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Anaerobic Degradation of Halogenated Aromatic Compounds

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Abstract. Recent microbiological findings show how compounds, regarded hitherto as unusual substrates for anaerobic bacteria, are degraded under anaerobic conditions. The complete conversion of halobenzoic acids and halophenolic compounds to methane by lake sediment and sewage sludge microorganisms has been demonstrated. Since haloaromatic compounds are widely used and may be found in such effluents as those from the forest industry, these studies could stimulate a broader interest in anaerobic treatment of industrial waste waters which contain unusual organic compounds.

Introduction

In recent years, anaerobic treatment of industrial waste water not only from food manufacturing but also from other sources such as the forest industry has gained increased interest. For example, Hakulinen and Salkinoja-Salonen investigated kraft pulping bleachery waste waters [6]. These authors found that about 600–2,000 mg of chlorophenolic compounds per m^3 were contained in this effluent. This may seem small when compared to the other constituents, but when entering rivers or lakes, haloaromatics finally accumulate in organisms like fish [11], and the deleterious effects of these compounds have been well publicized.

Only recently the groups of Tiedje and Boyd have demonstrated that sediment or sewage sludge microbes are able to completely metabolize halogenated benzoic acid or phenolic compounds to methane and carbon dioxide [1, 2, 17, 18]. We had previously isolated an organism which degrades the heteroaromatic furfural (a major constituent of sulfite evaporator condensate) to acetic acid in the strict absence of oxygen [3, 4]. When we began to study the anaerobic degradation of chlorine bleaching liquor from a paper mill, we also became interested in the anaerobic catabolism of haloaromatics. The intention of this article is to briefly summarize present knowledge of these microbial processes.

Occurrence of Haloaromatic Compounds

Haloaromatics, such as polychlorinated phenols, do not occur only in pulp bleaching effluents; they are also manufactured industrially on a large scale, as

Table 1. Halometabolite levels in marine organisms

Animals	5.1–9.6
Plants	19.3–19.8
Some red algae species	up to 2,400[a]

Average chloro- or bromometabolite concentrations in $\mu g \cdot g^{-1}$ wet weight according to data of Hager [5]
[a] Lipid halometabolite concentration

Table 2. Examples of aromatic compounds examined for reductive dehalogenation by anaerobic lake sediments and fresh and acclimated sludge

Substrates	Initial concentration	End products	References
Benzoate			
	Monohalogens		
2,3- or 4-Iodo-	0,1–0,82 mmol·l^{-1}	CH_4, CO_2	[15]
2,3- or 4-Bromo-	0,1–0,82 mmol·l^{-1}	CH_4, CO_2	[15]
2- or 4-Chloro-	0,1–0,82 mmol·l^{-1}	None	[15]
3-Chloro-	0,1–0,82 mmol·l^{-1}	CH_4, CO_2	[15]
3-Chloro-	2 mmol·l^{-1}	CH_4, CO_2	[a]
3- or 4-Fluoro-	0,2–0,82 mmol·l^{-1}	None	[15]
4-Amino-3-Chloro-	0,2–0,82 mmol·l^{-1}	4-Aminobenzoate	[15]
	Dihalogens		
2,4-Dichloro-	0,2–0,82 mmol·l^{-1}	None	[15]
2,5-Dichloro-	0,2–0,82 mmol·l^{-1}	2-Chlorobenzoate	[15]
2,6-Dichloro-	0,2–0,82 mmol·l^{-1}	None	[15]
3,4-Dichloro-	0,2–0,82 mmol·l^{-1}	4-Chlorobenzoate	[15]
3,5-Dichloro-	0,2–0,82 mmol·l^{-1}	CH_4, CO_2	[15]
4-Amino-3,5-Dichloro-	0,2–0,82 mmol·l^{-1}	4-Amino-3-Chlorobenzoate	[15]
	Trihalogen		
2,3,6-Trichloro-	0,2–0,82 mmol·l^{-1}	2,6-Dichlorobenzoate	[15]
Phenol	Mono- and dihalogens		
2- or 4-Chloro-	15–50 $\mu g \cdot l^{-1}$	CH_4, CO_2	[1, 2]
3-Chloro-	15–50 $\mu g \cdot l^{-1}$	(CH_4)	[1, 2]
2,4-Dichloro-	15–50 $\mu g \cdot l^{-1}$	CH_4, CO_2	[1, 2]
3,4-Dichloro-	15–50 $\mu g \cdot l^{-1}$	None	[1, 2]
3,5-Dichloro-	15–50 $\mu g \cdot l^{-1}$	None	[1, 2]

[a] Schoberth SM, Brunner M, Sahm H (1984) First IAWPRC Symposium on Forest Industry Waste Waters, Tampere, Finland, 11–15 June (see also [7, 18])

Suflita et al. [17, 18] point out. For example, pentachlorophenol is widely used as a wood preservative, and 2,3,6-trichlorobenzoic acid as a herbicide.

In a recent review, Hager [5] showed that some haloorganic compounds are natural products of living cells, though occurring only in traces. Relatively high levels may be found in marine organisms (Table 1). In some red algal species, an exceptional 5% of the dry weight consists of haloorganics. In general, naturally occurring haloaromatics have potent biological activity. Examples are the iodo aromatic hormone thyroxine found in mammals, and the antibiotics griseofulvin and aureomycin produced by actinomycetes.

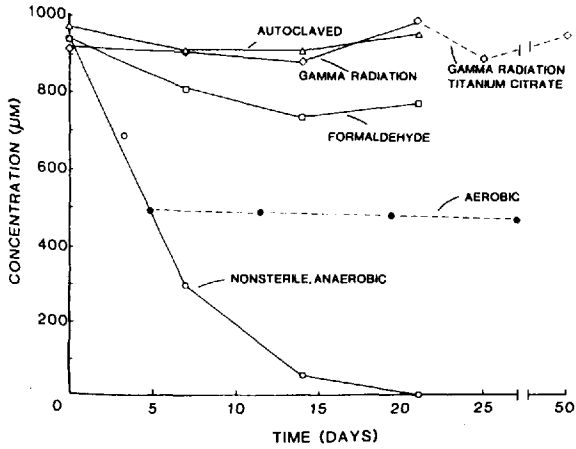

Fig. 1. The effect of various sterilization procedures and the addition of 20% oxygen on the dehalogenation of 4-amino-3, 5-dichlorobenzoic acid in acclimated sediment. Reprinted with permission of Horowitz, Suflita and Tiedje [7].

Thus microorganisms may have developed strategies for the degradation of haloaromatic compounds long before the chemical industry began their production, increasing the chance for biotechnology to find microbes which can solve some of today's pollution problems.

Present Knowledge of Anaerobic Microbial Degradation of Haloaromatic Compounds

Table 2 summarizes some recent results from the laboratories of Tiedje and Boyd [1, 2, 17, 18]. These authors demonstrated that microbial populations from lake sediments and sewage sludge mediated the anaerobic degradation to methane of some 19 chloro- bromo- and iodo-benzoate and phenolic compounds. From these results, it appears that meta halogens are more susceptible to attack by anaerobic bacteria, when compared to the ortho or para isomers. In addition, the bromo and iodo substituents seem to be more readily removed. However, whether a compound can be degraded may also be a matter of time and acclimation of the microbial flora: when 4-chlorophenol was incubated with digested sludge for 8 weeks, no degradation occurred; however after an additional 8 weeks, 4-chlorophenol was almost completely degraded [1]. Another example: 3,4- and 3,5-dichlorophenol were persistent in fresh sludge for 6 weeks, but were degraded within 14 days in sludge previously acclimated to 3-chlorophenol [1].

The following evidence demonstrates that these dehalogenation reactions are brought about by living anaerobic bacteria and not by abiotic photochemical reactions or chemical reductants [7]:

1. The reactions occur only in the dark and in the absence of oxygen. Sterilized sludge samples do not carry out any reaction even in the presence of the strong reductant titanium citrate (Fig. 1).
2. Upon increasing the temperature above 39°C, dehalogenation reactions in freshwater sediments are lost, since sediment microbes are not adapted to these conditions. On the contrary, a chemical mechanism would be enhanced by such temperatures.

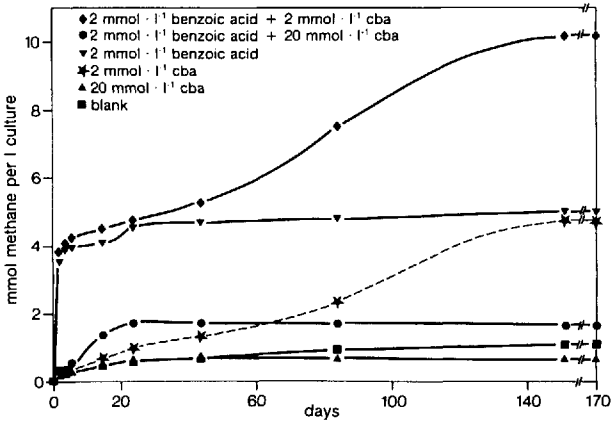

Fig. 2. Degradation of 3-chlorobenzoic acid (CBA) to methane in the absence and presence of benzoic acid by an anaerobic enrichment. Means of triplicates (see text).

3. Usually, when haloaromatics are added to unadapted sediments, long lag periods with a low background of methanogenesis are followed by a phase of rapid degradation. This indicates a specific biological adaptation, as does the acclimation of sediments by prior exposure to the haloaromatic compounds.

Little is known about the metabolic steps of these anaerobic degradations. Apparently, the primary event is the removal of halides from the aromatic ring. Kinetic studies with chlorobenzoates revealed apparent K_m values of 30–67 μmol·l^{-1} [18], i.e., the bacterial population has a high affinity for haloaromatic substrates and can degrade very low concentrations appropriately.

We have some experience with degradation of 3-chlorobenzoic acid to methane (S. M. Schoberth, M. Brunner and H. Sahm, 1st IAWPCR Symposium on Forest Industry Wastewaters, Tampere, Finland, 1984): when we began to investigate spent bleach liquor, 3-chlorobenzoate was chosen as a model compound to study anaerobic degradation of the various halogenated aromatics present in such an effluent [6, 11]. 3-Chlorobenzoate was chosen since its dehalogenated form, benzoate, has been well studied in terms of anaerobic degradation (see [12, 13] for references). However, initial attempts with 3-chlorobenzoate failed, as this substrate proved to be persistent under our conditions and even seemed to inhibit endogenous methanogenesis from the seed sludge. Finally, the following technique turned out to be successful: enrichments were set up using mineral media and techniques [8] similar to those described previously [15]. However, we applied either benzoate, 3-hydroxy- or 4-hydroxybenzoate as "main" substrates at initial concentrations of 2 mmol·l^{-1} (244–276 mg·l^{-1}). 3-Chlorobenzoate was added only in traces (3 mg·l^{-1}). The cultures were periodically supplied with fresh substrates. These conditions allowed for enrichment of a strong population of anaerobes able to convert the benzoic acids to methane.

After 18 months of incubation at 30°C, these cultures were able to attack 3-chlorobenzoate at concentrations of 2 mmol·l^{-1} (313 mg·l^{-1}), not only in the presence of the "cosubstrates" but also with 3-chlorobenzoic acid as sole carbon source (Fig. 2). Compared to the amount initially added, the conversion to methane was almost quantitative according to the equation:

$$C_7H_5O_2Cl + 5\ H_2O \rightarrow 3.5\ CH_4 + 3.5\ CO_2 + HCl \quad \text{(Equation 1)}$$

The degradation of 3-chlorobenzoate to methane was also substantiated using thin layer and high pressure liquid chromatography. 3-Chlorobenzoate at initial concentrations of 20 mmol·l^{-1} (3130 mg·l^{-1}) inhibited methane formation almost completely (Fig. 2). However, recent preliminary studies in our laboratory indicate that 3-chlorobenzoate may be anaerobically degraded up to concentrations of 11.5 mmol·l^{-1} (1800 mg·l^{-1}).

Discussion

Anaerobic degradation of halogenated benzoates and phenols is a new field of research in microbiology. The mechanisms of dehalogenation and cleavage of the aromatic ring structure are still unknown. Only recently, bacterial species have been described which are able to attack nonhalogenated aromatic ring structures in the absence of light and electron acceptors such as oxygen, nitrate, or sulfate: *Pelobacter acidigallici* ferments gallic acid, pyrogallol, 2,4,6-trihydroxybenzoic acid, and phloroglucinol to acetic acid [14]. *Syntrophus buswellii* produces acetate, CO_2, and H_2 (or formate) from benzoate and, possibly, from hydrocinnamate [12, 13]. *Syntrophus buswellii* requires coculture with an appropriate hydrogenotropic bacterium (sulfate reducer or methanogen), in order to get rid of hydrogen which inhibits its growth. Benzoate degradation in this organism is presumed to start with reductive cleavage of the benzene ring followed by a β-oxidation mechanism as proposed for the anaerobic catabolism of fatty acids [12]. Only very recently, the degradation of haloaromatics to methane under defined conditions could be demonstrated, as pointed out in this article. The microorganisms involved are scarcely known. Which of these organisms carry out the dehalogenating steps is not known. Shelton and Tiedje [16] report on an anaerobic rod able to dehalogenate 3-chlorobenzoic acid when growing in a complex medium containing pyruvate and rumen fluid. The dehalogenation product, benzoate, was not attacked by this organism but was used by other nondehalogenating species present in this methanogenic enrichment.

What will be the impact of these microbiological studies on biotechnology? It may turn out that anaerobic techniques are better suited to remove halogenated aromatic compounds from industrial effluents than aerobic biological treatment since:

1. Haloaromatic compounds tend to polymerize when degraded by ordinary aerobic bacteria. For example, chloropyrocatechol accumulates in cultures of *Alcaligenes eutrophus* grown on 3-chlorobenzoate. Consequently the autoxidation products of chloropyrocatechol polymerize and give the cultures a brownish-black color [10]. These polymerization products are rather resistant to further bacterial attack. However, under anaerobic conditions, oxidation, and hence polymerization, are not possible.
2. Theoretically, polyhalogenated aromatics should be more easily degraded by anaerobes than by aerobic bacteria since an increasing degree of haloge-

nation of an aromatic ring decreases the electron density of the aromatic nucleus. Thus electrophilic attack of oxygen on aromatic structures is more difficult. To the contrary, a decreased electron density of the aromatic nucleus should enhance anaerobic enzymatic attack by a reductive (nucleophilic) mechanism (Knackmuss, personal communication; see also [9]).

3. Since anaerobic enrichments have a high affinity for haloaromatics [18], anaerobic techniques seem to be well-suited to remove trace levels from industrial effluents.

Interest in the anaerobic treatment of effluents containing such compounds will increase. To achieve higher rates and better yields than those found in nature, it is necessary to gather more information on the organisms involved and to learn more about their nutritional requirements and metabolic pathways.

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