Mycopathologia vol. 57, 1, pag. 19–22, 1975

ISOLATION OF PENICILLIUM CORYLOPHIUM DIERCKX FROM ACID MINE WATER AND ITS OPTIMAL GROWTH ON HYDROCARBONS AT ACID pH

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Abstract

Penicillium corylophilum Dierckx was isolated from sludge collected at the interface of an aqueous, copper-bearing leachate and an organic, kerosene based, ion exchange solvent. The organism assimilated kerosene and various straight chain and cyclic hydrocarbons including dodecane, hexadecane, octadecane, toluene, benzene, and cyclohexane. Assimilation of kerosene and hexadecane was optimal at pH 2 and was stimulated by yeast extract.

Introduction

Hydrocarbonoclastic fungi are widespread in nature and have been isolated from soil, from fresh water, and from seawater (1, 3). Under optimal environmental conditions they may become the predominant microflora and produce extensive growth in hydrocarbon rich habitats. Such has been shown to be the case in the fouling of stored diesel (2) and kerosene based aviation fuels (5).

Microscopic examination of sludge formed in a kerosene-liquid ion exchange (LIX) copper extraction plant of a Southwestern copper mine revealed extensive mycelial development. A mold was isolated and subsequently identified as *Penicillium corylophilum* Dierckx by Dorothy B. Prest, Keuka College, Keuka Park, New York.

Factors influencing growth of this organism on hydrocarbons under acid conditions are the subject of this report.

Materials and methods

Organism source and isolation

Samples of sludge were collected in sterile bottles at the aqueous-organic interface of a liquid ion exchange copper recovery plant. The aqueous fraction had a pH of 2.7. The organic fractions consisted of a mixture of kerosene and LIX-64 (General Mills Chemicals, Inc.) in a ratio of 10 : 1 respectively. Portions of sludges amples were examined microscopically and plated directly onto Trypticase Soy Agar (BBL). The sludge was composed entirely of branched mycelial elements with diameters ranging in size from 5 μM to 10 μM. All colonies that formed were viscous, light yellow in color, morphologically homogeneous and were composed of mycelium. When transferred to Czapek Solution Agar (Difco) the organism produced velvety growth.

Media and cultural conditions

Hydrocarbon assimilation tests were carried out in 125 ml Erlenmeyer flasks containing 10 ml of a minimal salts-yeast extract medium having the following composition: $NaNO_3$, 3 g; K_2HPO_4, 1 g; $MgSo_4 : 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 0.01 g and yeast Extract (Difco), 0.5 g in 1 l distilled water. The medium was adjusted to pH 3.0 with N HCl. Hydrocarbons tested included dodecane, hexadecane, and octadecane (Eastman Organics); toluene, benzene and cyclohexane (J. T. Baker and Co.) and kerosene obtained locally and of unknown purity. One ml of test hydrocarbon was added to each flask.

The effect of pH on the assimilation of kerosene and hexadecane was determined using the medium described above adjusted with N HCl to pH values ranging from pH 1 to pH 7.

Stimulation of kerosene and hexadecane assimilation by yeast extract and vitamin-free casein hydrolysate (Nutritional Biochemicals Corp.) was studied as follows. The minimal salts solution described above was enriched with various concentrations of yeast extract ranging from 0 to 0.1 %; with 0.1 % vitamin-free casein hydrolysate; or with a vitamin mixture containing biotin, 2μg/l; Ca-panto-

thenate, 400 μg/l; folic acid, 2 μg/l; inositol, 2 mg/l; niacin, 400 μg/l; para-aminobenzoic acid, 200 μg/l; pyridoxine-HCl, 400 μg/l; riboflavin, 200 μg/l and thiamine-HCl, 400 μg/l.

Each flask was inoculated with a standard loopful of an aqueous spore suspension. No attempt was made to quantitate numbers or dry weight of spores in the inoculum.

Cultures were incubated in triplicate at 25 °C in the dark under stationary conditions. Maximum cell yields were produced within 14 days with hydrocarbons and within 3–4 days with sucrose. Extent of growth was measured by determining the dry weight of washed mycelial mats harvested in entirety from flask cultures by filtration onto 1.2 μM membrane filters (Millipore Corp.).

Results

Penicillium corylophilum grew well at both pH 7 and pH 3 in the minimal salts solution containing sucrose as the sole carbon source. Maximum dry cell yields of 151 mg and 143 mg were obtained at pH 7 and pH 3 respectively after 4 days incubation. Growth with kerosene under similar conditions however was scant. Enrichment of the minimal salts solution with 0.05 % yeast extract markedly stimulated assimilation of kerosene as well as other hydrocarbons at pH 3 (table 1). Most growth occurred with the paraffins dodecane, hexadecane, and octadecane all of which are normal constituents of kerosene. In addition the organism assimilated the cyclic hydrocarbons toluene, cyclohexane, and benzene. Least growth was produced on kerosene. Little growth was produced in cultures incubated at pH 7. These observations strongly suggested that assimilation of kerosene and other hydrocarbons was both pH dependent and required a growth factor.

Table 1. Assimilation of hydrocarbons by Penicillium corylophilum at pH 3.

Hydrocarbon	Yield of dry cells[1]
	mg
None added	2
Kerosene	37
Dodecane	81
Hexadecane	85
Octadecane	79
Toluene	60
Cyclohexane	63
Benzene	58

[1]average of triplicate cultures, 14 days incubation, 25C.

Table 2. Effect of pH on the assimilation of kerosene and hexadecane by Penicillium corylophilum.

pH	Yield of dry cells[1] Kerosene	Hexadecane
	mg	mg
1	2	5
2	30	90
3	25	85
4	8	45
5	4	20
6	2	15
7	2	10

[1]average of triplicate cultures, 14 days incubation, 25C.

These possibilities were further investigated by first determining the effect of initial pH on assimilation of kerosene and hexadecane. Results are shown in table 2. Most growth occurred at pH 2 and slightly less growth occurred at pH 3. Final pH values in all cases in which growth was evident varied little from pH 6.5 to pH 7. Thus initial pH of the medium markedly affects assimilation of hydrocarbons. Moreover since the organism produced little growth on kerosene and hexadecane at pH values approaching neutrality these data suggest that extremely acid conditions are required only for the initial reactions involved in the metabolism of hydrocarbons.

Enrichment of the minimal salts solution with yeast extract stimulates assimilation of kerosene by *P. corylo-*

Table 3. Effect of yeast extract on the assimilation of kerosene by Penicillium corylophilum at pH 3.

Per cent Yeast extract	Yield of dry cells[1] Kerosene	No kerosene
	mg	mg
0	7	<1
0.01	19	<1
0.03	26	<1
0.05	29	1.0
0.07	32	1.5
0.1	39	2.2

[1]average of triplicate cultures, 14 days incubation, 25C.

Table 4. Effect of yeast extract, casein hydrolysate and vitamins on the assimilation of hexadecane by Penicillium corylophilum at pH 3.

Addition	Total yield of dry cells[1]
	mg
None	38
Vitamin mixture	41
Yeast extract (0.05%)	87
Casein hydrolysate (0.1%)	81

[1] average of triplicate cultures, 14 days incubation, 25C.

philum as indicated by the data in table 3. In the absence of yeast extract, only 7 mg dry cells were produced after 14 days incubation. Addition of as little as 0.01% yeast extract stimulated a threefold increase in yield of dry cells. Moreover it is apparent that yeast extract alone supports little growth. With 0.1% yeast extract, the highest concentration tested, only 2.2 mg dry cells were produced. Yeast extract and casein hydrolysate also stimulate assimilation of hexadecane (table 4). After 14 days incubation yeast extract and casein hydrolysate stimulated an approximate two-fold increase in yield of dry cells above those levels attained in minimal salts alone or in minimal salts supplemented with a vitamin mixture. In control cultures containing growth factors alone without added hexadecane an average of only 3 mg dry cells were produced. Although the specific compounds were not identified our results strongly suggest that stimulation of assimilation of kerosene as well as hexadecane at pH 3 is owing to an amino acid or combination of amino acids.

Discussion

Penicillium corylophilum is well adapted to the habitat it occupies in the acid environment from which it was isolated. The organism was shown to assimilate kerosene and constituent hydrocarbons optimally at a pH which approximated that of the acid mine water flowing into the liquid ion exchange plant. Assimilation of hydrocarbons under extreme acid conditions appears to be a unique property of this mold and to our knowledge has not been reported previously. The requirement for low pH and the stimulation of assimilation by some non-vitamin component of yeast extract or casamino acids cannot be explained on the basis of the data presented. However, since the organism grows equally well at acid and neutral pH on sucrose alone it would appear that extreme acidity and growth factor requirements are peculiar to hydrocarbon assimilation only. The limited ability of our isolate to assimilate kerosene and hexadecane under mildly acid conditions, i.e. pH 5 and pH 6, agrees in general with the findings of Nyns et al. (4). These authors tested two stains of *P. corylophilum* for their ability to assimilate fuel oil at pH 4.5. Neither strain produced growth after four weeks incubation at 27 °C on Yeast Nitrogen Base (Difco) agar slants supplemented with fuel oil.

Whether *P. corylophilum* is indigenous to acid mine water or merely transient, being inoculated into the water from adjacent soil, is not known. The latter appears most plausible since according to Raper (6) the organism is fairly abundant and widely distributed in nature and has been isolated from soil. Moreover in a study of the aerobic heterotrophic microorganisms of acid mine water Tuttle et al. (7) found that yeasts and molds predominated. Furthermore these authors suggested that fungi were transient and not indigenous to the water.

The major significance of these investigations lies in the demonstration that *Penicillium corylophilum* assimilates hydrocarbons only under acid conditions and further attests to the remarkable adaptability of molds to colonize unusual and often harsh environments.

Acknowledgements

This research was supported in part by a University of Arizona Institutional Grant.

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