Available online at www.sciencedirect.com
ScienceDirect
Journal of Environmental Sciences 20(2008) 536–542
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

Characterization of the fate of lipids in activated sludge

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Received 23 July 2007; revised 15 October 2007; accepted 16 November 2007

Abstract

Experiments were carried out to characterize the transformation of lipids in activated sludge under aerobic conditions. Results showed that the overall lipid content in the effluent could not be reduced to values below 300 mg/L from an initial content of 2,000 mg/L. However, the contents of individual fatty acids underwent drastic decreases and increases during all microbial growth phases. These changes in contents of individual fatty acids showed that fatty acids were used as substrates by microorganisms as well as released into the wastewater as by-products. We have therefore suggested a novel model of transformation of lipids in activated sludge, showing that utilization of microbial activity for complete removal of lipids from wastewater is limited.

Key words: activated sludge; biodegradation; lipids/fatty acids; microbial by-products; wastewater treatment

Introduction

Although lipids (characterized as oils, greases, fats or long-chain fatty acids) are important organic components of municipal and industrial wastewater (Henze, 1992; Raunkjaer *et al*., 1994; Becker *et al*., 1999; Quemeneur and Marty, 1994), their exact behavior in wastewater treatment processes is not well understood. Lipids cause many problems in biological wastewater treatment systems, including sludge flotation and promotion of growth of filamentous microorganisms, which cause bulking and foaming (Jenkins, 1992; Duchene, 1994; Hwu *et al*., 1998; Andreasen and Nielsen, 1998).

Many methods have been used in an attempt to enhance the removal of lipids from wastewater. Hrudey (1981) studied the effects of emulsified lipids on activated sludge. Although Hrudey's study shows that activated sludge can efficiently remove lipids even at high lipid loadings, the study also shows that activated sludge process exhibits poor performance with increase in lipid-to-microorganisms ratio (lipid/MLSS; mixed liquor suspended solids). Young (1979) carried out studies by mixing activated sludge with lipids and the effluent biological oxygen demand (BOD) correlated with the amount of lipids added. The removal of lipids by activated sludge was equal or better than the BOD removal, suggesting that not only biodegradation occurred, but also adsorption of lipids to the biomass took place. The later causes the specific gravity of sludge to decrease, resulting in process failure (Chao and Yang, 1981). Becker *et al*. (1999) proposed that biological treatment of lipid-rich wastewater under thermophilic conditions (i.e., above 60°C) has some advantages, because both the diffusion coefficient and the solubility of lipids in aqueous media increase significantly with rising temperature. To show this advantage, Becker *et al*. (1999) studied the aerobic thermophilic degradation of olive oil using a pure culture of bacterial strain, *Bacillus thermoleovorans* IHI-91. They observed a lipid removal of more than 90% of the initial lipid concentration of 2 g/L at a residence time of 2 h. In subsequent experiments, they treated wool scouring wastewater, with 15–20 g/L of lipids, under aerobic conditions at 65°C using *B. thermoleovorans* IHI-91 as an inoculum. The lipid removal was only 20%–25% at a residence time of 10 h. Although these results show that aerobic thermophilic treatment of lipid-rich wastewater is possible, it is also clear that lipids present in industrial wastewater and common lipids such as olive oil respond differently to microbial degradation. They attributed the low degradation rates of lipids in industrial wastewater to negative effects of salinity and toxic materials on biological treatment process. Furthermore, Lefebvre *et al*. (1998) found that microbial growth on saponified lipid substrates followed an exponential growth pattern. However, complete removal of saponified lipids from the wastewater could not be achieved even though their biodegradation began as soon as they were mixed with activated sludge. To explain the cause of low degradation rate, Lefebvre and co-workers (1998) showed that the occurrence of foam caused the low degradation activity and, consequently, the low lipid removal efficiency.

Although the literature indicates that lipids are readily removed by activated sludge, effluents from wastewater treatment systems are reported to contain lipids (Chipasa and Mędrzycka, 2006; Strydom *et al.*, 1995; Barker and Stuckey, 1999; Dignac *et al*., 2000; Miron *et al*., 2000;

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Peira *et al*., 2002; Naidas *et al*., 2005). The presence of lipids in wastewater is still a challenge and new approaches are still required to fully understand the behavior of lipids in biological wastewater treatment processes. Studies aimed at characterizing the fate of lipids during biological treatment processes can give insight into understanding the source of residues of lipids in treated wastewater effluents. We hypothesized that the presence of lipids in wastewater effluents suggests two phenomena: (1) lipids pass through biological wastewater treatment systems or (2) lipids are produced by the activated sludge microorganisms. In this regard, the objective of this study was to examine the transformation of lipids/fatty acids in activated sludge. We used gas liquid chromatography (GLC) method to characterize fatty acid composition of lipids extracted from the influent as well as the effluent wastewater. Additionally, we monitored changes in oxygen utilization rate (OUR), lipid and MLSS contents to characterize the transformation of lipids in the wastewater.

1 Materials and methods

1.1 Source of microorganisms and growth medium

The activated sludge used in the experiment was collected from the biological reactors of the local municipal wastewater treatment plant (Gdansk, Poland). It was a mixture of activated sludge from all the biological reactors, which function according to the modified UCT process to enhance biological nutrient removal. A full description of this plant is given elsewhere (Chipasa, 2003). Activated sludge portions were washed several times with distilled water until the resulting supernatant was clear. The supernatant was discarded by decanting after sedimentation. Washing of activated sludge was necessary to reduce the amount of dissolved substances in the mixed liquor. Next, equal amounts (500 ml; MLSS, 1,200 ± 25 mg/L) of washed activated sludge were mixed with synthetic wastewater in bioreactors. The synthetic wastewater was composed of the following ingredients dissolved in distilled water: refined rapeseed oil (1 g/L), Tween 80 (1 g/L), $MgSO_4 \cdot 7H_2O$ (0.08 g/L), $NaNO_3$ (1 g/L), K_2HPO_4 (1.5 g/L), KH_2PO_4 (2 g/L), $NaHPO_4 \cdot 7H_2O$ (0.4 g/L), NH_4Cl (0.5 g/L), $(NH_4)_2SO_4$ (0.5 g/L) and $FeSO_4 \cdot 7H_2O$ (0.05 g/L); and trace minerals: $Na_2MoO_4 \cdot 2H_2O$ (0.025 g/L), $CaCl_2 \cdot 2H_2O$ (0.035 g/L), $CaSO_4 \cdot 7H_2O$ (0.02 g/L), $ZnSO_4 \cdot 5H_2O$ (0.015 g/L), $Fe_2(SO_4)_3$ (0.025 g/L) and $CuSO_4 \cdot 5H_2O$ (0.025 g/L). All the reagents were analytically pure. We obtained them from commercial sources (refined rapeseed oil–Olvit (Gdansk, Poland), Tween 80–Sigma-Aldrich (Munich, Germany), and all other reagents –POCH (Gliwice, Poland)). Refined rapeseed oil and Tween 80 (as an emulsifying agent) were the only added sources of carbon.

1.2 Bioreactor operation and sampling

In biodegradation tests, batch reactors are preferred because they are simple to operate and problems associated with reactor hydraulics are avoided (Kristensen *et al*., 1992). For these reasons, we used batch reactors in this experiment. Aerobic biodegradation tests were conducted in three 10-L plexiglass bioreactors each with a working volume of 5 L (Fig.1). We added 4500 ml of the prepared synthetic wastewater and 500 ml of washed activated sludge into each bioreactor. The bioreactors were fed with synthetic wastewater only once at mixing time (day 0). Air was continuously supplied at constant rate (1,500 cm^3/min) by using an aquarium-type pump and diffuser. The airflow rate was uncontrolled with respect to oxygen consumption requirements. The air movement also helped in keeping the contents of the bioreactors well mixed. The pH of the synthetic wastewater was not adjusted during the experiment. Therefore, any changes in pH and dissolved oxygen concentration were attributed to microbial activity. The bioreactors were covered with aluminum foil to prevent evaporation of water. The bioreactors were operated at ambient temperature (20±1°C) at sludge retention time (SRT) of 7 d. Samples were taken from the bioreactors for analysis daily. Before withdrawing samples for analysis, air supply was switched off and each bioreactor was thoroughly shaken by hand for a few seconds. Immediately, 50 ml of the mixed liquor were withdrawn for determination of biomass content (defined as mixed liquor suspended solids, MLSS) in accordance with Standard Methods (APHA *et al*., 1995). The contents of the bioreactors were then allowed to sediment before withdrawing 100 ml of the supernatant by using a pipette. Immediately, the supernatant was centrifuged for 15 min at 3,200 r/min to remove solid suspensions and microbial cells. All three bioreactors were run simultaneously and the results presented are mean values.

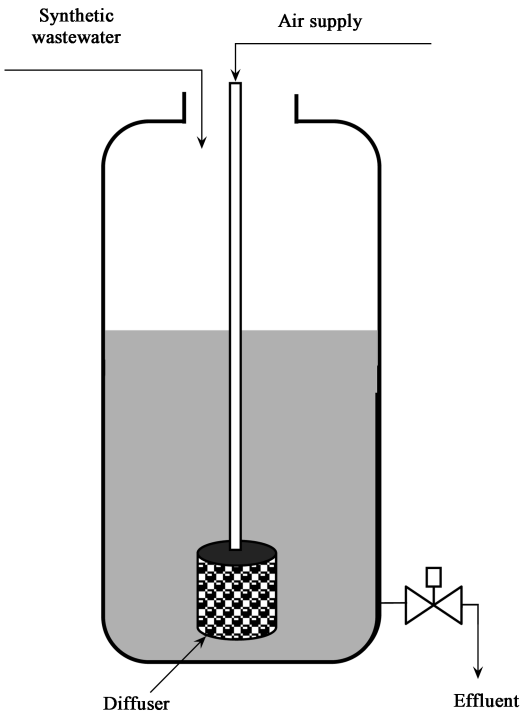

Fig. 1 Schematic illustration of the bioreactor.

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1.3 Determination of lipid content

The content of lipids was determined using the partition-gravimetric method (APHA *et al*., 1995), which depends on the extraction of dissolved or emulsified lipids from water by intimate contact with a solvent. We slightly modified the procedure. Briefly, 75 ml of the centrifuged samples were acidified to a pH of 2 with 1:1 HCl. Each acidified sample was then transferred to a separating funnel and 60 ml of chloroform was added. The mixture was then vigorously shaken for 5 min. Next, the mixture was allowed to separate and then the chloroform layer was drained through a funnel containing a filter paper and 10 g of anhydrous Na_2SO_4 into a clean pre-weighed flask. The remaining water layer was twice treated with 30 ml of chloroform. Later, the filter paper and Na_2SO_4 were rinsed with 20 ml of chloroform. Then all portions were combined in the flask. Chloroform was evaporated from the flask using a vacuum rotary evaporator and a glycerin bath at 65°C. To determine the content of lipid extracts, the flasks were weighed after cooling in desiccators. The gain in weight of each flask represented the weight of lipids, which we converted into mg of lipids/L.

1.4 Measurement of oxygen utilization rate

We measured OUR as described in method 2710 B of the Standard Methods (APHA *et al*., 1995) using an oxygen-sensing probe (Handy OxyGuard®, OxyGuard International, Birkerod, Denmark).

1.5 Determination of fatty acid compositions

The GLC method was used for the determination of the composition of fatty acids. After weighing the flasks, the lipid extracts were dissolved in chloroform and transferred into esterification ampoules. To avoid the oxidation of lipid extracts, chloroform was evaporated from the mixture using a moderate stream of nitrogen. We saponified and esterified the lipid extract samples as follows. Each ampoule containing lipid extracts was connected to an air cooler and inserted in a glycerin bath. Then 1 ml of 0.5 mol/L KOH in methanol was added and the sample was saponified at 80°C for 10 min. After the saponification step, the sample was esterified using 2 ml of 2% $SOCl_2$ in methanol at 80°C for 10 min. Next, the sample was allowed to cool at room temperature. After cooling, 2 ml of *n*-hexane was added to the sample and delicately agitated by tilting. When the sample separated into layers, a saturated solution of NaCl was added to the sample and gently mixed. The obtained fatty acid methyl esters (FAME) were in the top *n*-hexane layer.

Because lipid contents in the samples were different, it was necessary to obtain similar concentrations of FAME for each analyzed sample. Hence, *n*-hexane was partially evaporated using a moderate stream of nitrogen to regulate the concentration of the obtained FAME. Then 5 μl of *n*-hexane was withdrawn using a GLC syringe and injected into the GLC injector. The analysis was carried out using a Philips PYE Unicam PU 4550 gas chromatography equipped with a flame-ionization detector at 250°C, injector at 250°C, DB-23 capillary column (30 m × 0.25 mm, J & W Scientific) and an integrator (HP 3392A), which was programmed to start integrating after 2 min. Helium was used as the carrier gas. The oven temperature program was 60°C for 20 min, with a 20°C/min ramp for 5 min and a final hold at 180°C for 43 min. This procedure quantified short, medium and long-chain fatty acids. All the standard fatty acids used in the analysis were obtained from Sigma-Aldrich. Using a computer program (Microsoft Excel 2000), we converted the relative fatty acid distribution values (as recorded by the integrator) into mg/L to determine the amount of each fatty acid in the lipid extracts. In this case, we assumed that the extracted lipids were mainly composed of fatty acids. This assumption was supported by our preliminary thin layer chromatography analysis, which showed that the lipid extracts (except the initial sample that contained triacylglycerols and Tween 80) were composed of fatty acids.

1.6 Data analysis

All the results presented in this paper are average values of triplicate measurements. The data from the triplicate experiment were calculated to obtain the arithmetic mean values and standard deviations using computer programs (Microsoft Excel 2000 and Microcal Origin 6). The arithmetic mean values (n = 3) were used for plotting the graphs.

2 Results and discussion

2.1 Changes in biomass and lipid contents

Figure 2a shows the changes in both lipid and biomass contents. It shows a typical profile of microbial growth, which shows that after exponential growth and stationary phases the biomass enters endogenous phase and its content reduces because of depleted substrates. At the same time, we observed that oxygen consumption was still taking place despite the decrease in biomass content, especially after day 5 (Fig.2b), suggesting that some microorganisms in the sludge were still viable. A concept described in the literature can be used to explain these observations. According to the growth-death concept (Van Loosdrecht and Henze, 1999), the net biomass production is limited by the available substrates (including secondary substrates from cell lysis) that allow a part of the biomass to grow and the other part decays due to loss of cell viability. Dueholm *et al*. (2001) showed that addition of lipids to activated sludge was accompanied by an increase in oxygen consumption. With regard to our results (Fig.2b), this suggests that the observed decrease in OUR (after day 3) was caused by the decrease in the content of lipids.

Moreover, we observed that the lipid content could not be reduced to values below 300 mg/L from the initial content of 2,000 mg/L (Fig.2a). Other researchers have reported similar results. The results of Keenan and Sabelnikov (2000), who studied the biodegradation of lipids by bacterial strains (*Acinetobacter* sp., *Rhodococcus* sp. and *Caseobacter* sp.), show that biological treatment could not

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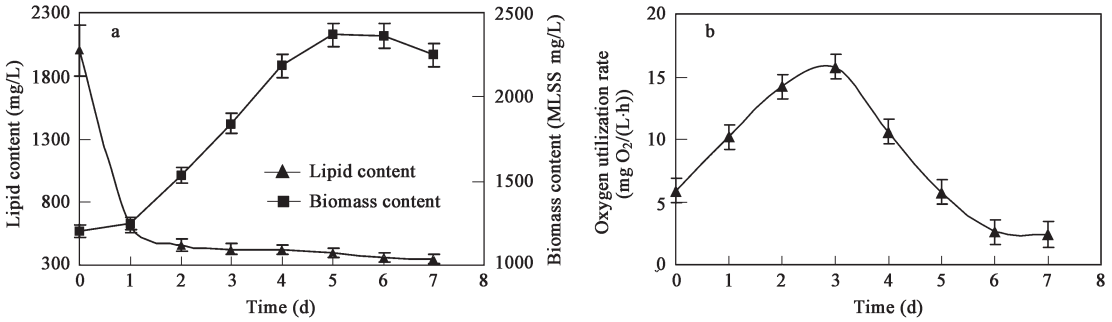

Fig. 2 Changes in lipid and biomass contents (a) and oxygen utilization rate (OUR) (b) during the experimental period of 7 days. Conditions: initial lipid content 2,000 mg/L; initial biomass (MLSS) content 1,200±25 mg/L; temperature 20±1°C; air supply 1,500 cm^3/min; initial pH 7.04±0.02. Error bars show three standard deviations of the data.

reduce the content of lipids in wastewater to values lower than 305 mg/L. Similarly, Wakalin and Forster (1997) reported that the content of lipids in wastewater could not be reduced to values below 100 mg/L even when acclimated microbial species (*Acinetobacter* sp.) was used for the degradation of lipids. Further, Lefebvre *et al.* (1998) reported that degradation of saponified lipids begun as soon as they were mixed with activated sludge and continued until the lipid content was 900 mg/L from the initial content of 3,500 mg/L (after 2–3 h). No further degradation occurred even after extending the SRT to 10 h.

Increases in the content of lipids in biological treatment systems have also been observed (Miron *et al.*, 2003; Naidas *et al.*, 2005). Therefore, we propose that the increase in biomass content causes the decrease in lipid content, whereas the decrease in biomass content causes the increase in lipid content in wastewater. In this regard, Fig.2a shows that in the early stages (from day 0–5) the decrease in lipid content was predominant (due to both phenomena of adsorption and biodegradation, Hwu *et al.*, 1998; Dueholm *et al.*, 2001), while in the late stages (from day 5) an equilibrium between the increase and decrease in lipid content was reached, resulting in an insignificant change in the overall lipid content. Other methods should be considered for characterizing the transformation of lipids in activated sludge. In this regard, application of fluorescence labeled lipids has been used to characterize the kinetics of hydrolysis of lipids in activated sludge (Duehom *et al.*, 2001; Frølund *et al.*, 1995). In this study, because the overall lipid content did not show the changes that they underwent, we decided to hydrolyze them (all lipids contain fatty acids in their structures) to liberate fatty acids and investigated the changes in the contents of individual fatty acids by GLC.

2.2 Changes in compositions and contents of fatty acids

Figure 3 shows changes in individual fatty acid contents during the course of the experiment. The initial fatty acids contents are represented by sample 1 (day 0), which was the actual blend of refined rapeseed oil, Tween 80 and washed activated sludge. Subsequent samples were taken every 24 h from day 1 to 7. The fatty acid composition of the synthetic wastewater used was very similar to that found in municipal wastewater, where palmitic, stearic, oleic, linoleic and linolenic acids make up approximately 80% of the long-chain fatty acids (Quemeneur and Marty, 1994). The decrease in the contents of these fatty acids (91%, C_{16}; 75%, $C_{18:0}$; 94%, $C_{18:1}$; 94%, $C_{18:2}$; and 96%, $C_{18:3}$) was very rapid during the first day (Figs.3e, 3g–3j). In the same period (day 1), the contents of C_{4-8} and C_{20} were moderately reduced by about 38% and 77% (Figs.3a and 3k), respectively, while the contents of C_{14} and C_{17} (Figs.3d and 3f) slightly increased. During the experimental period of 7 d, the contents of all individual fatty acids underwent both increases and decreases (Fig.3), suggesting that these substrates were not consumed by microorganisms at the same rate. These results are in agreement with those reported by Novak and Klaus (1973), who found that the utilization rate of fatty acids by activated sludge microorganisms is different and depends on the length and degree of unsaturation of their carbon chains.

Further, because the content of other long-chain fatty acids decreased during the first day (Figs.3e, 3g–3j), the drastic increase in the content of lignoceric acid (Fig.3m; C_{24} from 4 to 352 mg/L) shows that both biodegradation and biosynthesis of fatty acids occurred. After day 2, the content of lignoceric acid significantly decreased, which shows that the acid was subsequently biodegraded. Similarly, decreases and increases in contents of other individual fatty acids were also observed during the course of the experiment (Fig.3). Results of Dignac *et al.* (2000) also show increases in contents of fatty acids (palmitoleic, stearic, linoleic, linolenic, arachidic, behenic and lignoceric) in wastewater effluents after 24 h of a biological treatment process using activated sludge. Pereira *et al.* (2002) also reported the appearance of palmitic acid in wastewater after a biological treatment process. Because palmitic acid was absent from the wastewater at the beginning of the process, they concluded that it was a by-product of biodegradation of oleic acid that was initially added to the wastewater. Many other researchers have reported that fatty acids accumulate in biological wastewater treatment systems (Beccari *et al.*, 1998; Lalman and Bagley, 2001; Salminen *et al.*, 2000). Therefore, since biodegradation and biosynthesis of fatty acids occur inside microbial cells (Kunau *et al.*, 1995), the observed increases in contents of

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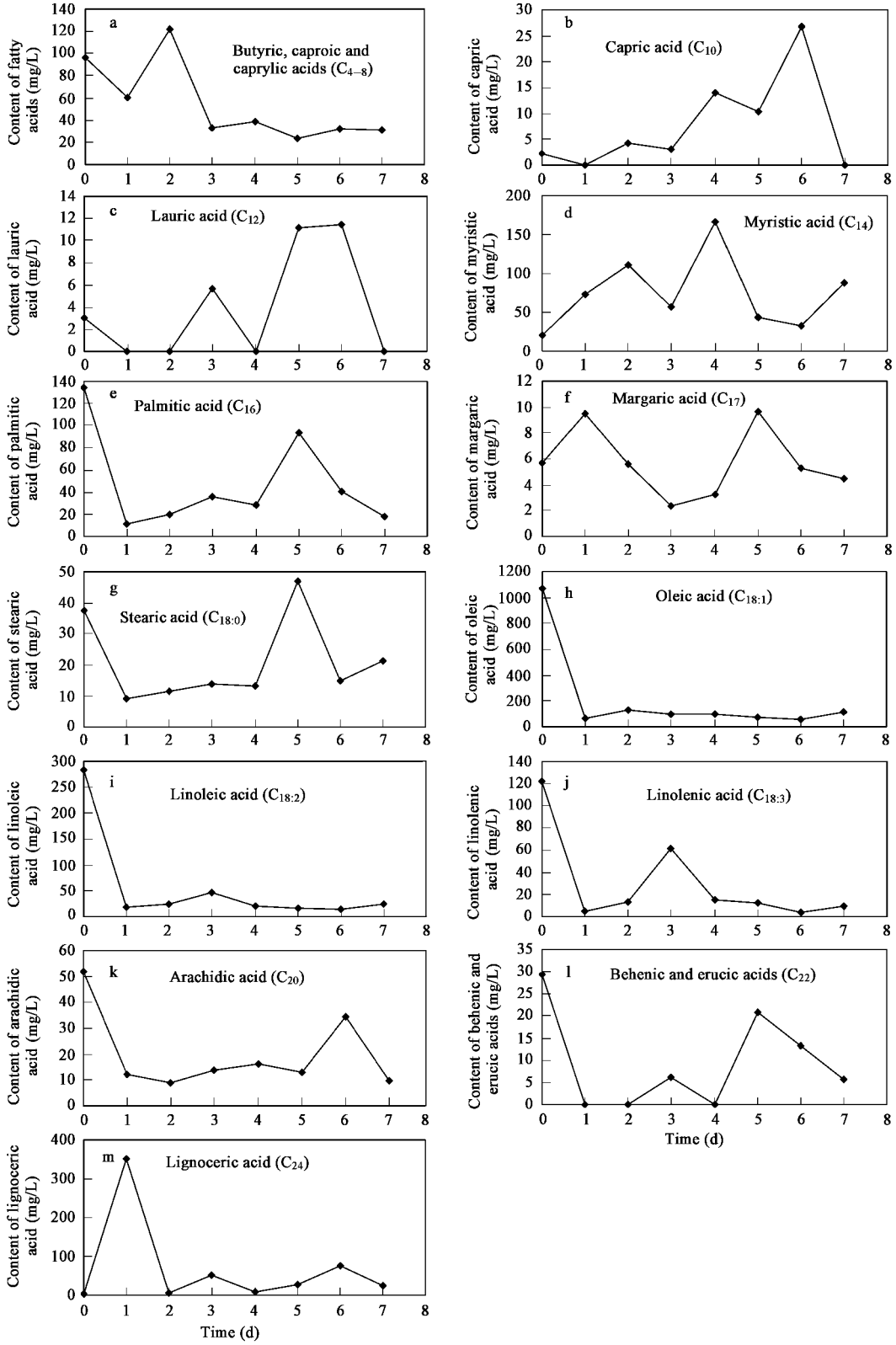

Fig. 3 Changes in content of butyric, caproic and caprylic acids (C_{4-8}) (a), capric acid (C_{10}) (b), lauric acid (C_{12}) (c), myristic acid (C_{14}) (d), palmitic acid (C_{16}) (e), margaric acid (C_{17}) (f), stearic acid ($C_{18:0}$) (g), oleic acid ($C_{18:1}$) (h), linoleic acid ($C_{18:2}$) (i), linolenic acid ($C_{18:3}$) (j), arachidic acid (C_{20}) (k), behenic and erucic acids (C_{22}) (l), and lignoceric acid (C_{24}) (m) during the experimental period of 7 d. Conditions are as in Fig.2.

fatty acids show that they were released into the wastewater as microbial by-products. At the same time, decreases in their contents show that fatty acids were subsequently consumed by microorganisms as substrates.

2.3 Conceptual model of transformation of lipids in activated sludge

The literature shows that biodegradation of lipids by activated sludge microorganisms involves at least three

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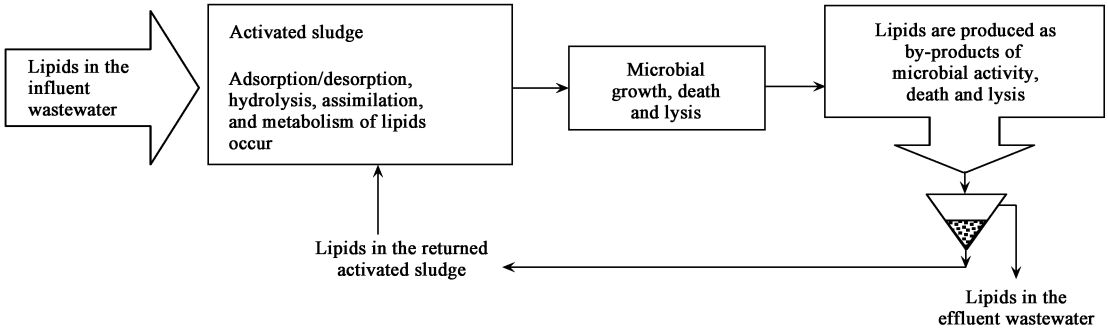

Fig. 4 A conceptual model of transformation of lipids by activated sludge microorganisms.

mechanisms (Hwu *et al*., 1998; Dueholm *et al*., 2001): (1) adsorption/desorption–lipids are adsorbed onto sludge flocs by DLVO forces (i.e., van der Waals interaction and electrostatic forces) and non-DLVO forces (i.e., hydrophobic interaction, steric and hydration forces). Results of Hwu *et al*. (1998) suggest that the accompanying desorption is a biologically mediated process; (2) hydrolysis of lipids–microorganisms produce extracellular enzymes, mainly lipases, to hydrolyze, for example, triacylglycerols to fatty acids; and (3) uptake of fatty acids–fatty acids are consumed by activated sludge bacteria after being transported into microbial cells. Transport mechanisms include active transport system mediated by specific proteins and direct diffusion into microbial cells. After transportation into microbial cells, fatty acids are degraded by sequential removal of two-carbon atoms via the β-oxidation pathway (Kunau *et al*., 1995; Maloy *et al*., 1981; Ratledge, 1992).

Based on the above-mentioned mechanisms, Dueholm *et al*. (2001) suggested a model of transformation of lipids in activated sludge. However, their model does not take into account the production of lipids by the activated sludge. In our earlier studies, we have shown that production of microbial by-products is one of the major mechanisms that affect both the quality of wastewater effluents and microbial activity (Chipasa and Mędrzycka, 2004). Further, taking the results reported in this paper into account, we suggest a novel model of transformation of lipids in activated sludge (Fig.4). The model shows that utilization of microbial activity for complete removal of lipids from wastewater is limited. This is the reason that the release of lipids into wastewater will always occur since production of microbial by-products, as a result of microbial activity, death and lysis, occurs in all phases of microbial growth (Van Loosdrech and Henze, 1999). Hence, new treatment methods, especially those that can optimize both microbial activity and removal of microbial by-products, are required to achieve complete removal of lipids from wastewater.

3 Conclusions

The GLC analysis showed that contents in individual fatty acids underwent drastic decreases and increases during all microbial growth phases, although the overall residual lipid content could not be reduced to values below 300 mg/L from an initial content of 2,000 mg/L. The changes in contents of individual fatty acids suggest that fatty acids were used as substrates by microorganisms as well as released into the wastewater as by-products.

Analysis of the suggested model of transformation of lipids in activated sludge shows that utilization of microbial activity for complete removal of lipids from wastewater is limited. Therefore, new treatment methods, especially those that can optimize both microbial activity and removal of microbial by-products, are required in order to achieve complete removal of lipids from wastewater.

Acknowledgements

This work was supported by the Polish Ministry of Education. The first author (KBC) would like to thank Dr. Pawłowicz and Dr. Tynek for their technical assistance in setting up the GLC equipment.

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