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Biodegradation and utilization of monomethyl sulfate by specialized methylotrophs

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Methylations in organic syntheses are often carried out with dimethyl sulfate (DMS) as the methyl donor. Usually in such reactions, only one methyl group of DMS is transferred to the methyl accepting group (hydroxyl, mercapto, amino or imino group) and monomethyl sulfate (MS) is formed as a by-product in stoichiometric quantities. Therefore, the production of large-scale chemicals such as agrochemicals or dyestuffs involving methylations with dimethyl sulfate yields large volumes of monomethyl sulfate containing mother liquors. Only in rare cases can MS be used for further methylations. The recycling and remethylation of MS to DMS is very expensive and the disposal of MS by hydrolysis can create security problems (formation of dimethyl ether by alkaline hydrolysis or formation of chloromethane by acid hydrolysis with HCl). One of the methods best suited for its disposal is the incineration of the MS-containing mother liquors. As the biodegradation of monomethyl sulfate would be an alternative to physical or chemical methods of disposal, we searched for MS degrading microorganisms in order to establish a biological waste treatment process for MS-containing mother liquors.

Monomethyl sulfate is a C_1-compound. We therefore decided to investigate and isolate methylotrophs (C_1-utilizing microorganisms). From the literature[1,5,12,18] it is known that the following C_1-compounds are utilized by specialized methylotrophs as the sole source of carbon and energy: methane, methanol, dimethyl ether, methylamine, dimethylamine, trimethylamine, trimethylamine-N-oxide, tetramethylammonium salts, trimethylsulfonium salts, formate, formaldehyde, methyl sulfides[21,7], dimethyl sulfoxide[7] and dichloromethane[3]. No data were available on the biodegradation or utilization of monomethyl sulfate.

Evaluation of methylotrophs from culture collections

More than 30 methanol and/or methylamine degrading methylotrophs from culture collections were tested for MS utilization under the following conditions:
50-ml shake flasks with 20 ml of mineral medium MV7 containing 5–10 g/l of methanol or methylamine (as hydrochloride) as the sole source of carbon were inoculated with lyophilized cells of the collection strains and then incubated at 28 or 37 °C at 250 rpm for 3–7 days. After several propagations with methanol or methylamine as the carbon source, cells of well-grown cultures were transferred into medium MV7 containing 1, 5 or 10 g/l of sodium monomethyl sulfate (prepared from DMS) as the sole carbon source and again incubated at 28 or 37 °C at 250 rpm for 7 days.
Mineral medium MV7: 2.0 g NH_4NO_3, 1.4 g Na_2HPO_4, 0.6 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2 \cdot 2H_2O$, 0.001 g $FeSO_4 \cdot 7H_2O$ and 1.0 ml of a trace element solution in 1 l of water, pH 6.5 after sterilization for 20 min at 120 °C.
Trace element solution: 20 mg $Na_2MoO_4 \cdot 2H_2O$, 20 mg $Na_2B_4O_7 \cdot 10H_2O$, 20 mg $ZnSO_4 \cdot 7H_2O$, 20 mg $MnSO_4 \cdot H_2O$, 20 mg $CuSO_4 \cdot 5H_2O$ in 1 l of water.
The carbon sources methanol, methylamine (in solution) and monomethyl sulfate (in solution) were added to the medium after sterile filtration. An addition of 1 ml of 1 M phosphate buffer pH 7 was made to the cultures with monomethyl sulfate as the carbon source in order to stabilize the pH (liberation of sulfuric acid by MS-hydrolysis).
The following strains were tested for MS utilization under the conditions described above: *Methylomonas methylovora* (ATCC 21369), *Methylobacillus glycogenes* (ATCC 29475), *Pseudomonas* MA (ATCC 23819), *Pseudomonas* MS (ATCC 25262), *Pseudomonas rosea* (NCIB 10601), *Paracoccus denitrificans* (NCIB 8944), *Pseudomonas aminovorans* (NCIB 9039), *Pseudomonas* M27 (DSM 1339), *Pseudomonas* AM1 (DSM 1338, ATCC 14718, NCIB 9133), *Protaminobacter ruber* (ATCC 8457), *Pseudomonas extorquens* (NCIB 9399), *Pseudomonas rhodos* (ATCC 14821), *Arthrobacter globiformis* (ATCC 8010), *Protaminobacter alboflavus* (ATCC 8458), *Pseudomonas* 6307 (ATCC 21439), *Alteromonas thalassomethanolica* (ATCC 33145), *Methylomonas thalassica* (ATCC 33146), *Protaminobacter thiaminophagus* (ATCC 21371), *Protaminobacter candidus* (ATCC 21372), *Pseudomonas* MC-7 (ATCC 21438), *Methylobacterium organophilum* (ATCC 27886), *Methylomonas methanolica* (ATCC 21704), *Pseudomonas methylotropha* = *Methylophilus methylotrophus* (NCIB 10510–10515 and 10592–10596), *Microcyclus eburneus* (ATCC 21373) and *Pseudomonas insueta* (ATCC 21276). None of these strains was able to utilize monomethyl sulfate at significant rates!
MS utilization is thus not a common property of methylotrophs.

Enrichment and isolation of monomethyl sulfate-utilizing microorganisms

Samples of sewage sludge and effluents from sewage treatment plants as well as samples of sediments and effluents from landfills were investigated for the presence of MS degrading microorganisms. By selective enrichment in mineral medium MV7 with 5 g/l of monomethyl sulfate as the sole source of carbon and energy (28 °C, 250 rpm, see above) and by plating diluted samples on MS containing MV7-agar MS utilizing microorganisms were obtained from samples of both sewage sludge from an industrial sewage treatment plant and sediment from a landfill. Pure cultures of MS degraders were obtained by repeated plating (on MV7-agar containing 5 g/l of MS) of the enrichment cultures.
Five of these pure cultures, designated MS 72, MS 75, MS 219, MS 223 and MS 246, were then further investigated and taxonomically characterized.

Taxonomical characterization of the isolated MS utilizing microorganisms

Two commercially available taxonomy kits were utilized for the taxonomical characterization of the 5 MS degrading pure cultures: Oxi/Ferm Tube (Roche) and API 20 E (API System Inc.).
Both systems are restricted to gram-negative bacteria and the numerical codes are limited to microorganisms of medical (clinical) importance. Nevertheless, the biochemical information provided by these two systems allows a reasonably good classification for organisms from soil or sewage treatment facilities if it is combined with information from microscopy, electron microscopy and staining procedures.
The biochemical data for the 5 MS utilizing strains (all of them are gram-negative) obtained with the two taxonomy systems are listed in table 1.
All the tests were performed in duplicate, once with inoculum from nutrient agar and once with inoculum

Table 1. Biochemical reactions of monomethyl sulfate utilizers

Strain	ONPG: Hydrolysis by β-galactosidase[A]	ADH: Arginine dihydrolase[A,O]	LDC: Lysine decarboxylase[A]	ODC: Ornithine decarboxylase[A]	CIT: Citrate utilization[A,O]	H_2S: H_2S from thiosulfate[A,O]	URE: Urease[A,O]	TDA: Tryptophan desaminase[A]	IND: Tryptophan degradation→formation of indole[A,O]	VP: Acetoin test[A]	GEL: Gelatine liquefaction[A]	GLU: Glucose utilization[A]	MAN: Mannitol utilization[A]	INO: Inositol utilization[A]	SOR: Sorbitol utilization[A]	RHA: Rhamnose utilization[A]	SAC: Saccharose utilization[A]	MEL: Melibiose utilization[A]	AMY: Amygdaline utilization[A]	ARA: L-Arabinose utilization[A]	AND: Dextrose, anaerobic degradation[O]	AD: Dextrose, aerobic degradation[O]	XYL: Xylose utilization[O]	OX: Oxidase[A]	CAT: Catalase[A]	NO_2/N_2: Nitrate reduction[A,O]
Hyphomicrobium MS 72	+	−	+	+	+	−	±	−	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±
Hyphomicrobium MS 75	+	−	−	−	−	−	−	−	−	±	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+	±
Hyphomicrobium MS 219	+	−	±	−	+	−	+	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
Hyphomicrobium MS 223	+	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	±	+	±
Hyphomicrobium MS 246	+	−	−	−	+	−	+	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	±	+	+

A, API 20 E. O, Oxy/Ferm Tube. A, O, reaction in both systems.
+, 3 or 4 of the parallel tests were positive[A,O], or both of 2 parallel tests were positive[A] or [O].
−, all parallel tests were negative, or only one of 4 tests was positive.
±, 2 of 4 or one of 2 parallel tests were positive.

from MS containing mineral agar. They were carried out according to the instructions in the test kits, but incubation was at 28 °C instead of 37 °C.

The biochemical data indicate that the 5 strains are not identical but can be divided into 3 distinct groups. Strain MS 72 is clearly different from all the other strains with respect to carbohydrate utilization and ornithine decarboxylase. Strain MS 75 is closely related to strain MS 223; the only significant difference between these two strains is the liquefaction of gelatin observed with MS 75 but not with MS 223. The strains MS 219 and MS 246 are almost identical, but strain MS 246, in contrast to MS 219, tends to aggregate in liquid cultures (flocculation).

According to the numerical code of the Oxi/Ferm Tube test the 5 MS utilizing strains would be tentatively classified as *Pseudomonas*-like, *Alcaligenes faecalis* or *Achromobacter* sp. However, this classification would be inconsistent with the typical morphology of these organisms (see below).

Morphology and growth parameters of the strains MS 72, 75, 219, 223 and 246 (parameters identical for all strains):

- Optimum temperature: 28–30 °C; growth at 22 °C or at 37 °C is significantly slower than at 28–30 °C.
- Doubling time (28 °C, with monomethyl sulfate as the sole carbon source, medium MV7 in shake flasks at 250 rpm): 8–10 h.
- Tolerance of electrolytes: osmotolerant, halotolerant, acid tolerant.
- Relation to oxygen: aerobic.
- Colonies on nutrient agar or MS-containing MV7-agar: small (1–2 mm diameter), circular, convex, smooth, opaque, cream in color.
- Light microscopy (identical for all strains): cells 1 × 2 µm, rodshaped with pointed ends, oval, egg- or bean-shaped forms; occurring singly, in pairs or in aggregates (MS 246); some of the cells are motile and some of them produce monopolar filamentous outgrowths (stalks) of variable length.
- Electron microscopy (negative staining with sodium phosphotungstate): on the basis of their indentical morphology the 5 MS utilizing strains belong to the same genus of microorganisms. All strains have a pleomorphic appearance. Cells of different age with and without stalks, but also polarly flagellated swarmers are present. The stalks are sometimes branched and buds are formed at the tips of the stalks. Mature buds become motile and break off (swarmers with one single polar or subpolar flagellum).

The typical morphology of the monomethyl sulfate utilizing microorganisms excludes their classification as *Pseudomonas*-like, *Alcaligenes faecalis* or *Achromobacter* sp. According to Bergey's Manual of Determinative Bacteriology (8th edn)[13] and other sources[2,6,14–16] the MS utilizing strains are to be

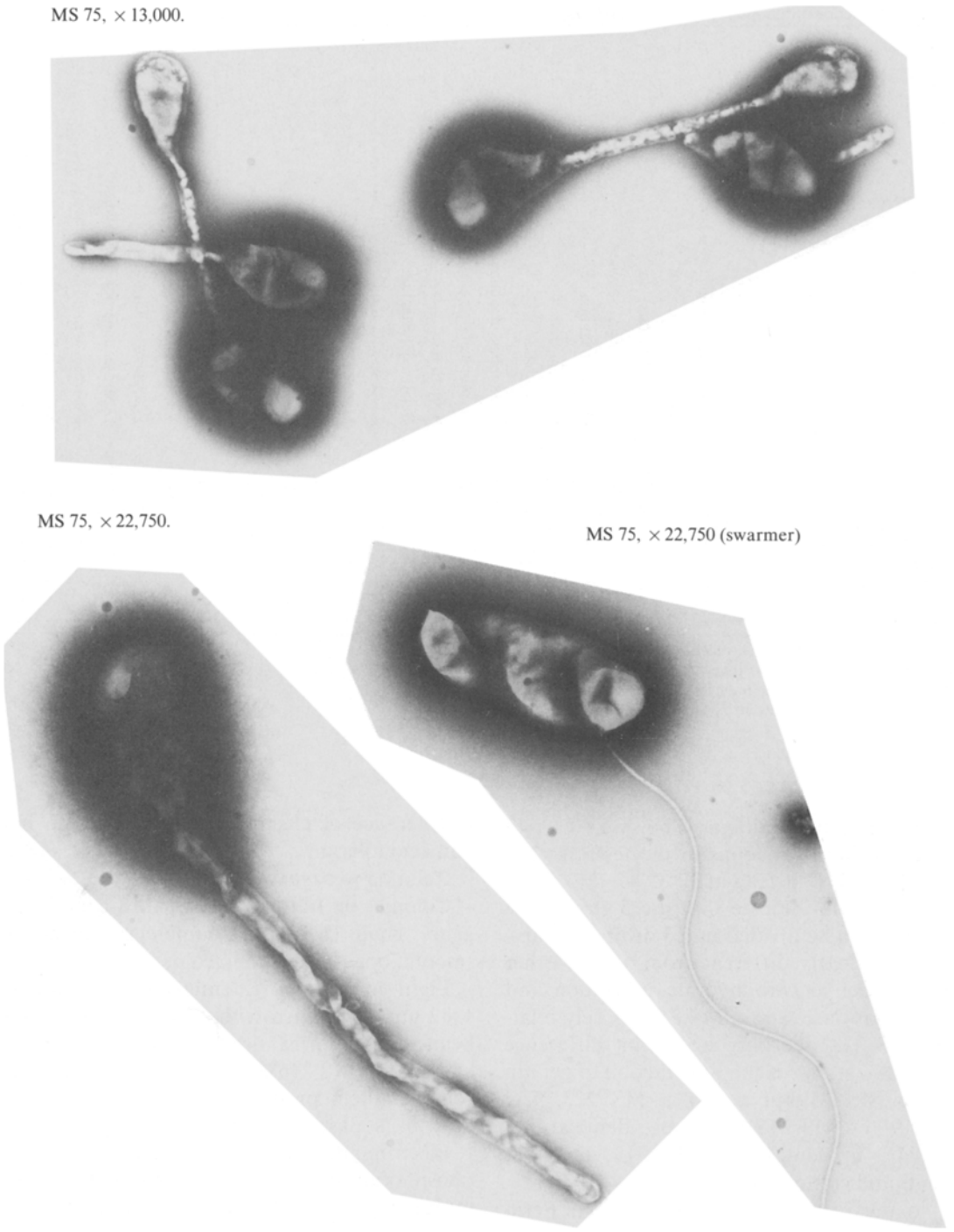

Figure 1. Electron micrographs of monomethyl sulfate utilizing *Hyphomicrobium* sp. (cells of strain MS 75 grown on MS).

classified as budding and/or appendaged bacteria. Morphology and biochemical data are in good agreement with the descriptions given for the genus *Hyphomicrobium*[2,6,13–16]. Therefore the MS utilizing strains are attributed to this genus.

Substrate spectra of the monomethyl sulfate utilizing hypomicrobium strains

The substrates utilized by the 5 strains of *Hyphomicrobium* described in the preceding section are listed in table 2. The list also comprises the substrates that are not utilized by the 5 strains. All the substrates were tested as the sole source of carbon and energy under the conditions described above starting from precultures grown on monomethyl sulfate. The 5 strains *Hyphomicrobium* MS 72, MS 75, MS 219, MS 223 and MS 246 are practically identical in their substrate spectra, but also with respect to the cell densities (OD_{650}) and growth rates attained with the individual substrates. Only with formate and ethyl-

methylamine did strains MS 219 and MS 246 grow significantly slower than the other 3 strains.

The substrate spectrum shows clearly that the MS utilizing *Hyphomicrobium* strains are specialized facultative methylotrophs.

The following observations are of special interest:

- Monomethyl sulfate is utilized as the sole source of carbon and energy but not monoethyl sulfate. Ethanol, the product expected from hydrolysis of monoethyl sulfate is easily utilized by these microorganisms. This indicates a rather narrow substrate specificity of the hydrolyzing enzyme (sulfatase) involved in the utilization of monomethyl sulfate.
- Dimethyl sulfate is also utilized as a carbon source, but in this case the chemical hydrolysis of DMS to MS and methanol seems to be the rate limiting step. Therefore, not DMS itself but only its hydrolysis product monomethyl sulfate acts as a substrate for these microorganisms. DMS is thus not listed as a true substrate.
- Methyl-sulfur compounds with an oxidation number different from that of MS are not utilized.
- Only primary and secondary methyl- or methylethylamines are utilized but not tertiary methylamines, quaternary methylammonium or methylethylammonium compounds.

Table 2. Substrate spectra of the monomethyl sulfate utilizing *Hyphomicrobium* strains MS 72, MS 75, MS 219, MS 223 and MS 246

Substrates utilized as the sole source of carbon and energy by all of the 5 strains (test concentrations in brackets):

Sodium monomethyl sulfate (5 g/l)
Methanol (5 g/l)
Ethanol (5 g/l)
Formate (2 g/l)
Acetate (2 g/l)
Methylamine (5 g/l)*
Dimethylamine (5 g/l)*
Ethylmethylamine (5 g/l)*
Dimethyl phosphite (0.5 and 1 g/l)
Trimethyl phosphite (0.5 and 1 g/l)

Substrates not utilized by the 5 *Hyphomicrobium* strains:

Sodium monoethyl sulfate (2, 5 and 10 g/l)
Trimethylamine (5 g/l)*
Tetramethylammonium chloride (5 g/l)
Trimethylethylammonium chloride (5 g/l)
Trimethylamine-N-oxide (5 g/l)
Ethyldimethylamine (5 g/l)*
Ethylamine (5 g/l)*
Formamide (2 g/l)
N,N-Dimethylformamide (2 g/l)
Urea (2 g/l)
Dichloromethane (1 g/l in sealed bottles)
1,2-Dichloroethane (1 g/l in sealed bottles)
Trimethyl phosphate (1 g/l)
Dimethylmethane phosphonate (3 g/l)
Methane – air (1:1 in sealed bottles)
Methanol anaerobic (5 g/l)
Dimethyl sulfoxide (2 g/l)
Dimethyl sulfide (2 g/l in sealed bottles)
Sodium dodecyl sulfate (2 g/l)
Dimethyl sulfite (2 g/l)
Dimethyl sulfone (2 g/l)

* Amines were added as hydrochlorides.
Conditions: medium MV7, buffered pH 7, incubation at 28 °C, 250 rpm for 6–10 days. The chemicals were bought from FLUKA chemicals or synthesized in the laboratories of Ciba-Geigy (Dr F. Rigamonti).

A comparison with data reported in the literature seems to indicate that our strains are different from other species of *Hyphomicrobium* described so far.

Hyphomicrobium neptunum[13] and *Hyphomicrobium indicum*[13] show different biochemical reactions and growth parameters and are not reported to utilize methanol or methylamines. *Hyphomicrobium vulgare* strains NQ[14,15,20], MEV[14,15], ZV[20] and 3[20] as well as *Hyphomicrobium* spp.[14,15] and *Hyphomicrobium* X[17] utilize methanol, methylamine, dimethylamine, trimethylamine, urea (not ZV and 3), formate, formamide or acetamide (not strain 3) as carbon sources. Acetate was reported to be a poor carbon source, at least for some of the strains.

Hyphomicrobium variabile[16] shows biochemical reactions different from our strains. *Hyphomicrobium* DM2[22] utilizes methanol, methylamine, dimethylamine, trimethylamine, acetate, formate, dichloromethane, ethanol, acetonitrile, ethyl acetate etc.

As our monomethyl sulfate utilizing *Hyphomicrobium* strains do not utilize trimethylamine, formamide, urea or dichloromethane, identity with one of the species listed above can definitely be excluded. The utilization of monomethyl sulfate, dimethyl phosphite and trimethyl phosphite, however, was not reported for those species.

Degradation pathway for monomethyl sulfate

The pathway proposed for the biodegradation and utilization of monomethyl sulfate by *Hyphomicrobium* MS strains is depicted in figure 2. As the spontaneous hydrolysis of MS to methanol and sulfate is extremely slow at low temperatures (30 °C) and under neutral conditions, an enzyme (sulfatase) is needed to achieve an efficient degradation of MS. This enzyme seems to

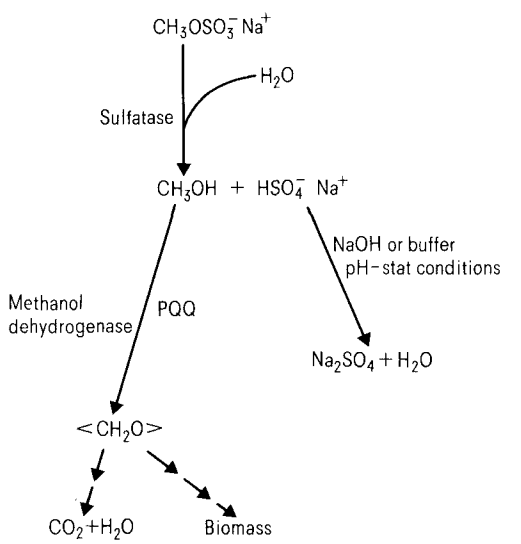

Figure 2. Proposed pathway for the biodegradation and utilization of monomethyl sulfate by *Hyphomicrobium* MS strains.

be rather specific for methyl groups as the homologous substrate monoethyl sulfate is not degraded and utilized by whole cells of our MS utilizers. However, the free enzyme has not yet been evaluated for its substrate specificity (work in progress). To our knowledge a methyl specific sulfatase has not been reported so far. The sulfatases described in the literature[8,9,19] are reported to accept substrates such as mono-, di- and trisubstituted sugars (glycosulfatase), chondroitin sulfate (chondrosulfatase), potassium myronate (myrosulfatase), steroid sulfates (steroid sulfatase), arylsulfuric acids (arylsulfatases, phenolsulfatases) and medium chain alkyl sulfates (alkylsulfatases). Typical substrates for alkylsulfatases are sodium 2-(2,4-dichlorophenoxy)ethyl sulfate[9,19], sodium dodecyltriethoxy sulfate[11] and n-pentyl sulfate up to n-dodecyl sulfate[4].

After enzymatic hydrolysis of monomethyl sulfate the methanol formed is then utilized as the carbon source by *Hyphomicrobium* MS strains. The assimilation (via formaldehyde) and dissimilation of methanol follows the general pathways found in all methanol utilizing bacteria described so far[1,5,12,18].

The biodegradation of dimethyl phosphite or trimethyl phosphite probably follows a similar route involving an enzymatic methyl phosphite cleavage as the first degradation step.

Fermentation kinetics and mass balances with monomethyl sulfate

In shake flask experiments with unbuffered medium MV7 and 5 g/l of sodium monomethyl sulfate as the carbon source a rapid decrease of pH (from 7 to 3.5–4) due to liberation of sulfuric acid was observed. At low pH-values the growth of *Hyphomicrobium* MS strains is inhibited and only moderate cell densities (OD_{650} 0.2–0.3) can be obtained. In buffered medium MV7 (see above) cell densities are significantly higher (OD_{650} 1.0–1.2, with a total consumption of the substrate).

For a more detailed investigation of the microbial growth on monomethyl sulfate stirred tank fermentations were carried out in a 14-l Chemap-fermenter using the *Hyphomicrobium* strain MS 72. A description of a typical example of such a fermentation follows. A preculture was prepared by growing the organism in 500-ml shake flasks containing 100 ml of medium MV7, 5 ml 1 M phosphate buffer, pH 7 and 5 g/l of pure sodium monomethyl sulfate. Incubation was on a rotary shaker at 250 rpm at 28 °C for 72 h. 500 ml of this preculture were then inoculated into the fermenter containing 10 l of sterilized medium MV7 and 5 g/l of MS (sterilized by filtration of a 10% solution). During the fermentation the pH level was controlled at 6.0 with 4 N NaOH and 1 N HCl, the temperature was kept at 28 °C and the aeration rate at 0.26 l air/l culture/min. Stirring was at 400–700 rpm with an agitator with 2 six-bladed turbines. Growth was followed by OD_{650} and dry weight determinations. The consumption of NaOH for neutralization and the concentrations of monomethyl sulfate and Na_2SO_4 (both determined by isotachophoresis and ion-chromatography) as well as the concentration of methanol (determined by gas chromatography) were also measured.

Exponential growth started after a lag-phase of about 20 h and stopped after 69 h of fermentation when practically all the substrate was utilized ($OD_{650} = 1.06$). When 160 ml of mother liquor from an industrial methylation process (composition in weight per volume: 31% sodium monomethyl sulfate, 10% methanol, 1% inorganic sulfate, $\leq 2\%$ organic by-products, 56% water) was added to the stationary culture at 79 h, exponential growth was restored immediately at the identical growth rate as with pure MS. The addition of mother liquor corresponded to concentrations of 5 g/l of MS plus 1.6 g/l of methanol in the culture liquor. The second phase lasted until 93 h when an OD_{650} of 2.48 was reached (dry weight 0.9 g/l) and more than 90% of both MS and methanol were degraded.

The following growth constants could be calculated from the growth experiments on monomethyl sulfate:
Maximum specific growth rate: $\mu_{max} = 0.05\text{–}0.06\ h^{-1}$,
Medium generation time: $\bar{g} \sim 12$ h,
Growth yield: $Y_{X/S} \sim 0.1$.
For methanol an $Y_{X/S}$ of ~ 0.43 was calculated.

The amount of NaOH consumed for pH-control was 93% of what was theoretically needed for the neutralization of HSO_4^- formed by complete enzymatic hydrolysis of MS. The NaOH consumption showed exact linearity in the semilogarithmic plot for both phases of the experiment (growth with pure MS and with MS containing mother liquor). It paralleled the concentration of Na_2SO_4 and was complementary to the curve of MS concentration. The methanol concentrations were below 5 mM throughout phase 1 and dropped to zero at the end of this phase. We conclude from these data that the methanol liberated by enzymatic hydrolysis of MS is rapidly utilized for cell growth and energy metabolism and that the hydrolysis of MS to methanol and HSO_4^- is the rate limiting step in MS utilization.

All the results of the growth experiments are in good agreement with the biodegradation pathway for monomethyl sulfate proposed in figure 2.

Disposal of monomethyl sulfate wastes under practical conditions – an outlook

Our fermentation studies have demonstrated that monomethyl sulfate can be degraded by *Hyphomicrobium* MS strains either as the pure substance or as a constituent of process effluents or mother liquors from methylation processes.

For the practical application of such specialized strains, different biological waste treatment processes could be envisaged:

- Biodegradation of MS-containing mother liquors is possible in a classical sewage treatment plant after inoculation with MS utilizers or after acclimatization. Experiments have shown that our *Hyphomi crobium* MS strains can survive in a model industrial sewage treatment plant, even under discontinuous exposure to MS, if the plant is stabilized by periodical addition of methanol or other C_1-compounds utilized by MS degraders. As methanol is a relatively cheap substrate it is often used for denitrification (correction of unfavorable C/N-ratios). An unstabilized plant with changing substrate composition would probably require a continuous inoculation. A disadvantage would be that direct biodegradation of MS-containing wastes in an industrial sewage treatment plant would require continuous pH-adjustment to prevent a significant pH-decrease
- As MS-containing mother liquors are produced in large quantities by big chemical companies a separate treatment of such defined process effluents (without mixing with effluents from other processes) in small- or medium-sized bioreactors using pure or enriched cultures of MS utilizers could be established. Such a process could operate at cell densities comparable to classical treatment systems.
- If the stability of methylsulfatase were good enough, one could consider a pretreatment of MS wastes with immobilized sulfatase or immobilized resting cells before delivery of the hydrolyzed and neutralized mother liquors to the sewage treatment plant.
- Growth inhibition problems with MS degraders can occur due to the presence of toxic by-products in the MS-containing effluents. We have identified chlorinated aromatics such as chloroanilines, aminochlorophenols or chloronitrophenols as potential growth inhibitors for our MS-utilizing *Hyphomicrobium* strains. But even under strongly inhibitory conditions (with concentrations of chlorinated aromatics >50 ppm) where practically no growth is observed, the *Hyphomicrobium* MS strains still hydrolyze monomethyl sulfate (behaving like a resting cell system) and degrade methanol. Thus only the assimilation but not the dissimilation of methanol is inhibited.

In ecologically integrated systems the sewage sludge (or single cell protein in the case of pure cultures) produced in the specific biodegradation facilities proposed above should be considered as a potential source for the production of chemicals, enzymes or energy. We will deal with such aspects in part III of this series of papers[10].

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