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Fate of Branched-Chain Fatty Acids in Anaerobic Environment of River Sediment

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ABSTRACT

The fate of six different branched-chain fatty acids (BCFAs) in an anaerobic environment of a river sediment was studied in vitro by culturing enrichment consortia. The anaerobic consortium of BCFA-degrading genus degraded BCFAs with tertiary carbons through β-oxidation, followed by methanogenesis by methane-producing anaerobic bacteria. The consortium could not degrade BCFAs with quaternary carbon. Degree of branching at the alpha or beta position along the carbon chain interfered with the beta-oxidation mechanisms of the branched-chain fatty acid.

Index Entries: Anaerobic consortium; β-oxidation; branched-chain fatty acid; river sediment.

INTRODUCTION

Branched-chain fatty acids (BCFAs) were first isolated from the preen gland waxes of birds, degras, and animal fats. BCFAs were also found as the constituents of various bacteria, namely *Sarcina* sp. and *Bacillus* sp. *(1)*. These compounds are of natural lipid origins that have single-methyl substitutions at up to four separate positions along the carbon (C) chain. In recent years, increased industrial applications of synthetic BCFAs and the somewhat uncertain degradation mechanisms have drawn much attention *(2–4)*. A number of BCFAs are produced as intermediate products through degradation of certain industrial wastes or directly discharged in other industrial effluents. For instance, 2-methylbutanoic acid (2-MBuA) and 3-methylbutanoic acid (3-MBuA) are produced through anaerobic degradation of a number of common amino acids, namely leucine, isoleucine, and valine *(5)*. On the other hand, xenobiotic BCFAs, such as 2,2,-

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dimethylpropanoic acid (2,2-DMPrA) and 2-ethylhexanoic acid (2-EHeA) are discharged in pharmaceutical wastewaters *(3,6)*.

There has been much controversy about biological degradability and mechanisms of BCFAs degradation. A number of synthetic BCFAs were believed to be persistent in microbial ecosystems *(3)*. In particular, 2,2-DMPrA and 2-EHeA in pharmaceutical wastewaters have been reported to be persistent in on-site aerobic effluent treatment facilities comprised of trickling filters, activated sludge basins, secondary clarifiers, and sludge return *(7)*. These compounds are not readily acclimatized by the microorganisms in biological processes of on-site pretreatment facilities and municipal sewage treatment works, and are discharged largely undegraded to the receiving water bodies. McInerney et al. *(8,9)* reported that an anaerobic bacterial consortium, which degraded straight-chain fatty acids up to C_8, could not degrade the branched-chain 2-MBuA. On the contrary, recent studies by Chua et al. *(10,11)*, Yap et al. *(12)*, and Jimeno et al. *(4)* showed that BCFAs were degradable in anaerobic filters under specific conditions. Although β-oxidation is widely accepted as the mechanism in biodegradation of straight-chain fatty acids *(13)*, the mechanisms in biodegradation of BCFAs are uncertain. Richardson et al. *(14)* isolated 2-MBuA-degrading cultures, composed of an obligate syntroph and methanogens, but the degradation mechanism was not described. β-oxidation was first assumed to be the mechanism of anaerobic degradation of 2-EHeA, and an anaerobic process treating a synthetic wastewater bearing 2-EHeA was mathematically modeled and verified *(15)*.

In this paper, the fate of six selected BCFAs in the anaerobic environment of a river sediment are investigated. In vitro investigation of the anaerobic microbial population, degradation mechanisms and the effect of branching on biological degradability are reported.

MATERIALS AND METHODS

Branched-Chain Fatty Acids

Six different BCFAs were separately used as the sole C source in enrichment cultures 1–6 (Table 1). 2-Ethylhexanoic acid (2-EHeA) represented BCFAs with an even number of C in the main chain and a branching at the alpha position. 2-Ethylpentanoic acid (2-EPeA) represented BCFAs with an odd number of C in the main chain and a branching at the alpha position. 3-Ethylhexanoic acid (3-EHeA) and 3-ethylpentanoic acid represented BCFAs with a branching at the beta position. All four of these BCFAs had a tertiary C. 2,2-Diethylhexanoic acid (2,2-DEHeA) represented BCFAs with two branchings at the alpha position; 3,3-diethylhexanoic acid (3,3-DEHeA) represented BCFAs with two branchings at the beta position, all of which had a quaternary C.

Table 1
Branched-Chain Fatty Acids in Enrichment Consortia

Culture number	Carbon source	Structural formula
1	2-Ethylhexanoic acid (2-EHeA)	$CH_3—CH_2—CH_2—CH_2—CH(C_2H_5)—COOH$
2	2-Ethylpentanoic acid (2-EPeA)	CH_3-CH_2-CH_2-$CH(C_2H_5)$-COOH
3	3-Ethylhexanoic acid (3-EHeA)	CH_3-CH_2-CH_2-$CH(C_2H_5)$-CH_2-COOH
4	3-Ethylpentanoic acid (3-EPeA)	CH_3-CH_2-$CH(C_2H_5)$-CH_2-COOH
5	2,2-Diethylhexanoic acid (2,2-DEHeA)	CH_3-CH_2-CH_2-CH_2-$C(C_2H_5)_2$-COOH
6	3,3-Diethylhexanoic acid (3,3-DEHeA)	CH_3-CH_2-CH_2-$C(C_2H_5)_2$-CH_2-COOH

Enrichment Consortia

A series of enrichment consortia were used to study the bacterial populations and the degradation of BCFAs in an anaerobic environment. The enrichment consortia medium was prepared in six 100-mL serum bottles, with the following formulation, in g/L: NH_4Cl, 0.0159; KH_2PO_4, 0.0037; $MgSO_4 \cdot 7H_2O$, 0.0200; $FeCl_3$, 0.0284; $MnCl_2 \cdot 2H_2O$, 0.003; $Al_2(SO_4)_3 \cdot 18H_2O$, 0.0022; $CaCl_2$, 0.0400; $CoCl_2 \cdot 6H_2O$, 0.0080; $NaSiO_3 \cdot 5H_2O$, 0.0040; H_3BO_3, 0.0040; $ZnSO_4 \cdot 7H_2O$, 0.0020; $CuSO_4 \cdot 5H_2O$, 0.0020; $(NH_4)_2MoO_4$, 0.0020; thiamine hydrogen chloride, 0.0080. Each of the six serum bottles was added with a different BCFA (analytical reagents, Fluka Chemie AG), to an initial concentration of 16 mmol/L. The BCFA acted as the sole C source in each bottle.

The bottles were then inoculated with 3 g of soft clay taken from a deep river sediment. This resulted in initial cell densities between 10^4 and 10^5 cells/mL in the culture medium. The inoculated consortia were maintained at 35°C. The entire procedure for preparing the enrichment consortia was carried out in the oxygen-free environment of an anaerobic chamber (Forma Scientific Model 1029). Redox potentials were not measured.

Bacterial Observation and Enumeration

The enrichment consortia were periodically sampled for observations of the bacterial populations by scanning electron microscopic techniques. One-mL sample from the enrichment consortia was filtered through a Nuclepore (13 mm × 0.4 μ) cellulose nitrate membrane, which was pre wetted with Triton-X 100 surfactant to ensure uniform distribution of bacteria on the membrane. The techniques were similar to that described by

Drier et al. *(16)*. The membrane with the fixed bacterial sample was coated with a 25-nm layer of gold-palladium mixture (Joel Fine-Coat Ion Sputter Type JFC-1100) and observed with a scanning electron microscope (Joel JSM-T220A) at 10 kV accelerating voltage and × 5 000–20,000 magnification. The bacterial cell density was calculated as $X (A_1/A_2)/v$, where X was the number of cells seen on the micrograph, A_1 and A_2 were the areas of filter membrane and field of micrograph, respectively, and v was the volume of sample.

Fluorescence microscopic techniques used for bacterial identification were similar to that described by Birk *(17)*. A Leitz Ortholux 2 microscope, with 4-Lambda Ploem Opak for incident light fluorescence excitation, 250s mirror house, and 250 lamp house were used.

Analytical Methods

The enrichment consortia were also periodically sampled for analysis of BCFA concentrations and intermediate volatile fatty acid (VFA) concentrations by a gas chromatograph (Shimadzu Model GC-14A) with a Chromosorb WAW 100/120 mesh (FFAP 15% and H_3PO_4 1%) column. A sample size of 3 μL was analyzed, and nitrogen (high-purity grade) at a flow rate of 20 mL/min was used as the carrier gas. Biogas quality was examined using a gas chromatograph (Varian Model 3300) with a 2 m Porapak Q 80/100 mesh column. A sample size of 0.5 mL was analyzed, and helium (high-purity grade) at a flow rate of 30 mL/min was used as the carrier gas.

RESULTS AND DISCUSSION

The anaerobic bacteria in enrichment consortia numbers 1–4 were similar and were composed of three morphologically distinctive species (Fig. 1): curved rods with rounded ends (0.3–2.0 μ in diameter and 1.5–5.0 μ in length); rods and filaments with distinctive truncated ends (0.3–0.8 μ in diameter and 3–15 μ in length); and cocci (0.5–1.2 μ in diameter), which autofluoresced when excited at 420 nm. The obligate syntrophic behavior between the first and third species in interspecies hydrogen transfer and the fluorescent property of the cocci, because of the presence of intracellular coenzyme F_{420} for electron transfer in the H_2 reduction of carbon dioxide, were previously described in detail by Chua et al. *(2)*. The three species were respectively identified as the BCFA-degrading and VFA-producings *Syntrophomonas* sp., decarboxylating *Methanothrix* sp., and H_2-utilizing *Methanococcus* sp.

Figure 2 shows the concentration profiles of fatty acids in the 2-EHeA enrichment consortia. Presence of butanoic and ethanoic acids in the 2-EHeA enrichment suggested that 2-EHeA was β-oxidized to butanoic acid (butyric acid) by a cleavage between the α- and β-C along the main chain of the 2-EHeA molecule (Fig. 3). Cleavage at the ethyl side chain

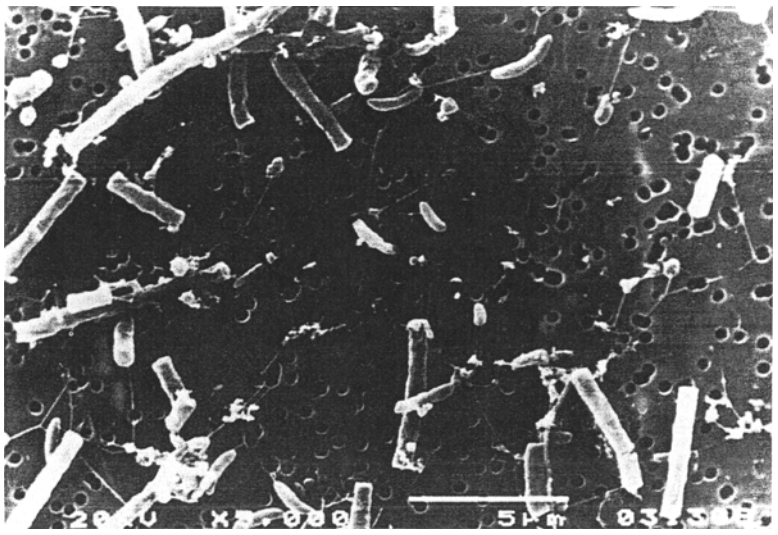


Fig. 1. Scanning electron micrograph of enrichment (20KV, ×5000).

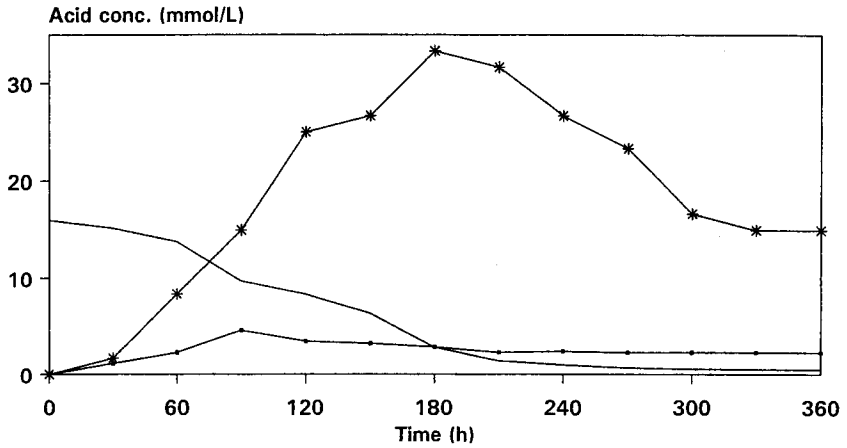


Fig. 2. Fatty acid concentration profiles in 2-EHeA enrichment: 2-EHeA (—), Butanoic acid (—■—), and Ethanoic acid (—✳—).

would have otherwise produced hexanoic acid as an intermediate product. The overall 2-EHeA degradation mechanism is shown in Fig. 4. Butanoic acid produced was further β-oxidized to ethanoic acid. Stoichiometrically, degradation of each mole of 2-EHeA should have generated 2 mol of butanoic acid and 4 mol of ethanoic acid (acetic acid) as the intermediate VFAs. However, the generation of VFAs and their subsequent degradation into final products, namely methane and carbon dioxide, were in a dynamic equilibrium. These resulted in the VFA concentrations in the culture medium being lower than the theoretical values calculated from chemical

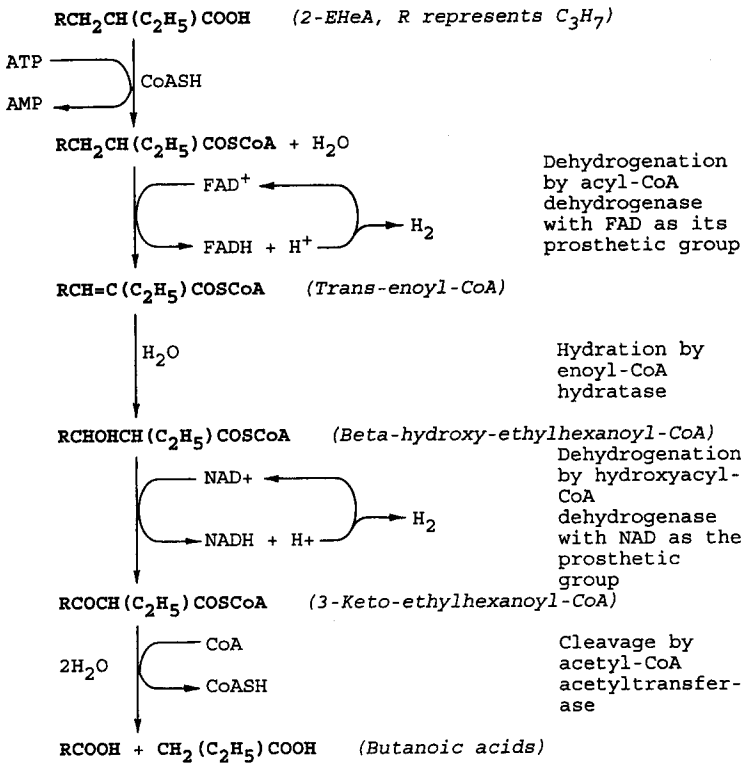


Fig. 3. Beta-oxidation of 2-EHeA.

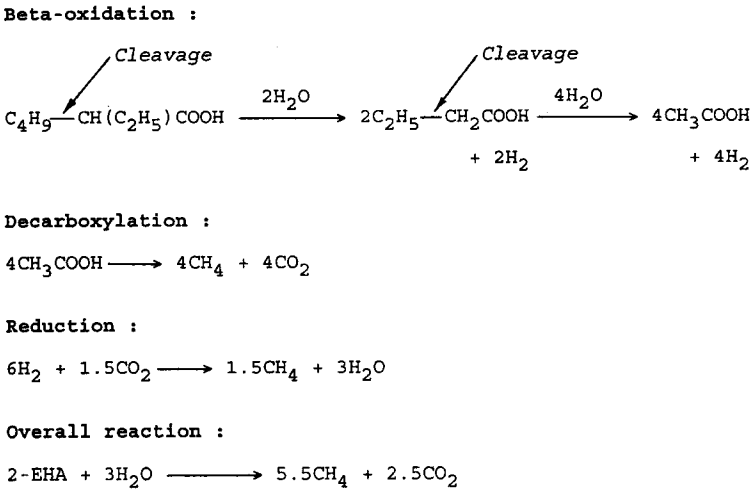


Fig. 4. Mechanism of 2-EHeA degradation.

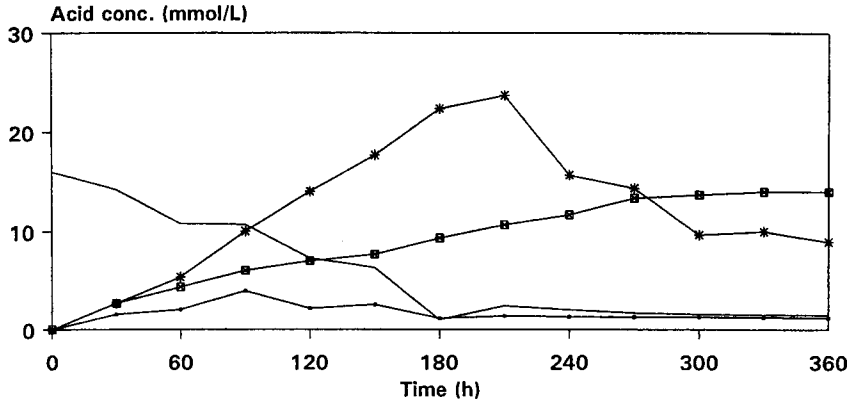
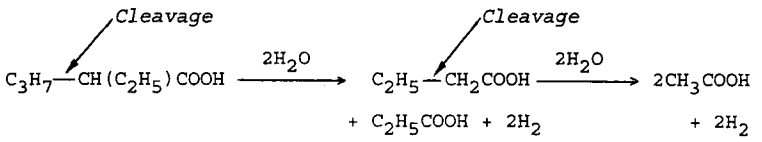


Fig. 5. Fatty acid concentration profiles in 2-EPeA enrichment: 2-EHeA (—), Butanoic acid (—■—), Propanoic acid (—□—), and Ethanoic acid (—✳—).

Beta-oxidation :



Decarboxylation :

$$2CH_3COOH \longrightarrow 2CH_4 + 2CO_2$$

Reduction :

$$4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O$$

Overall reaction :

$$2\text{-EPeA} + 2H_2O \longrightarrow 3CH_4 + CO_2$$

Fig. 6. Mechanism of 2-EPeA degradation.

reaction stoichiometry (Fig. 2). Because about 16 mmol/L of 2-EHeA was degraded in the enrichment culture, a maximum of only 4 mmol/L of butanoic acid and 33 mmol/L of ethanoic acid were detected in the culture medium. Methane and carbon dioxide were detected at between 3:1 and 2:1 mol ratios in the head space of the enrichment bottle.

Figure 5 shows the concentration profiles of fatty acids in the 2-EPeA enrichment consortia. The concentration profiles of 2-EPeA, butanoic, and ethanoic acids were similar to that in the 2-EHeA consortia. In addition, an accumulation of propanoic acid in the culture medium was also observed. This suggested that 2-EPeA was β-oxidized to butanoic and propanoic acids by cleavages between the α- and and β-C along the main chain of the 2-EPeA molecule (Fig. 6). Consequently, the butanoic and ethanoic

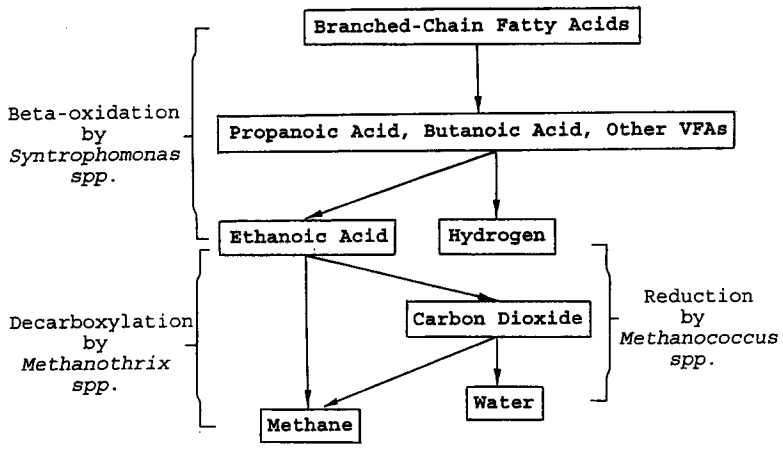


Fig. 7. Fate of branched-chain fatty acids in anaerobic environment.

acid concentrations in the culture medium were lower than that observed in 2-EHeA consortia. Butanoic acid produced was further β-oxidized to ethanoic acid; propanoic acid remained largely undergraded by the anaerobic consortium. *Syntrophomonas* sp. in the consortia could not effectively utilize propanoic acid as a C source. Stoichiometrically, degradation of 2-EPeA and generation of propanoic acid were in a one-to-one ratio, which agreed with the profiles observed in Fig. 5. As 16 mmol/L of 2-EPeA was degraded in the enrichment consortia, a maximum of about 15 mmol/L of propanoic acid was detected in the culture medium.

The degradation of 3-EHeA and 3-EPeA showed similar patterns. In the 3-EHeA culture medium, hexanoic, butanoic, and ethanoic acids were detected as the intermediate VFAs. In the 3-EPeA culture medium, pentanoic, propanoic, and ethanoic acids were detected as the intermediate VFAs. Produced propanoic acid accumulated in the culture medium without being further degraded. These observations agreed with the degradation pathway proposed in Figs. 4 and 6. The fate of the 4 BCFAs with a tertiary C in an anaerobic environment and the roles of various bacterial groups are as summarized in Fig. 7. BCFAs were β-oxidized by the *Syntrophomonas* sp., via intermediate VFAs, to ethanoic acid with concomitant H_2 production. Ethanoic acid was decarboxylated by the *Methanothrix* sp. to CH_4 and CO_2; H_2 was utilized by the *Methanococcus* sp. to reduce CO_2 to CH_4.

The maximum cell densities in enrichment consortia 1 to 4, using 2-EHeA, 2-EPeA, 3-EHeA, and 3-EPeA, respectively, as the sole C, ranged between 4.9 and 5.9 $\times$ 10^5 cells/mL (Table 2). These values were more than an order lower than that in the enrichment consortia on similar BCFAs and inoculated with anaerobic biofilms taken from a BCFA-degrading biofilter (2). The maximum degradation rates between 5.0 and 8.5 $\times$ 10^{-3} mmol/h in enrichment consortia 1 to 4 were also about an order lower

Table 2
Cell Densities and Degradation Rates

Enrichment cultures	Maximum cell density[a] ($\times 10^5$ cells/mL)	Maximum degradation rate ($\times 10^{-3}$ mmol/h)	Maximum specific degradation rate[b] ($\times 10^{-10}$ mmol/h-cell)
2-EHeA	5.0	6.5	1.335
2-EPeA	5.9	8.5	1.410
3-EHeA	4.9	5.0	1.030
3-EPeA	5.7	6.0	1.095
2,2-DEHeA	1.2	0.4	0.378
3,3-DEHeA	1.2	0.3	0.233

[a] Highest cell count observed in each enrichment consortia.
[b] Calculated based on the maximum degradation rate divided by the maximum total number of cells.

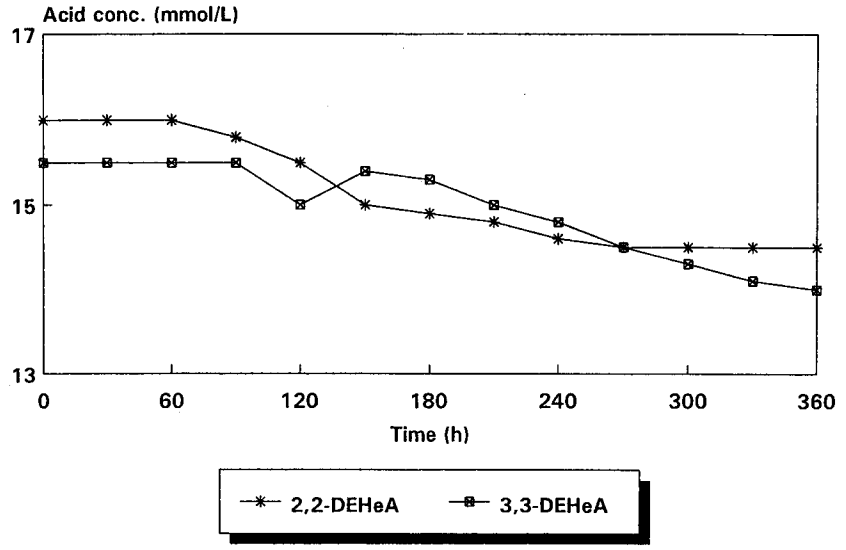


Fig. 8. Fatty acid concentration profiles in 2,2-DEHeA and 3,3-DEHeA enrichments. 2,2-DEHeA (—✳—) and 3,3-DEHeA (—□—).

than that reported by Chua et al. *(2)*. These results agree with the findings of Jimeno et al. *(4)* and Richardson et al. *(14)* that persistent BCFAs with a tertiary C could be degraded, although at slow degradation rates, by anaerobic consortia isolated by enrichment techniques.

On the other hand, 2,2-DEHeA and 3,3-DEHeA in enrichment consortia 5 and 6 were not readily degradable by, and could hardly support, cell growth in the anaerobic consortia (Table 2). The initial acid concentrations of 16 mmol/L only decreased by less than 1.5 mmol/L during the 360-h culture (Fig. 8), which were equivalent to degradation rates that were an order lower than that in enrichment consortia 1–4.

CONCLUSIONS

In assessing the fate or biodegradability of BCFAs in the anaerobic environment of a river sediment, 2-EHeA, 2-EPeA, 3-EHeA, and 3-EPeA formed a class of persistent BCFAs. These BCFAs have alkyl substituent at the α- or β-C from the carboxylic end of the C chain, resulting in a tertiary C, thus differentiating the compounds from the natural lipid-origin anteiso fatty acids described by Smith *(1)*, which are substituted at the antepenultimate position (third C from the alkyl end). The substituents at the α or β-positions are believed to interfere with the dehydrogenation and cleaving mechanism in β-oxidation, thus slowing down the degradation rates.

2,2-DEHeA and 3,3-DEHeA, on the other hand, form another class of multiple-branching, recalcitrant BCFAs. These recalcitrant BCFAs are different from the those isolated from preen gland waxes, which have single-methyl substitutions at up to four separate positions in the C chain *(1)*. The recalcitrant BCFAs are substituted with two alkyl groups at the α- or β-positions, resulting in a quaternary C. The recalcitrance of these BCFAs was attributed to the presence of quarternary C, which rendered the dehydrogenation and cleavage by β-oxidation impossible.

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