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Isolation and Identification of a Morpholine-Degrading Bacterium

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A gram-positive, slowly growing rod effectively utilizing morpholine as the sole source of organic carbon, nitrogen, and energy was isolated from a mixed culture in a laboratory reactor. The strain was tentatively identified as *Mycobacterium aurum*. Its growth characteristics at 20°C and pH 6.5 were as follows: maximum specific growth rate, 0.052 h^{-1}; half-velocity constant, 1.3 mg/liter; and yield, 0.37 g/g. The optimum temperature and pH were 31°C and 6.0, respectively.

Morpholine (C_4H_9ON) and its derivatives are industrial solvents widely used in the production of rubber, plastics, pesticides, drugs, etc. For many years, it was considered biologically nondegradable (6, 12). Present in numerous industrial wastewaters, it can pass through a biological treatment system unchanged and get into surface waters in which it can be modified to dangerous *N*-nitrosamine and *N*-nitroamine (3). Recently, however, it has been reported to be biodegradable by an immobilized mixture culture (5). This indicates that a microorganism degrading morpholine might belong to a group of slow growers. The aim of our research was to isolate and identify a morpholine-degrading microorganism and to determine its growth characteristics and optimum cultivation conditions.

An easily settled mixed culture (activated sludge, 3 g of dry solids) from the Prague Central Wastewater Treatment Plant, Prague, Czechoslovakia, was transferred into a 1-liter calibrated cylinder, and the system was operated semicontinuously on a once-a-day feeding schedule (22 h of aeration with compressed air, dissolved-oxygen concentration above 2 mg/liter, 2 h of sedimentation). The aeration volume was 1 liter. After the sedimentation, 0.5 liter of supernatant was siphoned off and replaced by 0.5 liter of fresh medium MP during the adaptation period or medium M during the postadaptation enrichment period. Morpholine of a technical grade (BASF, Ludwigshafen/Rhine, Federal Republic of Germany) was purified by distillation at atmospheric pressure. The fraction with a boiling point of 128 to 130°C was collected and used for preparation of media. Medium M contained 230 mg of morpholine, 55 mg of KH_2PO_4, 130 mg of Na_2HPO_4, and 100 to 300 mg of H_2SO_4 (depending on the pH in the reactor) in 1 liter of tap water. Medium MP was prepared by adding 100 mg of Bacto-Peptone (Difco Laboratories, Detroit, Mich.) to 1 liter of medium M.

During the adaptation period, which lasted 2 months, no biomass except that in the supernatant (10 to 20 mg/liter) was removed from the laboratory reactor. When almost all morpholine was removed from the solution during the 1-day cycle, as indicated by the chemical oxygen demand (COD) (1) value, medium MP was replaced by medium M, so that morpholine was the sole source of organic carbon, nitrogen, and energy. At the same time the mean cell residence time was adjusted to 10 days (1/10 of the cultivation mixture was removed daily from the reactor). The real mean cell residence time was calculated on the basis of biomass balance. Parameters of the cultivation under steady-state conditions are listed in Table 1. All chemical analyses were carried out by standard methods (1). Morpholine concentrations were calculated from COD values (specific COD of morpholine, 1.839).

The performance of the reactor was stable for the duration of the experiments, which lasted 7 months. The mixed culture formed easily settleable bacterial flocs also containing protozoa and rotifera. Complete nitrification also took place in the reactor (Table 1). Bacterial isolates were obtained as follows. Excess biomass from the reactor was treated in a glass homogenizer to destroy large bacterial flocs. The dispersion obtained was further sonicated for 2 min with a sound generator at 20 kHz (Ultrasonic Ltd., Shipley, England). The absorbed power was 3 W/ml of the treated sample; the temperature was maintained in a range from 20 to 25°C by a water jacket. After appropriate dilution, the cell suspension was streaked on plates of NA, NDA, and AMO media. NA consisted of 28 g of nutrient agar (Difco) in 1 liter of distilled water. NDA consisted of 0.28 g of nutrient agar and 15 g of Bacto-Agar (Difco) in 1 liter of distilled water. AMO plates were prepared by the addition of 15 g of Bacto-Agar to medium MO. Medium MO contained 230 mg of morpholine, 220 mg of KH_2PO_4, 530 mg of Na_2HPO_4, 240 mg of H_2SO_4, 50 mg of $CaCl_2$, 30 mg of $MgCl_2$, 3 mg of $FeCl_3$, 0.12 mg of $ZnSO_4$, 0.11 mg of $MnCl_2$, 0.15 mg of $Co(NO_3)_2$, 0.08 mg of $CuSO_4$, and 0.02 mg of Na_2MoO_4 in 1 liter of distilled water. Stock solutions of morpholine, phosphates with sulfuric acid, and the remainder of the chemicals were autoclaved separately before being mixed together.

The mean number of CFU growing on NDA plates was 9.5×10^7 in 1 ml, and an average of 27% of the colonies were capable of growing in medium MO. The colony counts on NA plates were always lower than those in NDA plates. Moreover, slow-growing colonies were often overgrown by fast-growing ones on NA plates because the slow-growing colonies could be counted only after 2 weeks of incubation at 20°C. The colony counts on AMO plates were similar to those on NDA plates, but morpholine-degrading bacteria formed higher colonies than on NDA plates. The growth of morpholine nondestroyers on AMO plates led to the supposition that Bacto-Agar contains enough soluble substrates for colony formation. This was proved by cultivation on Bacto-Agar medium without any addition of organic substrates and is in good agreement with the findings of Olsen and Bakken (13).

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TABLE 1. Parameters of semicontinuous cultivation of mixed microbial culture

Parameter	Value[a]
Aeration vol (liter)	1.0
Concn (mg/liter) in dosed medium M	
Morpholine	230
Morpholine COD[b]	423
Volumetric loading (mg/liter per day)	
Morpholine	115
Morpholine COD[b]	211
Biomass concn (mg/liter)	387 ± 30
Mean cell residence time (days)	8.2 ± 0.5
Temp (°C)	20 ± 0.5
pH	
Start-of-day cycle	7.2 ± 0.1
End-of-day cycle	6.5 ± 0.2
Concn (mg/liter) in filtered supernatant	
Organic matter as COD[b]	27 ± 10
NH_4^+-N	1.4 ± 0.6
NO_2^--N	0.2 ± 0.2
NO_3^--N	23.5 ± 6.4

[a] Average values ± standard deviations from 22 daily analyses during 1 month.

[b] COD was measured by standard methods (1). The specific COD of morpholine is 1.839 g/g.

Isolates from colonies differing in appearance were further cultivated in liquid medium MO. The isolate capable of metabolizing morpholine was designated MO1. It formed gram-positive nonmotile rods. Isolated strain MO1 was tested by Czechoslovak standard procedures (11) for growth at different temperatures, colony morphology, pigment formation, and resistance to drugs. The other characteristics tested were acid phosphatase, β-galactosidase, acetamidase, benzamidase, urease, nicotinamidase, pyrazinamidase, and succinamidase (7), arylsulfatase (10), β-glucosidase (4), nitrate reductase (18), Tween 80 hydrolysis (17), growth in Ogawa medium with hydroxylamine, *p*-nitrobenzoate, and hydrazide of thiophene-2-carboxylic acid (15), *p*-aminosalicylate degradation (15), and iron uptake (14). Screening tests with strain MO1 (strong acid fastness, arylsulfatase activity, no endospores, no mycelium forming, no branching, etc.) indicated that it belongs to the genus *Mycobacterium* (15, 19). Because of its scotochromogenicity and ability to grow in Löwenstein-Jensen medium in 7 days, the most suitable tests capable of distinguishing among 18 presently known fast-growing and pigment-forming *Mycobacterium* strains were used (Table 2). On the basis of 31 characteristics (Table 2), strain MO1 was tentatively identified as *Mycobacterium aurum*. The results are in good agreement with published data (15, 16, 19), except for the negative growth at 37°C. However, this phenomenon has been observed in some other taxonomic studies (11). Of the pigmented species, only *M. sphagni* and *M. komossense* have optimum growth at 31°C, but they differ from the MO1 strain in many other characteristics (8, 9, 19). Owing to the fact that cell wall and mycolic acid content analyses have not been conducted, the identification of strain MO1 is considered tentative.

The growth kinetics of strain MO1 were measured in an automatic respirometer (Sapromat; Voith, Heidenheim, Federal Republic of Germany). The inoculum was taken from strain MO1 growing exponentially in a stock batch culture in medium MO at 20°C and pH 6.5 and transferred into respirometric flasks containing medium MO, in which different pH values were obtained by changing the $KH_2PO_4/Na_2HPO_4/H_2SO_4$ ratio. About 10^6 cells per ml were present in one respirometric flask immediately after inoculation, and this number increased more than 100-fold during each run of tests. The maximum specific growth rates were computed from an increase of respiration rates over time. The dependence of the maximum specific growth rate of isolate MO1 growing exponentially in medium MO on temperature and pH is shown in Fig. 1. The optimum temperature and pH were 31°C and 6.0, respectively. Biomass balance determined during respirometric measurement in the Sapromat showed a yield of 0.37 g/g. The maximum rate of

TABLE 2. Characteristics of strain MO1

Characteristic	Result	Characteristic	Result
Acid fastness	+	Growth in presence of:	
Pigment formation	+	Hydroxylamine	
Growth in less than 7 days	+	250 mg/liter	+
Growth at:		500 mg/liter	−
22°C	+	*p*-Nitrobenzoate (500 mg/liter)	+
31°C	+	Hydrazide of thiophene-2-carboxylic acid (1 mg/liter)	+
37°C	−	Resistance to:	
42°C	−	Isoniazid (1 mg/liter)	−
Enzymatic activity		Streptomycin (100 mg/liter)	−
Acid phosphatase	−	*p*-Aminosalicylic acid (100 mg/liter)	+
Arylsulfatase		Ethambutol (5 mg/liter)	+
3 days	−	Rifampin (50 mg/liter)	+
14 days	+		
β-galactosidase	−		
β-glucosidase	+		
Nitrate reductase	+		
Tween hydrolysis (5 days)	+		
Acetamidase	+		
Benzamidase	−		
Urease	+		
Nicotinamidase	+		
Pyrazinamidase	+		
Succinamidase	−		
p-Aminosalicylate degradation	−		
Iron uptake	+		

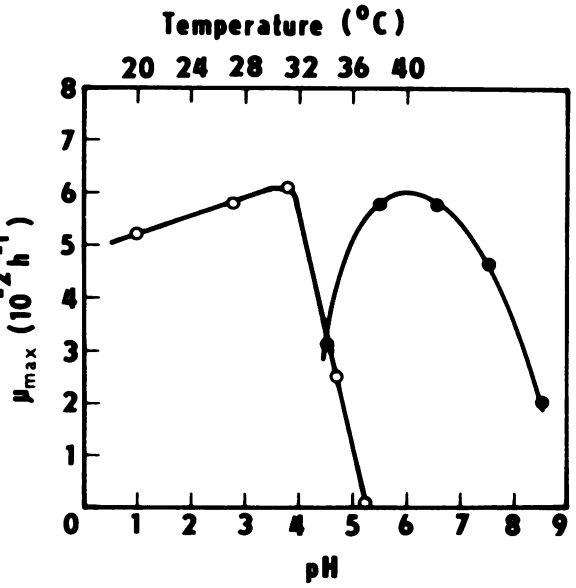

FIG. 1. Dependence of maximum specific growth rate (μ_{max}) of strain MO1 on temperature (○) (pH 6.5) and pH (●) (temperature, 27°C).

biodegradation of morpholine by strain MO1 was determined from the maximum specific growth rate and yield. Under optimum conditions, the maximum biodegradation rate was 160 mg/h per g (dry weight) of biomass.

The half-velocity constant was measured at 20°C and pH 6.5 by a respirometric method described by Čech et al. (2) using the suspension of strain MO1 taken from the Sapromat after the logarithmic growth phase. The half-velocity constant was found to be 1.3 mg/liter. The measurement made by the respirometric method (2) showed no inhibitory effects of morpholine on the metabolic activity of strain MO1 up to a concentration of 5 g/liter.

Data presented in this paper could help water technologists to effectively remove morpholine from industrial wastewaters during biological purification.

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