Anaerobic degradation of phenol in wastewater at ambient temperature

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Abstract Treating a synthetic wastewater containing phenol as the sole substrate at 26°C, an upflow anaerobic sludge blanket reactor was able to remove over 98% of phenol up to 1,260 mg/l in wastewater with 12 h of hydraulic retention time, corresponding to 6.0 g-COD/(l·day). Results showed that benzoate was the key intermediate of phenol degradation. Conversion of benzoate to methane was suppressed by the presence of phenol. Based on DNA cloning analysis, the sludge was composed of five groups of microorganisms. *Desulfotomaculum* and *Clostridium* were likely responsible for the conversion of phenol to benzoate, which was further degraded by *Syntrophus* to acetate and H_2/CO_2. *Methanogens* lastly converted acetate and H_2/CO_2 to methane. The role of epsilon-*Proteobacteria* was, however, unclear.
Keywords Ambient temperature; anaerobic; degradation; phenol; UASB; wastewater

Introduction

Phenol is the raw material for the commercial production of a wide variety of resins, including phenolic resins as construction materials for automobiles and appliances, epoxy resins as adhesives, and polyamide for various applications (Kirk-Othmer, 1978). In addition, phenolic compounds are often found in wastewaters from coal gasification, coke-oven batteries, refinery and petrochemical plants (Blum *et al*., 1986), and other industries, such as synthetic chemicals, herbicides, pesticides, antioxidants, pulp-and-paper, photo-developing chemicals, etc. (Young and Rivera, 1985; Wang *et al*., 1986). The level of phenol in some of the effluents from these industries can be as high as 6,000 mg/l (Cross *et al*., 1982).

Young and Rivera (1985) found that phenol could be stoichiometrically converted to methane and carbon dioxide by anaerobic sludge from a municipal digester. More recently, it was found that phenol (Fang *et al*., 1996) and cresols (Fang and Zhou, 2000) could be removed from wastewater in an upflow anaerobic sludge blanket (UASB) reactor (Lettinga *et al*., 1980) under the mesophilic condition of 37°C. This study was conducted to demonstrate that phenol could also be effectively removed from wastewater in a continuous UASB reactor even at ambient temperature without any organic co-substrate. The phenol degrading characteristics of such sludge were further analyzed using a batch reactor, and the microbial community of such sludge were analyzed using the RNA/DNA based molecular techniques.

Materials and methods

Degradation of phenol in UASB reactor

A phenol-containing wastewater was treated in a 2.8 l UASB reactor (Fang *et al*., 1996), which was operated at pH 7.0–7.5 and 26±1°C. The reactor was seeded with 1.0 l of anaerobic sludge from a previous study treating phenol at 37°C (Fang *et al*., 1996). Prior to operating at the experimental condition of 26°C, the sludge was first re-activated at 37°C

fed with wastewater containing phenol and glucose, as the co-substrate, at respective concentrations of 100 mg/l and 1,000 mg/l, plus balanced nutrients. The concentration of phenol was gradually increased with the corresponding decrease of glucose, until the wastewater was composed of 1,200 mg/l of phenol without any glucose. The ambient temperature experiment began on day 1 by lowering the reactor temperature to 26°C, and feeding it with a wastewater containing phenol as the sole substrate, plus nutrients. The reactor effluent was partially recycled with a flowrate equaling that of the incoming wastewater. The phenol concentration in wastewater increased gradually from the initial 300 mg/l. Starting on day 61, it was controlled at the constant level of 1,260 mg/l while the hydraulic retention time (HRT) was gradually lowered from 40 h to 4 h by day 165. This resulted in the deterioration of phenol removal efficiency. Consequently, the HRT was gradually increased while keeping the phenol concentration at 1,260 mg/l. The reactor was operated at 12 h of HRT for most of the remaining experiment, corresponding to a COD (chemical oxygen demand) loading rate of 6.0 g-COD/(l·d). Compositions of effluent and biogas were analyzed following the methods established from the previous study (Fang *et al.*, 1996). The amount of biogas produced was recorded daily using the water displacement method, and the methane content was analyzed by a gas chromatograph.

Degradation of phenol in batch reactors

The phenol-degrading sludge was sampled on day 356 from the UASB reactor which was treating wastewater containing 1,260 mg/l of phenol with 12 h of HRT. The phenol-degradation characteristics of the sludge were analysed in batch reactors by monitoring the residual phenol, benzoate, VFA and biogas content in each reactor at 26°C over 51 days. Eleven initial phenol concentrations from 200 to 2,200 mg/l were tested. Each reactor contained 1,900 mg-VSS/l of sludge, and the same levels of nutrients and pH as in the UASB reactor. Specific methanogenic activity (SMA) of the phenol-degrading granular sludge were measured based on the method of Dolfin and Mulder (1985) modified from the one proposed originally by Owen *et al.* (1979).

DNA extraction, PCR, DGGE and cloning-sequencing

The DNA of the microbial community of the sludge was first extracted. The extracted DNA was then amplified by polymerase chain reaction (PCR) using the *Eubacteria* specific primer set of Eub968F-GC and Uni1392R (Zhang and Fang, 2000). The amplified 468 base-pair DNA fragments were used in denaturing gradient gel electrophoresis (DGGE) to obtain the profile of the microbial populations, as described previously (Zhang and Fang, 2000). The extracted DNA was also amplified using the another *Eubacteria* specific primers set of GM3F (5′-AGAGTTTGATCMTGGCTCAG) and GM4R (5′-GGTTAC-CTTGTTACGACTT). The amplified 1501 base-pair DNA fragments were cloned using the TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA) and sequenced. Details of these procedures were described previously (Fang *et al.*, 2002; Zhang and Fang, 2001). The obtained DNA sequences were aligned to construct phylogenetic trees with ARB 2.5 (Strunk and Ludwig, 1996; Fang *et al.*, 2002).

Fluorescence in situ hybridization (FISH)

The distribution of bacteria and methanogens in the phenol-degrading granules was analyzed by FISH using oligonucleotide probe EUB338 targeting *Bacteria* and ARC915 targeting *Archaea*. EUB338 was labeled in the 5′-end with Cy3 while ARC915 was labeled with 6-carboxy-fluorescein (FAM) by Integrated DNA Technologies (Coralville, Iowa).

Samples of UASB granules were washed with a pH 7.2 phosphate-buffered saline solution (PBS; 0.13 M NaCl, plus 10 mM Na_2HPO_4), fixed in PBS containing 4% (w/v)

paraformaldehyde at 4°C overnight, and washed three times with PBS. The fixed granular sludge was subjected to five cycles of freeze-and-thaw (from –80°C to +40°C) to improve the penetration efficiency of oligonucleotide probes (Sekiguchi *et al.*, 1999). The fixed sample was then embedded with OCT compound (Miles, Elkhart, IN, USA), and sectioned into 20 µm slices with a rotary cryo-microtome (Leica CM1510, Leica). The cross-sections of sludge granules were collected on the wells of the glass slide coated by 0.01% poly-L-lysine solution and air-dried. The cryo-sectioned samples were then dehydrated by sequentially immersing them in 50%, 80% and 96% ethanol solutions for 3 min each. After air drying at room temperature, hybridization was conducted following the established method. The formamide concentration for the combination of EUB338/ARC915 was 20%. The hybridization image was captured using a Zeiss LSM 5 Pascal laser scanning confocal microscope (Zeiss, Jena, Germany) with the multi-track mode, which excited the sample sequentially with a laser at 488 nm and 543 nm.

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Results and discussion

Continuous phenol degradation in a UASB reactor

Throughout the study, phenol was the sole organic substrate. The effluent contained only residual phenol without any detectable intermediates, such as benzoate or volatile fatty acids. Table 1 summarizes the operating conditions, phenol concentrations in wastewater and the effluent, and the removal efficiency of the UASB treatment at room temperature. It shows that there was no detectable phenol in the effluent in the first 91 days during which the phenol concentration was step increased from 300 mg/l to 1,260 mg/l, HRT was shortened from 48 h to 24 h, corresponding to an increase of COD loading rate from 0.4 to 3.0 g-COD/(l·day). During days 61–439, the phenol concentration was kept at 1,260 mg/l with changing HRT. Table 1 shows that the phenol removal was lowered slightly from 100% at 24 h of HRT to 95% at 12 h during days 118–135. It further decreased to as low as 33%, due to the bioactivity inhibition by the increased phenol concentration in the mixed liquor, when HRT was shortened stepwise to 4 h during day 165–170. An effort was then made to recover by gradually increasing the HRT back to 12 h. This resulted in a slow recovery of phenol removal efficiency back to 94–98% during days 232–439. The removal efficiency dropped again to 78% when phenol concentration was raised to 1,470 mg/l while keeping HRT at 12 hr during days 440–493. But, it recovered to 98% at the end of the experiment when the phenol concentration returned to 1,260 mg/l and a HRT of 12 h.

Overall, results in Table 1 show that 98% of phenol at a concentration as high as 1,260 mg/l could be effectively degraded in a UASB reactor at 26°C with 12 h of HRT, corresponding to 6.0 g-COD/(l·day). Increasing loading above 6.0 g-COD/(l·day), either by increasing the phenol concentration or by lowering the HRT, resulted in deterioration of removal efficiency. Similar observations were reported for phenol degradation at 37°C (Fang *et al.*, 1996).

Degradation of phenol in batch reactors

Figure 1 illustrates the decrease of phenol concentration in the mixed liquors of batches with various starting phenol concentrations, from 400 to 2,200 mg/l. It shows that the time of complete phenol degradation increased with time. It took over 20 days for the complete degradation for the batch started with 1,800 mg/l of phenol. Figure 1 shows that about 50% of phenol was degraded after 24 days for the batch started with 2,000 mg/l of phenol; the remaining phenol took another 30 days to be degraded. However, there was no phenol degradation at all for the batch started with 2,200 mg/l of phenol. This shows that phenol-degrading bioactivity was completely suppressed at 2,200 mg/l. Figure 1 further illustrates that degradation of concentrated phenol was considerably slower when the mixed liquor

concentration remained over 1,000 mg/l; the degradation rate accelerated substantially when the phenol concentration in the mixed liquor dropped below 1,000 mg/l. Using the batch with the initial phenol concentration of 1,800 mg/l as an example, the maximum degradation rate was 72 mg/(l·day) when the phenol concentration was over 1,000 mg/l, but it increased to 155 mg/(l·day). The corresponding rates for the batch with the initial phenol concentration of 1,400 mg/l were 94 and 177 mg/(l·day).

Figure 2 illustrates the mixed liquor concentrations of phenol and benzoate, and methane in biogas in the batch of degrading phenol with an initial concentration of 2,000 mg/l. It shows that during the initial stage nearly all the phenol was converted to benzoate with little production of methane. The benzoate accumulation slowed down when the phenol concentration in the mixed liquor dropped below 1,000 mg/l, accompanied by a substantial increase of methane production. Benzoate reached the maximum concentration of 1,040 mg/l, when phenol reached 343 mg/l in the mixed liquor, and decreased rapidly thereafter.

Results in Figure 2 reveal two key characteristics of phenol degradation. Firstly, benzoate was the key intermediate of anaerobic phenol degradation. Secondly, phenol at a concentration over 1,000 mg/l severely suppressed the benzoate conversion to methane. The benzoate degradation rate increased substantially when the phenol concentration dropped below 343 mg/l. Similar observations were also found in batches degrading phenol with initial concentrations of 1,200, 1,400, 1,600 and 1,800 mg/l.

Table 1 Operation conditions and phenol removal efficiency at 26°C

| Day	HRT (hours)	Phenol in feed (mg/l)	COD loading (g-COD/(l·day))	Phenol removal (%)
1–60	48	300–840	0.4–2.4	100
61–79	40	1,260	1.8	100
80–91	24	1,260	3.0	100
92–117	16	1,260	4.5	97
118–135	12	1,260	6.0	95
136–153	8	1,260	9.0	89
154–164	6	1,260	12.0	77
165–170	4	1,260	18.0	33
171–198	7	1,260	10.3	70
199–231	8	1,260	9.0	70
232–323	12	1,260	6.0	88
324–371	12	1,260	6.0	94
372–413	15	1,260	4.8	98
414–439	12	1,260	6.0	96
440–460	12	1,470	7.0	78
461–493	24	1,470	3.5	100
493–512	12	1,260	6.0	98
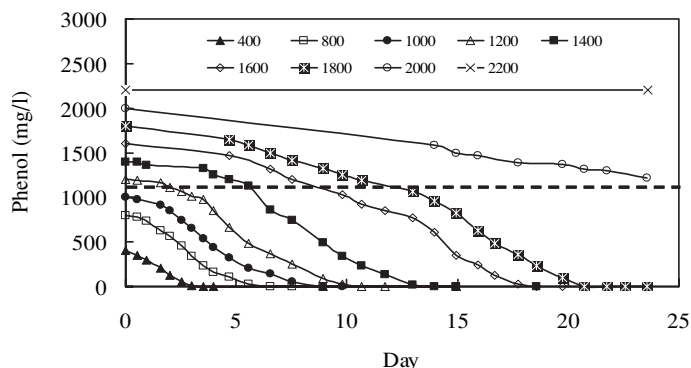
Figure 1 Degradation of phenol at various initial concentrations in batch reactors
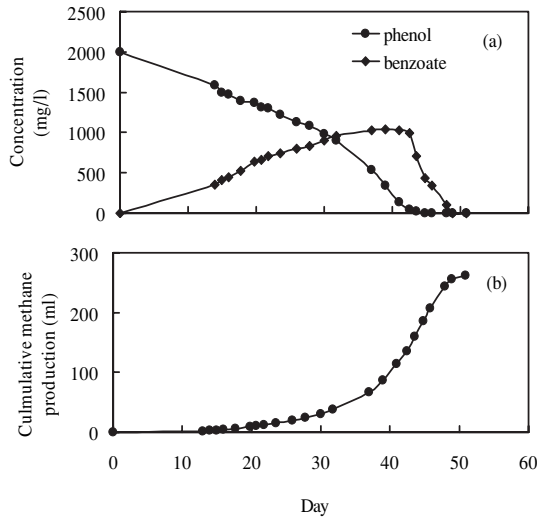
Figure 2 Batch degradation of phenol with initial concentration of 2,000 mg/l: (a) phenol and benzoate concentration, and (b) methane production

Figure 3 illustrates that the specific methane production activity (SMA) of phenol increases with phenol concentration. The maximum SMA was 0.18–0.19 g-COD/(g-VSS·day) for phenol concentrations in the range 1,200–2,000 mg/l.

Microbial population analysis

Figure 4 illustrates the DGGE profiles of microbial community of the phenol-degrading sludges sampled at days 14, 117, 135 and 153 when the reactor was operated at the respective HRTs of 48, 16, 12 and 8 h, and loading rates of 0.4, 4.5, 6.0 and 9.0 g-COD/(l·day). The number of detectable bands decreased from 16 bands for the day 14 sample to 9 bands for day 117 and 135 samples, to only 6 bands for the day 153 sample. This indicates that microbial diversity was decreased with the increase of loading rate.

Figure 5 illustrates the phylogenetic tree of the microbial community reported in previous studies (Sekiguchi *et al.*, 1998; Wu *et al.*, 2001a,b; Liu *et al.*, 2002) and those of the phenol-degrading sludge found in this study. It shows that four groups that had been identified in other UASB sludges were also found in phenol-degrading sludge. It is likely that *Desulfotomaculum* and *Clostridium* converted phenol into benzoate (Letowski *et al.*, 2001), which was then further degraded by *Syntrophus* (Li *et al.*, 1995) to acetate and H_2/CO_2. *Methanogens* lastly converted acetate and H_2/CO_2 into methane. However, the
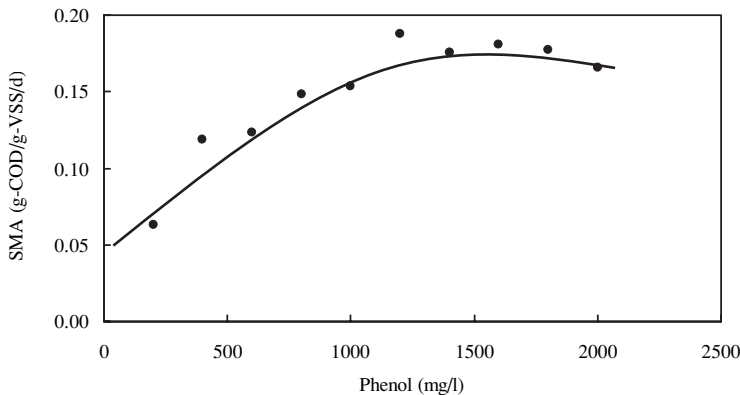

Figure 3 SMA of phenol degradation

40%   60%

d14;   HRT 48h

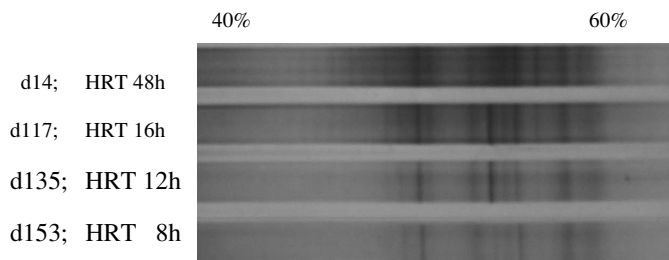d117;   HRT 16h

d135;   HRT 12h

d153;   HRT 8h

Figure 4 The DGGE images for *Bacteria* species in UASB phenol-degrading from day 14 to day 153 at four HRTs
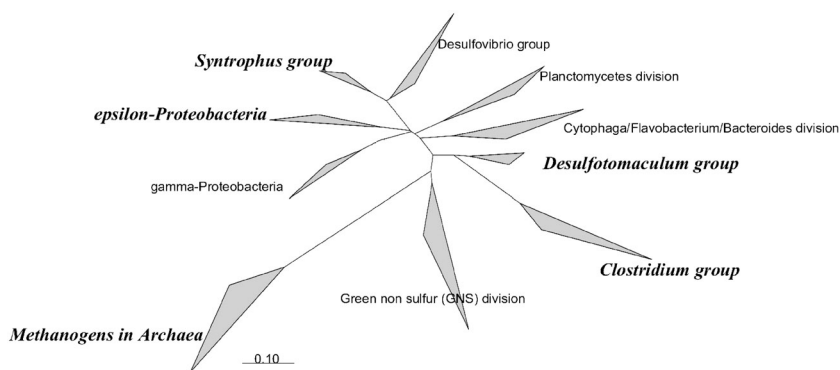

Figure 5 Phylogenetic tree of microbial groups found in various UASB granular sludges. (Those in bold-italic are detected in the phenol-degrading granules of this study.)

role of epsilon-*Proteobacteria,* which was only found in the phenol-degrading sludge of this study, was unclear.

FISH profiles of phenol-degrading granules

Figure 6 illustrates the FISH images of a phenol-degrading granule sampled on day 135, when the reactor was operated with 12 h of HRT and a loading rate of 6.0 g-COD/(l·day). The granule was simultaneously hybridized with the EUB338, a *Bacteria* domain specific probe, labeled with Cy3 (Figures 6a and 6c) and the ARC915, an *Archaea* domain specific probe, labeled with FAM (Figures 6b and 6d). Comparison of the cross-section images of the granule in Figures 6a and 6b shows that the granule comprised more methanogenic *Archaea* (74.1±8.5%) than *Bacteria* (27.3±5.8%). This is different from the terephthalate-degrading granular sludge, in which bacterial population was 52.9±5.4% of the microbial community (Wu *et al*., 2001b). Furthermore, Figures 6a and 6b illustrate that phenol-degrading sludge did not have a layered microstructure. This is consistent with previous observations of UASB granules degrading recalcitrant substrates (Fang, 2000).

Comparison of close-up images of the same granule in Figures 6b and 6c shows that both *Bacteria* and *Archaea* formed separate clusters. The archaeal clusters were significantly larger than the bacterial ones. This is similar to those observed in the terephthalate-degrading granular sludge (Wu *et al*., 2001b), but different from those of the sludge treating brewery wastewater (Liu *et al*., 2002) and those degrading yeast extract and a mixture of sucrose, acetate, propionate, and peptone (Sekiguchi *et al*., 1999). Although the discrepancy could be attributed to the substrate characteristics and degradation mechanisms (Wu *et al*., 2001b), further investigation is warranted.
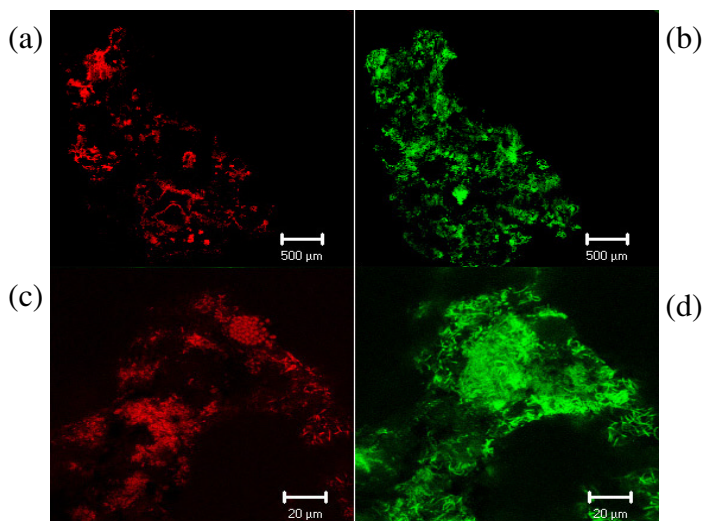
Figure 6 FISH images of a cross-section of phenol-degrading granular sludge: distributions of bacteria (a) and methanogens (b) in the granule; and distribution of bacteria (c) and methanogens (d) in close up

Conclusions

UASB reactor was able to remove over 98% of phenol at a loading rate of 6.0 g-COD/(l·day) treating a wastewater containing 1,260 mg/l of phenol at 26°C with 12 h of HRT and a recycle ratio of 1:1. Increasing loading rate above 6.0 g-COD/(l·day), either by increasing phenol concentration in wastewater or by shortening the HRT, resulted in drastic deterioration of phenol removal efficiency. Results of batch tests showed that benzoate was the key intermediate of phenol degradation. Conversion of benzoate to methane was suppressed by the presence of phenol. Based on DNA cloning analysis, the sludge was composed of five groups of microorganisms. *Desulfotomaculum* and *Clostridium* were likely to be responsible for the conversion of phenol to benzoate, which was further degraded by *Syntrophus* to acetate and H_2/CO_2. *Methanogens* lastly converted acetate and H_2/CO_2 to methane. The role of epsilon-*Proteobacteria* was, however, unclear.

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References

Blum, D.I.J., Hergenroeder, R., Parkin, G.F. and Speece, R.E. (1986). Anaerobic treatment of coal conversion wastewater constituents: biodegradability and toxicity. *J. Wat. Poll. Control Fed.*, **58**, 2122–2131.

Cross, W.H., Chian, E.S.K., Pohland, F.G., Harper, S., Kharkar, S. and Lu, F. (1982). Anaerobic biological treatment of coal gasification effluent. *Proc. Biotechnol. Bioengng. Symp.*, **12**, 349–363.

Dolfing, J. and Mulder, J.W. (1985). Comparison of methane production rate and coenzyme F_{420} content of methanogenic consortia in anaerobic granular sludge. *Appl. Environ. Microbiol.*, **49**, 1142–1145.

Fang H. P. (2000) Microstructure of UASB granules and its resulting effects. *Wat. Sci. Technol.*, **42**(12), 201–208.

Fang, H.H.P., Chen, T., Li, Y.Y. and Chui, H.K. (1996). Degradation of phenol in wastewater in an upflow anaerobic sludge blanket reactor. *Wat. Res.*, **30**, 1353–1360.

Fang, H.H.P. and Zhou, G.M. (2000) Degradation of phenol and p-cresol in UASB reactors. *Wat. Sci. Tech.*, **42**(5–6), 237–244.

Fang, H.H.P., Zhang, T. and Liu, H. (2002). Microbial diversity of a mesophilic hydrogen-producing sludge. *Appl. Microbiol. Biotechnol.*, **58**, 112–118.

Kirk-Othmer (1978). *Encyclopedia of Chemical Technology*. 3rd edn. Wiley Interscience, New York.

Letowski, J., Juteau, P., Villemur, R., Duckett, M.F., Beaudet, R., Lepine, F. and Bisaillon, J.G. (2001). Separation of a phenol carboxylating organism from a two-member strict anaerobic co-culture. *Can. J. Microbiol.*, **47**, 373–381.

Lettinga, G., van Velsen, A.F.M., Hobma, S.M., de Zeeuw, W. and Klapwijk, A. (1980). Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment. *Biotechnol. Bioengng.*, **22**, 699–734.

Li, Y.Y., Fang, H.H.P., Chui, H.K. and Chen, T. (1995). UASB treatment of wastewater with concentrated benzoate. *J. Environ. Engng.*, **121**, 748–751.

Liu, W.T., Chan, O.C. and Fang, H.H.P. (2002). Characterization of microbial community in granular sludge treating brewery wastewater. *Wat. Res.*, **36**, 1767–1775.

Owen, W.F., Stuckey, D.C., Healy, J.B., Young, L.Y. and McCarty, P.L. (1979). Bioassay for monitoring biochemical methane potential and anaerobic toxicity. *Wat. Res.*, **13**, 485–492.

Sekiguchi, Y., Kamagata, Y., Syutsubo, K., Ohashi, A., Harada, H. and Nakamura, K. (1998). Phylogenetic diversity of mesophilic and thermophilic granular sludges determined by 16S rRNA gene analysis. *Microbiol.*, **144**, 2655–2665.

Sekiguchi, Y., Kamagata, Y., Nakamura, K., Ohashi, A. and Harada, H. (1999). Fluorescence in situ hybridization using 16S rRNA-targeted oligonucleotides reveals localization of methanogens and selected uncultured bacteria in mesophilic and thermophilic sludge granules. *Appl. Environ. Microbiol.*, **65**, 1280–1288.

Strunk, O. and Ludwig, W. (1996). ARB: A software environment for sequence data. Technische Universität München, Germany.

Wang, Y.T., Suidan, M.T. and Rittman, B.E. (1986). Anaerobic treatment of phenol by an expanded-bed reactor. *J. Wat. Poll. Control Fed.*, **58**, 227–233.

Wu, J.H., Liu, W.T., Tseng, I.C. and Cheng, S.S. (2001a). Characterization of a 4-methylbenzoate-degrading methanogenic consortium as determined by small-subunit rDNA sequence analysis. *J. Biosci. Bioengng.*, **91**, 449–455.

Wu, J.H., Liu, W.T., Tseng, I.C. and Cheng, S.S. (2001b). Characterization of microbial consortia in a terephthalate-degrading anaerobic granular sludge system. *Microbiol.*, **147**, 373–382.

Young, L.Y. and Rivera, M.D. (1985) Methanogenic degradation of four phenolic compounds. *Wat. Res.*, **19**, 1325–1332.

Zhang, T. and Fang, H.H.P. (2000). Digitization of DGGE (denaturing gradient gel electrophoresis) profile and cluster analysis of microbial communities. *Biotechnol. Lett.*, **22**(5), 399–405.

Zhang, T. and Fang, H.H.P. (2001). Phylogenetic diversity of a SRB-rich marine biofilm. *Appl. Microbiol. Biotechnol.*, **57**, 437–440.