APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1978, p. 694-697
0099-2240/78/0035-0694$02.00/0
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Vol. 35, No. 4
Printed in U.S.A.

Growth of *Prototheca* Isolates on *n*-Hexadecane and Mixed-Hydrocarbon Substrate

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Received for publication 7 December 1977

Prototheca zopfii, an achlorophyllous alga, was capable of using hydrocarbons as sole carbon and energy source. The ability of *P. zopfii* to use hydrocarbons did not correlate with source of isolation. Seventy-five percent of the *P. zopfii* cultures recovered from sewage, plants, or animals utilized hydrocarbons. Other *Prototheca* species and *P. zopfii* that did not utilize hydrocarbons were isolated simultaneously from several sources with isolates that did use hydrocarbons. Species type rather than source of isolation was the predominant factor that determined hydrocarbon utilization.

Masters and Zajic observed myxotrophic growth of algae on hydrocarbons (3). However, it was only recently that heterotropic growth of algae on hydrocarbons was reported. Kockova-Kratochvilova and Havelkova (2) described *n*-hexadecane utilization and Walker et al. (8) described crude oil degradation by the achlorophyllous alga *Prototheca.*

To determine the extent of hydrocarbon utilization by *Prototheca,* 138 isolates were examined for their ability to grow on *n*-hexadecane and the mixed-hydrocarbon substrate (MHS) of Walker and Colwell (7). The latter contains a mixture of aliphatic, cyclic, and aromatic hydrocarbons. Results were compared with source of isolates and taxonomic species in order to determine if either were correlated with hydrocarbon-using ability.

MATERIALS AND METHODS

Isolates. *Prototheca* isolates were obtained from a number of different sources. The taxonomic disposition of each isolate was made utilizing the guidelines of Sudman (6). Isolates assimilating trehalose were classified as *P. wickerhamii,* whereas those that could not were classified as *P. zopfii.* Since no isolates were able to assimilate sucrose, *P. stagnora* was not identified. *P. filamenta* has been removed from the genus by Pore et al. (4). In this manner, 122 of 138 isolates were classified as either *P. wickerhamii* or *P. zopfii,* whereas the remaining 16 isolates bear the designation *Prototheca* sp.

Media. Medium I contained 0.7 g of KH_2PO_4, 0.3 g of $K_2HPO_4 \cdot 0.3$ g of $MgSO_4 \cdot 0.3$ g of $FeSO_4$, 1 ml of "A_5" solution (1), 10 μg of thiamine, and 5 g of glycine in 1 liter of distilled water at pH 6.3 (5). Medium II was medium I containing only 0.1 g of glycine and 10 g of glucose per liter(5). Medium III was medium I with 1 g of NH_4NO_3 per liter substituted for 5 g of glycine per liter.

Culture conditions. Preliminary experiments to assess hydrocarbon utilization were conducted by transferring a minimal amount of each isolate from a Sabouraud slant to two screw-cap test tubes (16 by 150 mm), containing, respectively, 5 ml of medium I, supplemented with 0.2 ml of filter-sterilized *n*-hexadecane, and 5 ml of medium II. Final experiments were conducted by transferring 0.2 ml of inoculum from stationary-phase cultures in medium II, washed four times by centrifugation, to 5 ml of medium I supplemented with 0.2 ml of *n*-hexadecane or MHS and 5 ml of medium II. Growth was monitored by visual inspection of turbidity in the aqueous or hydrocarbon phase for up to 80 days.

Additional experiments were conducted to assess growth in the presence and absence of glycine by transferring 0.2 ml of inoculum from stationary-phase cultures in medium II to 5 ml of medium I or III contained in 13- by 100-mm cuvettes. Final experiments were conducted by transferring 0.2 ml of washed or unwashed inoculum from stationary-phase cultures in medium II to 5 ml of medium I, 5 ml of medium I plus 0.2 ml of *n*-hexadecane, 5 ml of medium III, and 5 ml of medium III plus 0.2 ml of *n*-hexadecane contained in 13- by 100-mm cuvettes. Growth was monitored at 600 nm. All growth experiments were conducted at 25°C in triplicate.

RESULTS AND DISCUSSION

Growth in quiescent culture is a convenient method for assaying hydrocarbon-using ability for a large number of isolates. Preliminary experiments revealed that quiescent culture produced greater growth of *Prototheca* than did shaken culture.

One hundred and thirty-eight isolates of *Prototheca* were compared for their ability to grow on hydrocarbon substrates under quiescent incubation. Preliminary experiments to determine which isolates would utilize hydrocarbons were

conducted by transferring each isolate from Sabouraud slants to medium I plus *n*-hexadecane and to medium II. All of the isolates grew on the glucose-containing medium II within 4 days, attesting to their viability. Only 75% of *P. zopfii* isolates (48 of 64) and one *Prototheca* sp.

TABLE 1. *Days required for growth on hydrocarbons by unwashed and washed cultures of Prototheca in media containing glycine or NH_4NO_3*[a]

Isolate no.	Environmental source	Inoculum		
		Unwashed in medium containing NH_4NO_3 (C16)	Washed in medium containing glycine	
			C16	MHS
NF 8658	Protothecosis	—[b]	14	28
NRRL YB-4826	Unknown	—	4	14
CBV IV 7311	Unknown	—	7	21
CBV IV 7342	Unknown	—	7	21
CBV IV 7300	Unknown	—	7	14
MF 179-A	Onychopathy	6	3	14
RSP 858	Morgantown, W.Va., STP	6	3	14
RSP 870	Morgantown, W.Va., STP effluent	6	3	7
RSP 871	Morgantown, W.Va., STP effluent	9	56	—
RSP 872	Morgantown, W.Va., STP influent	6	7	14
RSP 874	Morgantown, W.Va., STP influent	—	56	—
RSP 878	Morgantown, W.Va., STP sediment	9	3	7
RSP 879	Morgantown, W.Va., STP sediment	—	56	—
RSP 890	Morgantown, W.Va., STP sediment	—	3	14
RSP 892	Morgantown, W.Va., STP sediment	—	3	14
RSP 893	Connellsville, Pa., stream	28	7	14
RSP 899	Uniontown, Pa., STP clarifier	—	3	14
RSP 901	Uniontown, Pa., STP clarifier	—	14	35
RSP 904	Uniontown, Pa., STP clarifier	—	35	14
EAB 946	*Morus rubra* slime flux	28	7	21
EAB 948	Lake Erie	—	19	14
EAB 954	Morgantown, W.Va., STP	—	21	35
WCB 968	Tarlac, Phillipines, sewer water	9	4	14
WCB 969	Clark AFB, Phillipines, raw sewage	24	4	14
WCB 970	Clark AFB, Phillipines, Imhoff tank	5	35	35
WCB 971	Clark AFB, Phillipines, Imhoff tank	28	4	14
WCB 975	Manila, Phillipines, sewer canal	5	14	7
WCB 976	Manila, Phillipines, sewer canal	5	4	7
WCB 977	Manila, Phillipines, Pasig River	5	7	7
WCB 985	Marcos Village, Phillipines, draining ditch	—	21	35
WCB 992	Angeles City, Phillipines, sewage	5	4	7
WCB 1003	Angeles City, Phillipines, Oasis Motel salad	9	4	14
WCB 1004	Angeles City, Phillipines, royal true orange	9	4	14
WCB 1006	Thailand, 56th USAFB, sewage lagoon	5	14	35
NRRL Y-7676	Oily muck	5	5	7
UTEX 328	Unknown	—	—	—
UTEX 1434	Unknown	—	21	21
FB 1030	Port au Prince, Haiti, Pasture soil	—	28	28
FB 1031	Port au Prince, Haiti, street sewage	—	28	28
FB 1034	Port au Prince, Haiti, irrigation ditch	—	28	28
ATCC 16524	Frass of *Populus tremuloides*	5	3	14
ATCC 16532	Locust tree	—	21	56
RSP 1084	Slime flux	—	7	7
RSP 1085	Slime flux	—	7	14
RSP 1087	Slime flux	—	7	14
RSP 1089	Slime flux	—	7	7
RSP 1090	Slime flux	—	3	14
RSP 1091	Slime flux	—	7	7
CDC B 1270	Bovine mastitis	6	3	14
NRRL-YB-990	Mulberry tree slime flux	28	7	14
DGA-17	Unknown	—	14	28
DL	Unknown	6	7	14

TABLE 1—*Continued*

Isolate no.	Environmental source	Inoculum		
		Unwashed in medium containing NH_4NO_3 (C16)	Washed in medium containing glycine	
			C16	MHS
SAM-W105	Eastport, Md., STP effluent	21	56	7
ATCC 16525	Unknown	6	3	7
ATCC 16527	Sludge	6	3	7
ATCC 16533	Unknown	—	7	28
ATCC 30253	Baltimore harbor sediment	5	4	14

[a] All isolates were *P. zopfii* except RSP 892, which was *Prototheca* sp. Abbreviations: ATCC, American Type Culture Collection; DGA, D. G. Ahearn, Georgia State University; RSP, R. S. Pore, W. Virginia University; SAM, S. A. Meyer, ATCC; NFC, N. F. Conant, University of North Carolina; CBV, C. B. Van Niel, Hopkins Marine Station; UTEX, The Culture Collection of Algae at the University of Texas; NRRL, Northern Region Research Laboratory; MF, Mildred Feo, Universidad Central de Venezuela; EAB, E. A. Barnett, W. Virginia University; CDC, Center for Disease Control, Atlanta, Ga.; WBC, Walter C. Barnes, USAF, Brook Air Force Base; FB, Frank Baker, Indiana University, Pa.; DL, David Lloyd, University College, Cardiff; STP, sewage treatment plant.

[b] —, No growth.

(RSP892) grew on *n*-hexadecane. None of the 58 isolates of *P. wickerhamii* or the other 15 *Prototheca* sp. was capable of growing on hydrocarbons. The *Prototheca* isolates were obtained from several different continents and from such diverse sources such as slime flux, tissue culture, sewage, sputum, drinking water, salads, and oil-polluted soil, as exemplified by the environmental sources of hydrocarbon-using *Prototheca* (Table 1).

To determine whether *n*-hexadecane was being cometabolized at the expense of growth on glycine, all of the isolates were screened on medium III, containing NH_4NO_3 instead of glycine, for their ability to grow on *n*-hexadecane. About 50% (28 of 57) of the *n*-hexadecane-using *Prototheca* were unable to grow on the hydrocarbon when NH_4NO_3 was the nitrogen source (Table 1). This suggested that glycine was necessary to support *n*-hexadecane utilization by some of these isolates, but this was not sufficient evidence to prove it was serving as a carbon source. Assessing growth by measuring change in turbidity indicated that there was significant nutrient carry-over in the inocula to support growth in the presence of glycine. When washed inocula were used, turbidimetric measurements showed that the glycine would not serve as a carbon source.

All isolates were washed by centrifugation and then used to inoculate medium I containing glycine and *n*-hexadecane or MHS. All of the *P. zopfii* isolates used *n*-hexadecane as sole carbon and energy source except UTEX 328, suggesting that both glycine and *n*-hexadecane were necessary for hydrocarbon utilization (Table 1). Three other isolates did not use MHS as sole carbon and energy source, even though it contained 8% *n*-hexadecane, probably because some hydrocarbons in the mixture inhibited growth.

One of the distinguishing characteristics of the *Prototheca* isolates that utilized hydrocarbons was the difference in time required for noticeable growth, even for isolates recovered from the same source (Table 1). Each pair of isolates (870 and 871, 872 and 874, 878 and 879) was recovered from the same source; e.g., isolates 870 and 871 were obtained from the effluent of the Morgantown, W.Va., sewage treatment plant during January 1973. However, one isolate of each pair grew very rapidly (3 days), whereas the other took considerably longer (56 days). The pairs of isolates with dissimilar growth times are all *P. zopfii*. The isolates that required 56 days before visual growth was observed on *n*-hexadecane were the only isolates that did not utilize MHS. We are examining these isolates to determine why their growth times were significantly different.

The *Prototheca* isolates were categorized by species type and source of isolation and compared for hydrocarbon-utilizing ability (Fig. 1). *P. wickerhamii* did not utilize hydrocarbons despite the fact that they were recovered from the same sources as the hydrocarbon-utilizing *P. zopfii*. All of the hydrocarbon-utilizing isolates were *P. zopfii* except for one *Prototheca* sp. (Table 1). Source of isolation did not significantly influence the ability of *P. zopfii* to use hydrocarbons. The proportion of total isolates of *P. zopfii* that utilized hydrocarbons was about the same (70 to 80%) for cultures recovered from sewage, slime flux, or diseased tissue.

ACKNOWLEDGMENTS

We are grateful to those individuals and organizations that were kind enough to allow us to examine their *Prototheca*

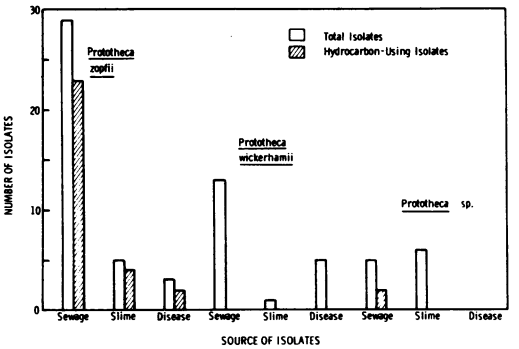

FIG. 1. *Number of total and hydrocarbon-using isolates of Prototheca from various sources of isolation.*

cultures and without whose assistance this study would not have been possible. We give special thanks to Walter Barnes, who supplied the largest number of isolates from diverse sources. J.D.W. acknowledges the excellent technical assistance of Katherine L. Aiello.

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