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Inhibition of the methanogenic fermentation of *p*-toluic acid (4-methylbenzoic acid) by acetate

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Abstract. The potential inhibitory effect of acetate on *p*-toluic acid methanogenic fermentation was studied during the continuous operation at 5.3 days hydraulic retention time of an upflow anaerobic sludge blanket reactor fed with a synthetic waste-water containing 3.67 mM *p*-toluic acid as sole carbon and energy source. In the absence of acetate, a chemical oxygen demand removal efficiency of 56.8% and an estimated *p*-toluic acid removal efficiency of 62.8% were achieved. Immediately after the addition of 58.3 mM acetate into the reactor influent, *p*-toluic acid degradation stopped while most of the acetate was consumed. The inhibition is explained by thermodynamic considerations. It is emphasized that such phenomena could occur during the treatment of waste-waters containing high concentrations of acetate and aromatic compounds that require a syntrophic association to be degraded to acetate and H_2.

Introduction

Recently, anaerobic treatment has been applied successfully to chemical industrial waste-waters containing phenol or benzoate (Borghans and van Driel 1988; Frankin et al. 1991) as well as to petrochemical effluents resulting from the production of terephthalic acid (1,4-benzenedicarboxylic acid) (Guyot et al. 1990a; Noyola et al. 1990; Macarie et al. 1992) and dimethylterephthalate (DMT) (Reule 1990; Frankin and Koevoets 1991). Beside mono-substituted benzenes and benzoic acids, the major pollutant in these wastes is acetic acid. For instance, the chemical oxygen demand (COD) of DMT waste-water (20–50 g O_2/l) is 49–57% due to acetic acid and only 16–19% to aromatic compounds (terephthalic, phthalic, *p*-toluic, benzoic acids, etc.) (Leenheer et al. 1976; Reule 1990).

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It is generally admitted that the methanogenic fermentation of mono- or bi-substituted but mono-hydroxylated benzenes depends on a syntrophic association between (1) acetogenic bacteria that convert the aromatic compound to acetate and H_2 and (2) hydrogenophilic methanogens that convert H_2/CO_2 to methane (Holliger et al. 1988). The cleavage of these aromatic compounds to acetate is highly endergonic under standard conditions, as illustrated for benzoic acid (Eq. 1) [the variation of Gibbs free energy (ΔG) was calculated from ΔG_{f° tabulated by Thauer et al. (1977) except for benzoate (Thauer and Morris 1984)].

$$\text{benzoate}^-_{(aq)} + 7\,H_2O_{(l)} \longrightarrow 3\,\text{acetate}^-_{(aq)} + 3\,H^+_{(aq)} + HCO^-_{3(aq)} + 3\,H_{2(g)} \quad (1)$$
$$\Delta G^{\circ\prime} = +58\ \text{kJ/reaction}$$

Hydrogen (H_2) is the key compound that controls the thermodynamics of these reactions. It must be continuously removed by a hydrogenotroph in order to maintain an H_2 partial pressure for which the $\Delta G'$ of the reaction becomes exergonic (i.e. $pH_2 < 10^{-4}$ atm for benzoate, $pH_2 < 10^{-2}$ atm for phenol). Quantitatively, acetate is, after H_2, the second product of the metabolism of acetogenic aromatic degraders. Its role in the thermodynamics of the reactions that these bacteria catalyse has been generally neglected. However, Dolfing and Tiedje (1988), using a batch syntrophic culture of a benzoate degrader and a hydrogenophilic methanogen (*Methanospirillum hungatei*), showed that Eq. 1 was inhibited by addition of high concentrations of acetate, whereas the H_2 partial pressure was adequate. They concluded that acetate was thermodynamically equivalent to H_2. The effect of acetate concentration on the ΔG of Eq. 1 calculated with the in situ conditions used by Dolfing and Tiedje (1988) confirms that the energy available for the growth of the benzoate-degrader partner decreases when the acetate concentration increases (Fig. 1).

Inhibition by acetate can be expected for all aromatic compounds the methanogenic fermentation of which involves a syntrophic association. As acetate is often pres-

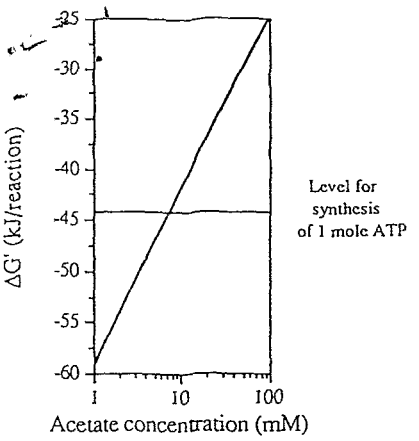

Fig. 1. Effect of acetate concentration on the Gibbs free energy of benzoate degradation to H_2 and acetate (see Eq. 1). The curve was calculated via the equation $\Delta G' = \Delta G^\circ + RT \ln (([\text{acetate}]^3[H^+]^3[HCO_3^-][P_{H_2}]^3)/[\text{benzoate}])$ with the assumption that [benzoate] = 10 mM, $[HCO_3^-] = 28.5$ mM, H_2 partial pressure (P_{H_2}) = 10^{-4} atm, pH = 7.0 and temperature = 25° C

ent in large amounts in chemical industrial effluents containing this kind of aromatic molecules, inhibition could occur during their anaerobic treatment, resulting in mineralization of the readily biodegradable COD, but not of the aromatics. In order to verify this hypothesis, we investigated the effect of acetate addition on the anaerobic treatment of a synthetic waste-water containing a benzenic derivative (*p*-toluic acid) as sole carbon and energy source. *p*-Toluic acid is a by-product formed during the manufacturing of DMT and terephthalic acid. Its biodegradability to CH_4 and CO_2 was reported by Horowitz et al. (1982). We have previously demonstrated the feasibility of the anaerobic treatment of synthetic effluent containing *p*-toluic acid (Macarie and Guyot, unpublished data). In this paper, we report the inhibition by acetate of the methanogenic fermentation of *p*-toluic acid in upflow anaerobic sludge blanket (UASB) reactors.

Materials and methods

Experimental apparatus. The study was carried out with two UASB laboratory reactors. Reactor 1 was made of a glass column 45 cm high with an internal diameter of 9.6 cm and working volume of 3.6 l. Reactor 2, also made of a glass column, was 35 cm high and had an internal diameter of 9 cm and a working volume of 2.75 l. The top of the reactors was equipped with a liquid-gas-solid separator. Biogas was collected in a column filled with a saturated solution of NaCl, pH < 4. Both reactors were thermostatically controlled at 34 ± 1° C and continuously fed using peristaltic pumps.

Synthetic waste-water. The defined medium was prepared with tap water and had the following composition in mg·l^{-1}: *p*-toluic acid (500), $NaHCO_3$ (500), $FeSO_4 \cdot 7H_2O$ (100), NH_4Cl (660), $(NH_4)_2SO_4$ (250), K_2HPO_4 (130), KH_2PO_4 (100), $CaCl_2$ (200), $MgCl_2 \cdot 6H_2O$ (100), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (10), $ZnCl_2$ (1), $CoCl_2 \cdot 6H_2O$ (1), $MnCl_2 \cdot 4H_2O$ (1), $NiCl_2 \cdot 6H_2O$ (0.2), $AlK(SO_4)_2 \cdot 12H_2O$ (0.1) and $CuCl_2 \cdot 2H_2O$ (0.05). Sodium bicarbonate, ammonium chloride and ammonium sulphate were of industrial grade; all the other chemicals were of analytical grade. *p*-Toluic acid was dissolved in boiling water to obtain, after cooling, a concentration of 1 g·l^{-1}. This solution was then diluted twice with the concentrated solution containing all the mineral salts. When acetate was added to the medium, $NaHCO_3$ was omitted because the medium maintained a convenient pH and alkalinity.

Reactor operation. Both reactors were inoculated with an anaerobically adapted activated sludge from the conventional waste-water treatment plant of the National University campus at Mexico city. They were fed for 9 months with *p*-toluic acid synthetic medium before starting the study. The sludge of the reactors presented high counts of *p*-toluic-acid degraders, hydrogenophilic, and acetoclastic methanogens.

During the whole experiment (day 285 to day 474 of operation), reactor 1 was operated at 5.3 days (SD = 0.8) of hydraulic retention time (HRT), with a feeding stop between days 380 to 420. On day 321, 4.78 g·l^{-1} of industrial anhydrous sodium acetate (58.3 mM acetate) was added to the *p*-toluic acid synthetic medium. This concentration was maintained till the end.

Reactor 2 was operated at three different HRTs: 1 day (SD = 0.02) from days 280 to 295, 7.46 days (SD = 1.44) from days 296 to 358, and 5.1 days (SD = 0.46) from days 403 to 455. A feed stop was applied between days 359 and 402 of operation. This reactor was used as control.

Analytical methods. Influent and effluent *p*-toluic acid concentration was determined after centrifugation and dilution (1/5) by measurement of UV absorbance at 255 nm with a Beckman model 25 spectrophotometer.

Methane content in the biogas was determined with a Gow-Mac gas chromatograph (series 550) equipped with a thermal conductivity detector and a stainless steel column (1.8 m × 3.2 mm) packed with carbosphere Q (80–100 mesh) (column temperature, 140° C; carrier gas, He (45 ml·min^{-1}); injection temperature, 150° C; detector temperature, 160° C; current of the filament, 150 mA). Gas sampling and injections were made with a Hamilton pressure lock syringe.

Volatile fatty acids were measured with a Hewlett Packard 5890A flame ionization detector gas chromatograph and a wide-bore column superoxFA (10 m × 0.53 mm) (column temperature, 120° C; carrier gas, N_2 (33 ml·min^{-1}); injection temperature, 125° C; detector temperature, 120° C).

Sludge oxidation-reduction potential was determined with a Pt, Ag/AgCl combination electrode (Cole-Parmer N-05990-55) and converted to the potential relative to the hydrogen reference (E_h) by addition of +216 mV (valid for 35 ± 2° C) to the measured values.

Other analytical determinations [COD, pH, temperature, volatile suspended solids (VSS)] were performed according to APHA (1985).

Sludge microbial activity. Consumption kinetic of acetate was measured according to Guyot et al. (1990*b*). The mineral solution was, however, changed for that of medium 1 of Balch et al. (1979). Four milliliters of sludge (0.10 g VSS for reactor 1 sludge, 0.16 g VSS for reactor 2 sludge) and 16 ml mineral solution was distributed in each serum bottle. At time zero, the substrate was added in known amount (≈ 10 mM).

Results and discussion

The operation of reactor 2 allowed the validity of the estimation of *p*-toluic acid concentration to be checked by measurement of the UV absorbance at 255 nm. Since *p*-toluic acid was the only organic compound present in the influent of that reactor, COD was also a measurement of its concentration and should correlate with ab-

Table 1. Characterization of the steady-state periods for reactor 2 and the difference between chemical oxygen demand (COD) removal and estimated *p*-toluic acid removal efficiencies

Days of operation	280–295	296–358	403–455
Influent COD ($mg \cdot l^{-1}$)	1020	1014	1010
HRT (days)	1.0	7.5	5.1
Organic Load ($kg\ COD \cdot m^3 \cdot day$)	1.02	0.14	0.20
E% COD	18.2	67.8	62.6
E% *p*-toluic acid	29.3	74.0	71.6
E% *p*-toluic acid − E% COD	11.1	6.2	9.0

HRT, hydraulic retention time; E, Removal efficiency

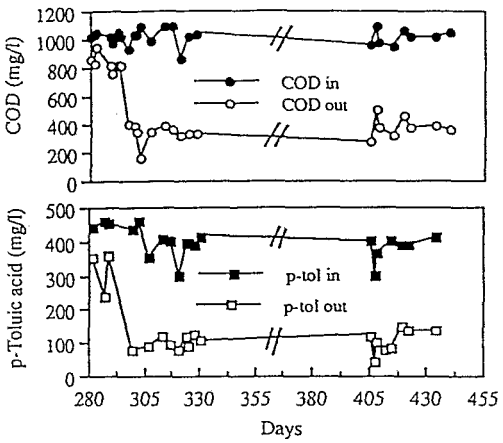

Fig. 2. Evolution of influent and effluent chemical oxygen demand (COD) and influent and effluent *p*-toluic (*p*-tol) acid concentration during the operation of reactor 2

sorbance. The mean *p*-toluic acid concentration estimated in the reactor influent at 255 nm was 402 $mg \cdot l^{-1}$ (SD = 37), which is 20% lower than the real concentration (500 $mg \cdot l^{-1}$). In contrast, the influent experimental COD was very close to the theoretical COD (1058 mg O_2/l) (Table 1). Despite this difference, the curves of COD and *p*-toluic acid concentration presented the same fluctuations with time and HRT (Fig. 2). Moreover, the variation between COD removal and the estimated *p*-toluic acid removal from UV measurements ranged only from 6 to 11% depending on the period of operation (Table 1). These results show that absorbance measurements allowed an estimation of *p*-toluic acid concentration with sufficient accuracy to determine the rate of its degradation.

During the operation of reactor 1, a stationary state with 55.8% COD removal was achieved from day 285 to 305 (Table 2, Fig. 3D). *p*-Toluic acid removal efficiency calculated from absorbance at 255 nm was 7% higher (Table 2). On day 306, a hydraulic shock (HRT = 0.71 day) was accidently applied to the reactor for a few hours. COD removal and *p*-toluic acid removal decreased quickly (Fig. 3D, 3E) and subsequently recovered to the former steady state. No biogas production was monitored during this period (Fig. 3C); this is explained by the very low theoretical biogas production

Table 2. Characterization of the steady-state periods for reactor 1

Days of operation	285–320	321–474
Organic load ($kg\ COD \cdot m^3 \cdot day$)	0.19	0.86
Influent COD ($mg \cdot l^{-1}$)	1004	4524
Effluent COD ($mg \cdot l^{-1}$)	436	1131
Influent pH	7.26	7.30
Sludge pH	7.20	8.05
Sludge E_h (mV)	−124.0	−261.5
E% COD	55.8	73.2
E% *p*-toluic acid	62.8	0.0
E% *p*-toluic acid − E% COD	7	—
Methane yield ($N \cdot m^3 \cdot CH_4 \cdot kg^{-1}\ COD_{rem}$)	—	0.28

N, Normal temperature and pressure (0° C, 1 atm); E_h, redox potential relative to hydrogen reference

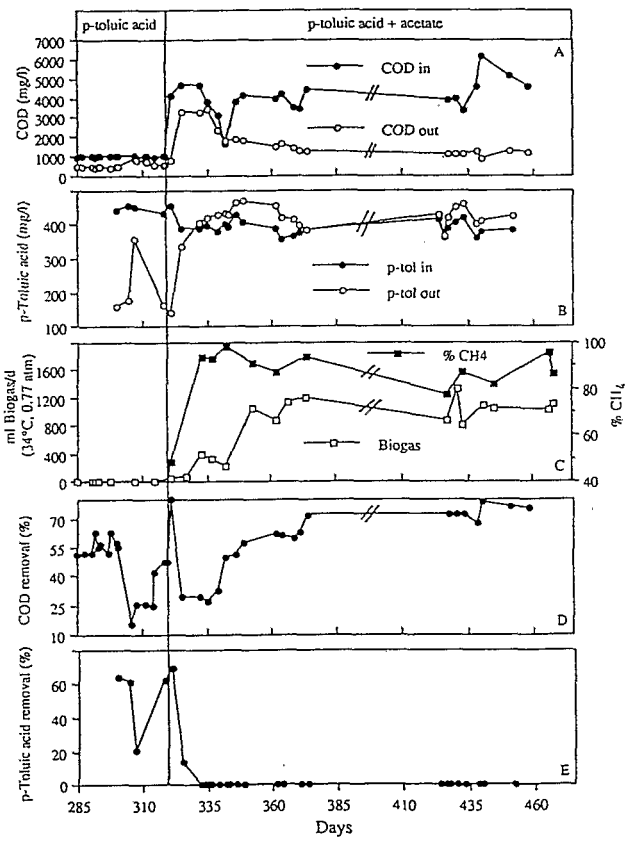

Fig. 3. Evolution of influent and effluent COD (**A**), influent and effluent *p*-toluic acid concentration (**B**), biogas production (**C**), percentage of CH_4 in the biogas (**C**), COD removal efficiency (**D**) and *p*-toluic acid removal efficiency (**E**) during the operation of reactor 1

(66 $N\ ml \cdot l^{-1}_{reactor} \cdot day$) and the poor sensitivity of the gas collection device.

Immediately after the addition of acetate on day 321, the *p*-toluic acid concentration in the reactor effluent increased sharply to finally reach that measured in the influent; the *p*-toluic acid removal efficiency dropped to zero (Fig. 3B, 3E). Biogas production with a high content of methane also increased quickly (Fig. 3C) after

the addition of acetate and indicated that it began to be degraded. In parallel, the COD of the effluent decreased. From day 374 to the end of reactor operation, a steady state with high COD removal and fairly good methane yield was achieved (Fig. 3D, Table 2). This COD removal was only caused by acetate consumption. *p*-Toluic acid remained undegraded, as indicated by Fig. 3E and by the residual COD of the effluent close to the theoretical COD of the *p*-toluic acid initially present in the influent (Fig. 3A, Table 2). Only 2–4 mM acetate was detected in the reactor effluent.

At the end of the experiment, the specific activities of acetate consumption of the sludges from both reactors were determined. The results were 0.184 mmol·g^{-1} VSS·h^{-1} for reactor 1 and 0.032 mmol·g^{-1} VSS·h^{-1} for reactor 2. The operating conditions applied to reactor 2 during its last feeding period (days 403–455) were similar to that applied to reactor 1 during its first feeding period (days 285–320) (same HRT and composition of the effluent). Therefore the acetate consumption measured for reactor 2 sludge can be considered an estimate of the sludge activity of reactor 1 during its first period of operation. Then, the difference of activity between the reactors suggests, after acetate addition, an increase in the activity of the methanogenic acetoclastic population in reactor 1.

The results clearly show that acetate has an inhibitory effect on the methanogenic fermentation of *p*-toluic acid. Based exclusively on thermodynamic considerations, this inhibition should have been reversed when acetate was almost exhausted in the digestor effluent. However, the acetate concentration in reactor 1 effluent dropped in around 49 days from 58.3 mM to a value lower than 5 mM. This time was long enough to cause a reduction in the *p*-toluic acid degrader population, which could explain the absence of reversibility. Such a change in population is suggested by a lowering of the reactor sludge oxidation-reduction potential, as well as by the increase in acetoclastic activity. Actually, the increase in pH (Table 2), due to the degradation of acetate to CH_4 and CO_2 and the related removal of protons from the aqueous phase, is not sufficient to explain the E_h variation. An increase of one pH unit makes the E_h more negative by only −60 mV (Srinivas et al. 1988) while the E_h variation observed was of −137 mV (Table 2).

The inhibition of the methanogenic fermentation of mono- or bi-substituted but mono-hydroxylated benzenes during the anaerobic treatment of waste-waters containing these compounds and high amounts of acetate has not yet been reported in the literature. Nevertheless, the anaerobic treatment of a DMT plant effluent with 24.5 g·l^{-1} (409 mM) acetate, 2 g·l^{-1} (14.7 mM) *p*-toluic acid and 1.5 g·l^{-1} (12.3 mM) benzoic acid using a fluidized bed reactor and 2 days HRT resulted in only 10% *p*-toluic acid and 33% benzoic acid removal (Reule 1990). This occurred while acetic acid was mostly degraded, its residual concentration being 140 mg·l^{-1} (2.3 mM). The weak *p*-toluic acid removal reported in that experiment might be due to the low HRT applied. As shown in Table 1 for reactor 2, *p*-toluic acid has a slow degradation kinetic and its removal decreases with HRT. At 1 day HRT, less than 19–30% *p-toluic acid was effectively degraded.* However, because of its different hydraulic regime pattern and its higher exchange surface, a fluidized bed reactor presents better mass transfer characteristics than a UASB reactor. As a consequence, even at lower HRT than a UASB digestor, it generally treats waste-water with a higher COD removal efficiency. Hence, an inhibitory effect of acetate in this case should not be discounted.

No inhibition was observed during the anaerobic treatment with UASB reactors of a chemical waste-water containing 6–7 g·l^{-1} of benzoic acid (49–57 mM; 59–69% of COD) and 4–6 g·l^{-1} of acetic acid (66–100 mM; 21–31% of COD) (Frankin et al. 1991), and of other chemical effluents containing mainly phenol (0.45 g·l^{-1}; 4.8 mM; 3.5% of COD) and acetic acid (19.5 g·l^{-1}; 325 mM, 68% of COD) (Borghans and van Driel 1988): 50–74% of benzoic acid (estimated from the reported 80–95% COD removal) and 95% of phenol was eliminated. This absence of inhibition is easily explained: the Gibbs free energy of benzoate and phenol degradation to acetate was exergonic ($\Delta G' = -24.6$ kJ/reaction for benzoate, $\Delta G' = -40.07$ kJ/reaction for phenol) when calculated with the concentrations of the compounds in the rough waste-waters, a temperature of 25° C, a pH of 7.0 and an H_2 partial pressure of 10^{-4} atm, which is commonly observed in digestor sludges (Archer 1983). The absence of inhibition in the case of phenol even for the very high acetate concentration found in the waste-water studied is not surprising, since the cleavage of phenol to acetate is only slightly endergonic under standard conditions ($\Delta G°' = +5.2$ kJ/reaction) as compared to that of benzoic acid ($\Delta G°' = +58$ kJ/reaction). A much higher H_2 or acetate concentration is then necessary to inhibit phenol acetogenic degradation.

The previous considerations suggest that simple thermodynamic calculations would allow estimation of the possibility of the occurrence of such inhibition when treating effluents containing both acetate and benzenic compounds, the methanogenic degradation of which requires syntrophy. The use of a two-stage anaerobic reactor can be recommended to avoid the inhibition. The first stage would be used to reduce the acetate concentration at a short HRT. The second stage would be used to degrade the aromatics at a higher HRT. The second stage should be started up once the acetate concentration in the first stage effluent was sufficiently low.

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