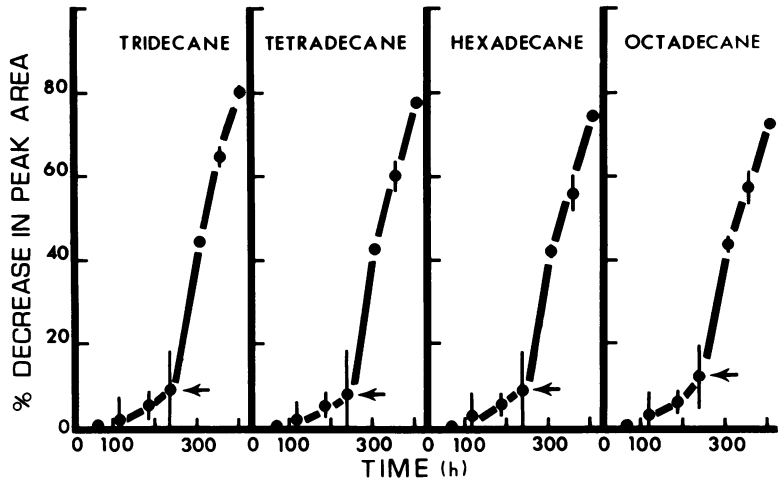

FIG. 2. Degree of utilization of *n*-alkanes by UMK-13 grown on MAS at 21°C. Arrows designate the point of emulsification at an O.D. of approximately 3.0. Vertical lines represent 1 standard deviation from the mean of three replicates.

persisted beyond 3 days of culture. After 22 h of culture (pH 5, 30°C), suspensions of strain UMK-13 had reached an O.D. of approximately 0.65, whereas ATCC 30253 suspensions measured an O.D. of approximately 0.11 and did not reach 0.65 until after approximately 40 h of growth. At 25°C and pH 5, strain UMK-13 grew nonexponentially at a steady rate of about 1.5 O.D. units day^{-1}.

Strain UMK-13 grew almost as well at pH 5 and 25°C on selected *n*-alkanes (Fig. 1) as on acetate, but only after a long lag time. We found that by using MAS-grown starter cultures, we could shorten the lag time compared with cultures started from acetate-grown cells. Strain UMK-13 grew best on MAS or on hexadecane, in which case we observed shorter lag times than those for the tridecane and tetradecane cultures. All cultures had similar growth rates (approximately 1.0 O.D. units day^{-1}) after a point of rather sudden emulsification (arrows, Fig. 1) occurring at approximately O.D. 0.30 to 0.35. The inoculum controls never attained an O.D. greater than 0.5; we attribute growth in that case to residual *n*-alkanes introduced with the inoculum. At 30°C, no growth occurred on any *n*-alkane. This observation was puzzling inasmuch as best growth occurred at 30°C on acetate. For 25°C cultures (Fig. 1), gas chromatographic analysis of extracts at an O.D. of approximately 1.5 revealed that 30 to 45% of *n*-alkanes had been removed as growth entered a plateau phase after 4 days. These values are within the ranges reported by Walker et al. (12) for ATCC 30253 grown at 25°C on mixed hydrocarbons for 14 days. When we tested *n*-alkane utilization by strain UMK-13 grown on MAS at 21°C, we found that 75 to 80% of each *n*-alkane had been removed as growth slowed at an O.D. of 3 to 3.5 after approximately 15 days of culture (Fig. 2). For strain UMK-13 at least, temperature had a considerable effect on utilization of the *n*-alkanes tested. The rate of utilization for each *n*-alkane increased dramatically at the point of emulsification (arrows, Fig. 2). Microscopic examination of preemulsification suspensions revealed that groups of cells appeared to adhere to the large *n*-alkane droplets formed by agitation. After emulsification, individual cells appeared to have one or more small *n*-alkane droplets adhering to the cell surface. Cell-free *n*-alkane suspensions did not emulsify. We interpreted these observations to mean that strain UMK-13 caused the emulsification phenomenon and that possibly physical contact was required for utilization of the hydrophobic substrates.

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