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ANAEROBIC DEGRADATION OF FAT PARTICLES IN SLAUGHTERHOUSE WASTEWATER WITH AND WITHOUT HYDROLYSIS PRETREATMENT

BY

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A thesis submitted under the supervision of

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and

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in partial fulfilment of the requirements

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ABSTRACT

Lipids represent an important fraction of the particulate organic charge in slaughterhouse wastewater. Anaerobic treatment of slaughterhouse wastewater has been reported to be slowed down or impaired because of high concentrations of suspended solids, particularly fats. However, the fate of lipids during anaerobic digestion has been poorly defined, especially for wastewaters from the meat processing industry. The objectives of this thesis were thus (1) to evaluate the effect of hydrolysis pretreatment on the anaerobic digestion of fat particles in slaughterhouse wastewater; (2) to characterise and quantify neutral fat hydrolysis and long-chain fatty acid (LCFA) oxidation during anaerobic degradation of slaughterhouse wastewater with and without hydrolysis pretreatment; and (3) to determine the effect of particle size on fat hydrolysis.

The efficiency of four pretreatments to hydrolyse and reduce the size of pork and beef fat particles during mixing at room temperature was tested: NaOH and three commercial lipases of plant, bacterial and animal origins. The most promising pretreatment was the pancreatic lipase PL-250 that could significantly reduce the initial average particle size (D_{in}) of pork fat by a maximum of 40% after 4 h of mixing at room temperature. Approximately 35% of the neutral fat was hydrolysed after 5.5-h of pretreatment with 250 mg/l of PL-250 in a substrate containing approximately 2000 mg/l of pork fat particles. Most of the free LCFAs released during the hydrolytic pretreatment remained adsorbed on the fat particle surface.

The effect of pretreatment with PL-250 on subsequent anaerobic digestion of the substrate was evaluated by feeding control and enzyme pretreated slaughterhouse

wastewater containing pork fat particles to anaerobic sequencing batch reactors (ASBRs) operated at 25°C. The main conclusions from the experiment were:

- Pretreatment with PL-250 only had a small effect on pork fat particle digestion at 25°C, marked by a decrease of about 5% in digestion time to achieve 80% reduction in initial neutral fat and free LCFA concentrations.
- Anaerobic degradation of pork fat particles is mainly controlled by free LCFA oxidation and, in ASBRs operated at 25°C, near maximum oxidation rate is reached at low free LCFA concentration. Consequently, increasing the initial free LCFA concentration by prehydrolysing the substrate will have limited effect on fat degradation rate.
- At D_{in} ranging from 60 to 450 μm, pork fat hydrolysis rate in anaerobic reactors is not a function of particle size. The fat particles became more filamentous and plate-like as their size was increased. Bacteria could probably colonise the inside as well as the outside of the particles. Consequently, specific surface area (m^2/m^3) available for hydrolysis was not significantly increased by decreasing the pork fat particle size.
- Neutral fat hydrolysis and free LCFA oxidation rates can be adequately modelled using first-order and Monod-type kinetics, respectively. The first-order hydrolysis rate constant averaged 0.63 ± 0.07 d^{-1}, while the maximum oxidation rate (k_{max}) and half-saturation concentration (K_s) were estimated at 164 ± 37 mg free LCFA /l/d and 35 ± 31 mg free LCFA/l, respectively.
- Fat hydrolysis rate will be underestimated if based on the increase in soluble compounds with respect to particulate organics. An analytical method that removes bound LCFAs from solids surface must be used to measure lipid hydrolysis.

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SYMBOLS AND ACRONYMS

Symbol		Definition
a	=	Long-chain fatty acid deactivation or inhibition constant (day^{-1})
A	=	Constant ranging from 0 to 1 (Eq. 2.11)
A_{hrs}	=	Hydrolytic reaction surface (m)
A_r	=	Concentration of rapidly degradable particles (mg/l)
A_s	=	Concentration of slowly degradable particles (mg/l)
ASBR	=	Anaerobic sequencing batch reactor
b	=	First order coefficient for particle size reduction with respect to concentration of PL-250 (l/mg)
b_1	=	First order coefficient for particle size reduction with respect to time during pretreatment with PL-250 (day^{-1})
BOD	=	Biological oxygen demand (mg/l)
$C_{LG-1000}$	=	LG-1000 concentration (g/l)
C_{NaOH}	=	Sodium hydroxide concentration (meq/l)
COD	=	Chemical oxygen demand (mg/l)
C_{PL-250}	=	PL-250 concentration (mg/l)
CV	=	Coefficient of variation (%)
D	=	Geometric mass mean size (μm)
D_i	=	Nominal size of particles in size range i (μm)
D_{in}	=	Initial average particle size (μm)
D_{min}	=	Minimum average particle size (μm)
D_p	=	Particle diameter (mm)
$D_{treated}$	=	Average particle size after pretreatment (μm)
EPA	=	Environmental protection agency
ESP	=	EcoSystem Plus
f_{ns}	=	Fraction of neutral fat in the sludge bed at the beginning of each experiment (mg neutral fat/mg VSS)
$F_{(ns-p-\gamma_{pe},\ \gamma_{pe},\ 0.05)}$	=	5% point for the F-distribution at n_s-p-γ_{pe} and γ_{pe} degree of freedom
G	=	Methane production

G_F	=	Ratio of pure to model error used to verify the validity of non-linear models (Eq. 3.3)
HRT	=	Hydraulic retention time (d)
k_h	=	First-order hydrolysis constant (d^{-1})
k_h'	=	Linear rate of long-chain fatty acid release in solution (mg/l/day)
$k_{h,A}$	=	Constant for Eq. 2.11 (varying dimension)
k_{hr}	=	First-order hydrolysis constant for particulate fraction A_r (d^{-1})
k_{hs}	=	First-order hydrolysis constant for particulate fraction A_s (d^{-1})
k_{max}	=	Monod maximum LCFA oxidation rate (mg LCFA/l/d)
K_s	=	Monod LCFA half-saturation concentration (mg LCFA/l)
k_x	=	Long-chain fatty acid disappearance rate (day^{-1})
L	=	Long-chain fatty acid concentration (mg/l)
L_0	=	Long-chain fatty acid concentration in solution at time 0 (mg/l)
$L_{ox\,(t=t)}$	=	Free LCFAs oxidised between time 0 and time t (mg/l)
L_0	=	Average free LCFA concentration at time 0 (mg/l)
L_p	=	Free LCFA production between times 0 and t (mg/l)
L_r	=	Average free LCFA concentration remaining in the mixed-liquor at time t (mg/l)
LCFA	=	Long-chain fatty acid
LG-1000	=	Lipase G-1000
m_i	=	Mass of solids in size range i (g)
M_0	=	Total mass of particles (kg)
M^p_0	=	Mass of particles within size range n (kg)
n	=	degree coefficient
n_s	=	Total number of date points in a model
N	=	Concentration of neutral fat from the pork particles (mg/l)
N_T	=	Total neutral fat concentration in the mixed-liquor (mg/l)
N_0	=	Pork particle neutral fat concentration in the mixed-liquor after feeding (time 0) (mg/l)
OLR	=	Organic loading rate (g/l/d)
p	=	Number of parameters in a model

PCOD	=	Particulate chemical oxygen demand (mg/l)
PL-250	=	Pancreatic Lipase 250
r_{arh}	=	Hydrolysis rate of rapidly degradable particles (mg/l/d)
r_{ash}	=	Hydrolysis rate of slowly degradable particles (mg/l/d)
r_h	=	rate of hydrolysis (mg/l/d)
r_{ox}	=	LCFA oxidation rate (mg LCFA/l/d)
R_0	=	Radius of particles within a range n (m)
SCOD	=	Soluble chemical oxygen demand (mg/l)
$SCOD_{control}$	=	SCOD in control samples after mixing period (mg/l)
$SCOD_{treated}$	=	SCOD after pretreatment minus SCOD from the enzyme (mg/l)
SHW	=	Slaughterhouse wastewater
SRT	=	Solids retention time (d)
SS	=	Suspended solids (mg/l)
SS_{res}	=	Sum of square of residuals
S^2_{pe}	=	Pooled estimate of variance of replicates (pure error)
t	=	Time (day)
TCOD	=	Total chemical oxygen demand (mg/l)
WAS	=	Waste activated sludge
VFA	=	Volatile fatty acid
VS	=	Volatile solids
VSS	=	Volatile suspended solids
UASB	=	Upflow anaerobic sludge blanket
X	=	Concentration of biodegradable suspended solids (mg/l)
X_B	=	Biomass concentration (mg/l)
$X_{B,H}$	=	Heterotrophic biomass concentration (mg/l)
X_F	=	Concentration of influent biodegradable organic matter (mg/l)
X_h	=	Concentration of hydrolytic enzymes (mg/l)
X_s	=	Remaining concentration of degradable particulates
η	=	Fraction of hydrolysed starch mass
Φ_s	=	Sphericity factor
γ_{pe}	=	Degree of freedom of the pure error (S^2_{pe})

CHAPTER 1

INTRODUCTION

According to the American Environmental Protection Agency (EPA), slaughterhouses produce a wastewater that could be very harmful to the environment (Polprasert et al., 1992). The pollution potential of meat-processing and slaughterhouse plants has been estimated at over 1 million population equivalents in the Netherlands (Sayed, 1987), and 3 million in France (Festino and Aubart, 1986). In Québec and Ontario, slaughterhouse wastewater is usually discharged to municipal treatment plants following varying levels of primary and/or chemical pretreatment at the slaughterhouse (Massé et Masse, 2000a). These pretreatments, however, are somewhat costly and are not sufficient to totally eliminate the surcharge exacted by Municipalities to further treat the wastewater.

The characteristics of slaughterhouse wastewater - high organic strength, mostly from fats and proteins, sufficient alkalinity, adequate inorganic nutrient concentration, and absence of toxic materials - make it particularly suitable for anaerobic digestion (Hammer and Jacobson, 1970). Previous experiments indeed suggested that settled or filtered slaughterhouse wastewater can be efficiently treated by anaerobic digestion (Borja et al., 1993, 1994, 1995a, 1995b; Polprasert et al., 1992; Rudd et al., 1985; Stephenson and Lester, 1986). However, unsettled slaughterhouse and meat packing wastewater typically contains from 700 to 19 000 mg/l of undissolved solids, which may include fat, grease, hair, feathers, fleshing, manure, grit, and undigested feed (Arora and Routh, 1980; Bull et al., 1982; Rands and Cooper, 1966). Insoluble, slowly biodegradable suspended solids (SS) account for up to 50% of the polluting charge, while another 25% originate from

colloidal solids (Sayed et al., 1988). Research indicated that anaerobic digestion of unsettled slaughterhouse and meat packing wastewater requires longer hydraulic retention times (HRTs) and lower organic loading rates (OLRs) than that of settled wastewater (Campos et al., 1986; Kostyshyn et al., 1988; Massé et Masse, 2000b; Saxena et al., 1986; Tritt, 1992).

Lipids represent an important fraction of the particulate organic charge in slaughterhouse wastewater. Fat accounted for 40% of total chemical oxygen demand (TCOD) in beef slaughterhouse wastewater (Sachon, 1986). Lipids represented less than 1% of soluble COD (SCOD) but over 67% of particulate COD (PCOD) in the wastewater from a mixed slaughterhouse (Sayed et al., 1988). Lipid degradation has been suggested as the rate-limiting step during anaerobic digestion of slaughterhouse wastewater (Sayed et al., 1988). However, there is no quantitative study on anaerobic degradation of fat in slaughterhouse wastewater. Research on lipid anaerobic degradation has focused on fat as part of complex wastes, such as waste activated sludge (WAS), or on liquid (oil and milk) and emulsified lipids, as opposed to fat in particulate forms as in slaughterhouse wastewater. Additionally, studies have dealt with fat hydrolysis or with long-chain fatty acid (LCFA) oxidation, but there are no quantitative studies reporting on both aspects of lipid anaerobic degradation.

Sayed et al. (1988) suggested that the liquefaction rate of adsorbed insoluble materials and entrapped coarse suspended solids, mainly fats, was the overall controlling factor in the anaerobic degradation of unsettled slaughterhouse wastewater. Consequently, the application of a pretreatment to hydrolyse SS, more particularly fats, should accelerate the anaerobic digestion of slaughterhouse wastewater. However, most studies on

hydrolysis pretreatments dealt with complex waste, such as WAS or municipal waste, which contain at least ten times more SS than slaughterhouse wastewater, and the fate of lipids was not assessed. Hydrolysis pretreatments were also evaluated solely by their ability to increase SCOD, and not by their efficiency in decreasing the size of particles in solution. Smaller particles usually have a higher surface to volume ratio than larger particles, and thus offer a higher surface area for bacterial colonisation and reaction. A reduction in particle size should thus increase the hydrolysis rate in anaerobic reactors.

There is a need for research on hydrolysis pretreatments dealing with the lipid fraction of relatively dilute organic wastewaters, such as those found in the food industry, and with particle size reduction as well as solids solubilization. The efficiency of the pretreatment in hydrolysing fat particles as well as in increasing subsequent anaerobic digestion should be assessed. There is also a need for research on fat particle degradation during anaerobic treatment of slaughterhouse wastewater. Fat hydrolysis and LCFA oxidation should be modelled and quantified in order to determine the rate-limiting step during anaerobic digestion of slaughterhouse wastewater. The objectives of this research were thus:

- To evaluate and characterise the effect of various hydrolysis pretreatments on pork and beef fat particles in slaughterhouse wastewater;
- To evaluate and quantify the effect of the most promising hydrolysis pretreatment on anaerobic digestion of slaughterhouse wastewater containing pork fat particles of different average particle sizes;
- To characterise and quantify the kinetics of fat anaerobic degradation in slaughterhouse wastewater with and without hydrolysis pretreatment;

- To determine the relation between particle size and fat hydrolysis rate.

1.1 Organisation of the thesis

This thesis consists of a literature review and four individual articles followed by a summary and appendices. The articles were originally written for publication in scientific journals. Their format was slightly changed to make the thesis more uniform, particularly with respect to tables and figures, units, spelling, bibliographic references and titles. Nonetheless, the presentation of the thesis as a collection of articles has led to some unavoidable repetitions, namely in Material and Methods, Introduction, and Literature Review Sections.

Chapter 2 reviews previous research conducted on the anaerobic degradation of lipids. particle hydrolysis, and hydrolysis pretreatments. In Chapter 2, the sections dealing with lipids were published as a short literature review (Masse et al., 2000).

Chapter 3 deals with testing of alkaline and enzymatic pretreatments for fat particles in slaughterhouse wastewater (Masse et al., 2001a). The objective of this experiment was to determine which of four hydrolysis pretreatments would be the most efficient in hydrolysing and reducing the size of fat particles in slaughterhouse wastewater. Treatment time was set as 4 hours, since the main objective was to design an hydrolysis pretreatment that could be apply to the wastewater in a relatively short residence period in an equalising tank. A variety of doses were used for all hydrolysing agents, based on information gathered from published scientific articles or from company's information sheet.

Chapter 4 describes the effect of the most promising pretreatment, Pancreatic Lipase 250 (PL-250), on the hydrolysis and size reduction of fat particles in slaughterhouse wastewater (Masse et al., 2001b). Effects of treatment time, enzyme dose as well as pork fat particle size and concentration were considered. A few tests were also performed with beef fat to examine the effect of fat type on enzyme efficiency.

Chapter 5 reports on fat degradation during digestion of slaughterhouse wastewater in anaerobic sequencing batch reactors (ASBRs) operated at 25°C (Masse et al., 2001c; under review). The kinetics of fat degradation was characterised and quantified. Results were also used to identify the rate-limiting step during anaerobic degradation of fat.

Chapter 6 describes the effect of hydrolysis pretreatment with PL-250 on the anaerobic digestion of slaughterhouse wastewater in ASBRs operated at 25°C. The data from this experiment were also used to validate the kinetic equations developed in Chapter 5 for fat degradation in slaughterhouse wastewater. Chapter 6 is an expanded version of an article published in the Proceedings of the conference *Anaerobic Digestion 2001* held in Brussels on September 2-5, 2001 (Masse et al., 2001d).

A summary presenting general conclusions of the investigation is finally presented in Chapter 7. It includes a critical review of the experiments and the results and offers new research avenues.

Appendices A to C present the results that were not included in the articles. Appendices A and B contain unpublished materials from Chapters 3 and 4, respectively. They include most results pertaining to experimental set-up and basic work on particle size and LCFA measurements. Appendix C presents unpublished results from Chapters 5 and 6. It contains most basic work on fat extraction and the separation of neutral fat and

LCFAs from total fat. It also presents all data collected during the digestion experiment, including methane production and yield with respect to COD fed to the digesters (Tables C23 and C24). All appendices include a short introductory section describing presented materials.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

In this chapter, microbiology and metabolic processes of anaerobic digestion will first be briefly presented. The chemistry and anaerobic degradation pathways of fat particles, quantitative research on the kinetics of fat degradation, studies on the inhibition of the anaerobic process by long-chain fatty acids (LCFAs), and existing works on the digestion of fat particles in slaughterhouse wastewater will then be described and discussed. The second part of the literature review will be concerned with the hydrolysis process in an anaerobic environment. Finally, previous experiments on hydrolysis pretreatments will be reviewed and discussed.

2.2 Microbiology and metabolic processes of anaerobic digestion

During anaerobic digestion, complex organic materials, such as carbohydrates, proteins and lipids, are ultimately transformed into methane and carbon dioxide. The anaerobic process can be divided into seven parallel or series reactions (Fig. 2.1):

1. Hydrolysis of particulates and large soluble organic polymers into small soluble particles;
2. Fermentation of the amino acids and sugars resulting from protein and carbohydrate hydrolysis;

3. Anaerobic oxidation of the long-chain fatty acids (LCFAs) and alcohol resulting from lipid hydrolysis;

4. Anaerobic oxidation of the intermediary products (such as volatile fatty acids (VFAs), except acetate) resulting from the fermentation and oxidation processes;

5. Homeocetogenesis or acetate production from carbon dioxide and hydrogen;

6. Aceticlastic methanogenesis or conversion of acetate into methane;

7. Reductive methanogenesis or methane production by reduction of carbon dioxide with free hydrogen. (Pavlostathis and Giraldo-Gomez, 1991)

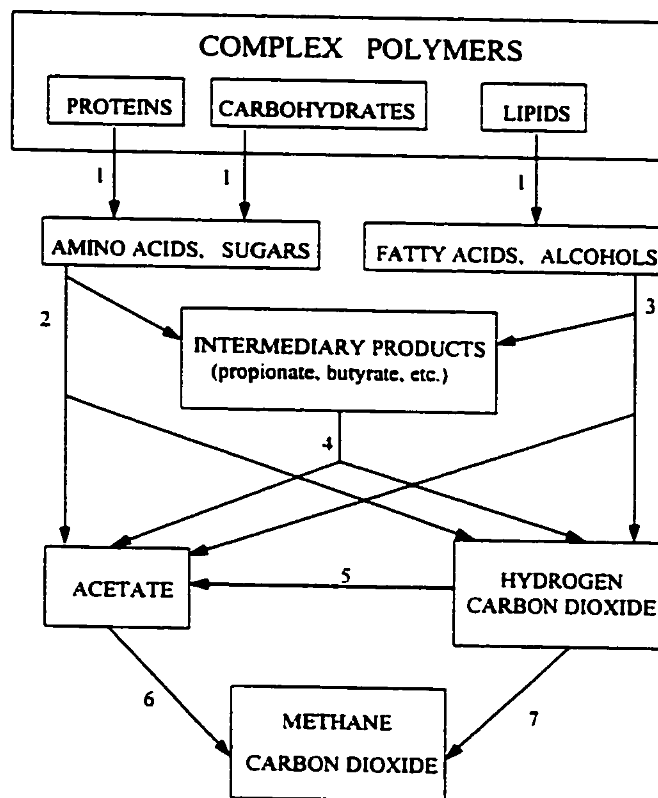


Figure 1.1 Reaction flowchart for anaerobic digestion of complex polymers. Numbers refer to processes described in the text (Adapted from Pavlostathis and Giraldo-Gomez, 1991)

Particulates and complex biopolymers are too large to permeate the cell membrane. Hydrolysis is the process by which these molecules are broken down into soluble compounds small enough to permit their diffusion across the membrane. Hydrolysis is catalysed by extracellular enzymes released by acidogenic bacteria (Angelidaki et al., 1990). Enzymes are compound specific such as cellulase for cellulose hydrolysis, protease for proteins, and lipase for lipids. The main products of hydrolysis are glucose and cellobiose for cellulose; pentoses, hexose and uronic acid for hemicellulose; polypeptides and amino acids for proteins; LCFAs, glycerol and phosphoric acid for lipids (Pavlostathis and Giraldo-Gomez, 1991). Factors influencing the extent and rate of hydrolysis are discussed in Section 2.4.

The sugars and amino acids resulting from carbohydrate and protein hydrolysis are degraded into VFAs (acetate, propionate, butyrate, etc.) and other small molecules by fermentative (acidogenic) bacteria. LCFA dissimilation proceeds through a different pathway called β-oxidation which is discussed in Section 2.3.2. Hydrolysis and fermentation constitute the acid phase of anaerobic digestion. The methanogenic phase consists in the oxidation of higher VFAs into acetate and carbon dioxide by hydrogen-producing acetogenic bacteria, and in the production of methane and carbon dioxide from acetate, hydrogen and carbon dioxide by methanogens. The oxidation of VFAs is a thermodynamically unfavourable process (free energy change, G'_0, greater than 0) unless a low hydrogen partial pressure is maintained in the reactor (Marty, 1986). This condition is made possible through the consumption by methanogens of the hydrogen produced during oxidation. Fatty acid oxidation and methanogenesis are thus considered as symbiotic processes.

2.3 Anaerobic degradation of lipids

2.3.1 Chemistry of lipid degradation

Lipids form an heterogeneous structural group broadly defined as a biological material extractable by apolar and slightly polar solvents such as benzene, ether and chloroform (Ferrato et al., 1997). They are classified as complex or saponifiable, if they become soluble in water upon reaction with hot alkali, or as unsaponifiable (Novak and Carlson, 1970). Saponifiable fats are the most common lipids and include triglycerides, phospholipids, shingolipids and waxes. Fat particles in animal and sewage sludge are mainly composed of triglycerides made of esters of fatty acids and glycerol, which have the following form:

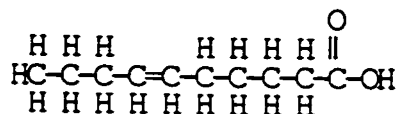
$$\begin{array}{l} R_1\text{-COO-CH}_2 \\ \qquad\qquad\ \ | \\ R_2\text{-COO-CH} \\ \qquad\qquad\ \ | \\ R_3\text{-COO-CH}_2 \end{array} \tag{2.1}$$

where R_1 , R_2 and R_3 are hydrocarbon chains of similar or different lengths (Heukelekian and Balmat, 1959).

Fatty acids consist of an hydrocarbon chain and a terminal carboxyl group. In *saturated* fatty acids, every carbon atom shares a single bond with the adjacent carbon as in:

$$\begin{array}{l} \quad\ \ \text{H H H H H H H H H} \ \overset{\text{O}}{\|} \\ \text{HC–C–C–C–C–C–C–C–C–C–OH} \\ \quad\ \ \text{H H H H H H H H H} \end{array} \tag{2.2}$$

Unsaturated fatty acids contain at least one carbon-carbon double bond:

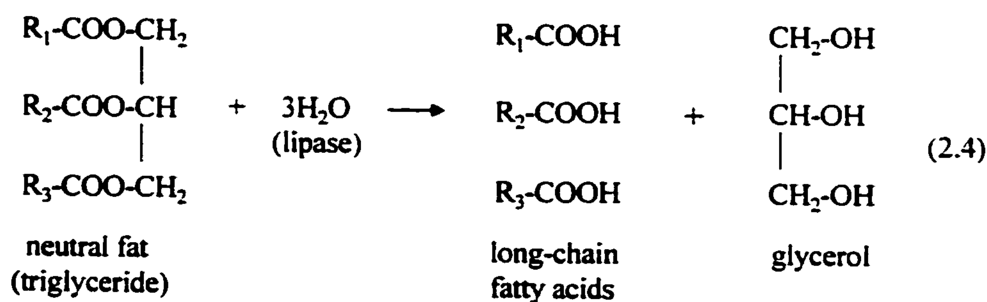


(2.3)

Most LCFAs contain an even number of carbon atoms, although LCFAs with an odd number of carbon atoms exist in small quantity (O'Rourke, 1968). Fatty acids are denoted by the number of carbon atoms and double bonds they contain. Oleic acid (18:1), which accounts for 30% to 50% of fat in most animals (Grinstone et al., 1986), has 18 carbons and one double bound. The saturated palmitic (16:0) and stearic (18:0) acids represent together approximately 34%, 40% and 65% of the fatty acids in beef, pork and mutton, respectively (Grinstone et al., 1986). Fatty acid composition of sewage sludge is similar to that of slaughterhouse wastewater. Oleic, palmitic and stearic acids accounted for 52%, 33% and 13% of total fat in sewage sludge, respectively (Viswanathan et al.,1962).

2.3.2 Processes of lipid anaerobic degradation

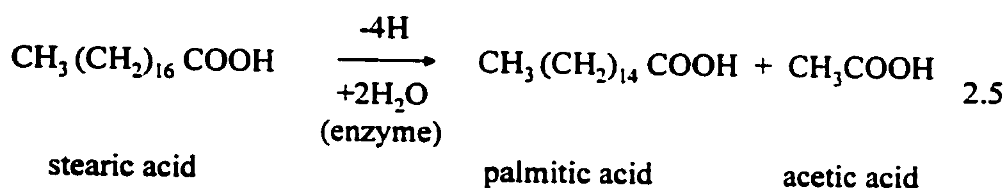
In an anaerobic environment, triglycerides are first hydrolysed (lipolyzed) to LCFAs and glycerol, as described below (Sayed, 1987):



(2.4)

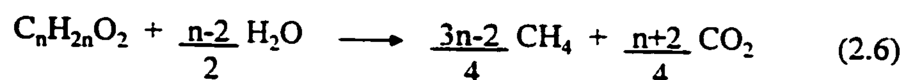
The LCFAs retain most of the triglyceride chemical oxygen demand (COD). In glycerol trioleate, for example, 95.6% and 4.4% of the COD are contained in the LCFAs and the glycerol molecule, respectively.

Free LCFAs are oxidised to shorter chain fatty acids by hydrogen-producing syntrophic bacteria similar to those degrading higher VFAs into acetate and carbon dioxide (McInerney, 1988), as follows:



$$\underset{\text{stearic acid}}{CH_3(CH_2)_{16}COOH} \xrightarrow[\substack{+2H_2O \\ \text{(enzyme)}}]{-4H} \underset{\text{palmitic acid}}{CH_3(CH_2)_{14}COOH} + \underset{\text{acetic acid}}{CH_3COOH} \qquad 2.5$$

The process is called ß-oxidation because the beta carbon (second from the carboxyl group) is oxidised (Elefsiniotis and Oldham, 1994). The ß-oxidation process is repeated (n-2)/2 times, where n is the total number of carbon atoms, until the acid is completely broken down to n/2 acetic acid molecules with the release of 2n-4 hydrogen atoms. The acetic acid and hydrogen atoms are subsequently transformed to methane and carbon dioxide by methanogens. The complete reaction is described as follows:



$$C_nH_{2n}O_2 + \frac{n-2}{2} H_2O \longrightarrow \frac{3n-2}{4} CH_4 + \frac{n+2}{4} CO_2 \qquad (2.6)$$

LCFA oxidation becomes thermodynamically unfavourable when the hydrogen partial pressure exceeds 10^{-4} atmosphere (Hanaki et al., 1987). The process thus relies on the ability of the methanogens to utilise the molecular hydrogen produced during oxidation

(Sayed, 1987). This dependence may explain why the dissimilation of lipids has not been successful in acid phase reactors where methane production has been suppressed (Eastman and Ferguson, 1981; Hanaki et al., 1987; Heukelekian and Mueller, 1958).

Based on bioenergetics and stoichiometric equations, the fraction of degraded substrate used for cell synthesis is 0.496 for carbohydrate, 0.290 for protein, and 0.052 for lipid (Pavlostathis and Giraldo-Gomez, 1991). At a similar substrate removal rate, the growth rate of lipid-degrading microorganisms will thus be almost one order of magnitude lower than that of protein- and carbohydrate-degrading bacteria. The fraction used for energy production (biogas) is 0.504, 0.710 and 0.948 for carbohydrates, proteins and lipids, respectively. Anaerobic digestion of lipids thus yields less biomass and produces more biogas than that of proteins or carbohydrates.

2.3.3 Lipid hydrolysis rate

Most data on lipid hydrolysis stem from studies on anaerobic digestion of complex waste performed under various conditions (temperature, pH, retention time) and inter-study comparisons are not always possible (Pavlostathis and Giraldo-Gomez. 1991). Gujer and Zhender (1983) calculated first-order apparent hydrolysis rates for various organic components during anaerobic treatment of sewage sludge in low rate mesophilic anaerobic reactors. For lipids, the first-order constant ranged from 0.08 to 1.7 d^{-1}, while it varied between 0.04 and 0.13 for cellulose, and between 0.02 and 0.03 for protein. Estimates were based on total biodegradability of the waste. an assumption which is probably more valid for lipid than for protein or cellulose. They were also based on the assumption that hydrolysis was the rate-limiting step of anaerobic digestion and that particle solubilization was proportional to methane production. This assumption may be

erroneous for lipids, since some experiments suggested that LCFA oxidation, and not hydrolysis, was the rate-limiting step of lipid degradation (O'Rourke, 1968).

Recent research on the hydrolysis of complex waste tends to show that lipids have a slower hydrolysis rate than other fractions of organic waste. Christ et al. (1999) estimated first-order hydrolysis rates for various fractions of municipal solid waste digested in thermophilic anaerobic reactors. The first-order hydrolysis constants ranged from 0.005 to 0.010 d^{-1} for lipids, from 0.015 to 0.075 day^{-1} for proteins, and from 0.025 to 0.200 day^{-1} for carbohydrates. However, lipid hydrolysis rates may have been underestimated since they were calculated from changes in the SCOD:TCOD ratio. Free LCFAs tend to adsorb onto solids surface, especially on cell membrane, and are not recovered by SCOD analysis (Gujer and Zhender, 1983). Shimizu et al. (1993) calculated anaerobic hydrolysis rates for the various components of mechanically lysed waste-activated sludge (WAS) digested at 37°C in continuously fed reactors. First-order hydrolysis constants were estimated at 1.3, 1.2, and 0.76 for proteins, carbohydrates and lipids, respectively. It seemed that the hydrolysis rates were also calculated from the concentrations of soluble substances and bound LCFAs were not taken into account. However, in continuously fed reactors, the adsorption sites on the solids may become saturated with LCFAs or other adsorbable compounds, such that free LCFAs produced during hydrolysis would then be released into solution.

All the above-mentioned studies did not consider particle size. Food waste particles will probably be much larger than biopolymers released from lysed WAS. This may partly explain the much lower hydrolysis rates obtained in the study of Christ et al. (1999) compared to those calculated by Shimizu et al. (1993), even if thermophilic

temperatures were used for the food waste. However, the effect of particle size on fat hydrolysis has not yet been established.

2.3.4 Fatty acid oxidation rate

O'Rourke (1968) used Monod-type kinetics to model LCFA oxidation during anaerobic degradation of sewage sludge. The maximum lipid degradation rates (k_{max}) averaged 6.67, 4.65, and 3.85 d^{-1} at 35°C, 25°C and 20°C, respectively. The Monod half-saturation concentration (K_s) was estimated at 2000, 3720 and 4620 mg/l at the three temperatures, respectively. These values regrouped all fatty acids with more than 5 carbons.

Novak and Carlson (1970) fed individual LCFAs to anaerobic reactors operated at 37°C. The value of k_{max} was estimated at approximately 1.0 d^{-1} for the 16- and 18-carbon saturates, while it averaged 4.0 and 5.0 d^{-1} for unsaturated oleic (18:1) and linoleic (18:2) acids, respectively. The K_s coefficient was 150 and 417 mg/l for palmitic and stearic acids, respectively, while it averaged 3180 and 1816 mg/l for oleic and linoleic acids, respectively. These estimates suggested that anaerobic oxidisers had a greater affinity for the saturated LCFAs (low K_s values), but maximum oxidation rate was higher for the unsaturated. The k_{max} and K_s values for the unsaturated LCFAs were similar to those obtained by O'Rourke (1968) at operating temperatures of 25°C-35°C. The degradation of unsaturates would thus be the limiting factor in sewage sludge degradation.

The Monod parameters reported by O'Rourke (1968) and Novak and Carlson (1970) were estimated using limited data sets of four to six values, and the error associated with the estimates was not provided. Angelidaki et al. (1999) reported much lower parameter values for the modelling of LCFA oxidation during anaerobic digestion of manure and

glycerol triolate in a thermophilic (55°C) digester. The maximum LCFA utilisation rate and the half-saturation concentration were set at 0.55 d^{-1} and 20 mg/l, respectively. However, the method used for parameter estimation and the error associated with the estimates were not reported.

Very small quantities of intermediate length LCFAs were measured in the mixed-liquor when myristic (14:0), palmitic (16:0) and stearic (18:0) acids were individually fed to anaerobic reactors (Novak and Carlson, 1970). It was concluded that the rate-limiting step in the degradation of saturated LCFAs was the activation of the ß-oxidation process. Once activated, oxidation was rapidly carried out until the LCFA was completely transformed into acetic acid. For unsaturates, however, significant amounts of intermediates were observed, and it was concluded that, for unsaturated LCFAs, the rate-limiting step was ß-oxidation rather than its activation. Rinzema et al. (1994), on the other hand, measured substantial concentrations of 4, 6 and 8-carbon fatty acid in the mixed liquor of overloaded anaerobic reactors fed caproic acid (10:0). They concluded that, at least under overloading conditions, the ß-oxidation process was rate limiting and not the activation step of the oxidation process.

2.3.5 Rate-limiting step

Controversy exists as to the rate-limiting step during fat particle degradation. Heukelekian and Mueller (1958) incubated seeded sewage sludge for 40 days at 20°C. The initial mixture contained 12.8 g/l of total lipids, of which 6.2 and 1.6 g/l were free fatty acids and triglycerides, respectively. Triglyceride concentration decreased by 50% within the initial five days of incubation, and was further reduced to 33% of the initial concentration in the next ten days. During this entire period, the free fatty acid

concentration remained almost constant. In the following ten days, the free fatty acid concentration decreased sharply to 14% of the initial value. Since there was no increase in free LCFA concentration during the entire digestion period, the authors concluded that the free fatty acids released by hydrolysis were oxidised as they were produced, and thus oxidation was not rate limiting.

O'Rourke (1968) conducted research on the degradation of fat during sewage sludge digestion in reactors operated at temperatures ranging from 15°C to 35°C. The sludge contained 4.3 g/l of lipids, of which about 0.9 g/l were triglycerides and 1.9 g/l were free fatty acids (C_5 and above). Triglyceride hydrolysis was virtually completed within 5, 7.5 and 10 days of treatment at 35°C, 25°C and 20°C, respectively. At all three temperatures, an accumulation of free fatty acids in the reactors suggested that the rate-limiting step of the anaerobic process was fatty acid oxidation.

There is no other research comparing neutral fat hydrolysis to LCFA oxidation rate.

2.3.6 Long-chain fatty acid toxicity

The inhibitory effect of LCFAs on the anaerobic biomass was first recognised by McCarty (1964). He suggested that wastes rich in LCFAs should be digested in continuously stirred and fed reactors in order to avoid overloading. Hanaki et al. (1981) observed a lag in VFA and methane production during the anaerobic treatment of a mixture of fatty acid salts. The lag increased as LCFA concentration was increased from 250 to 2000 mg/l. They concluded that high LCFA concentrations inhibited the ß-oxidation process as well as biomass methanogenic activity. In addition, the anaerobic biomass could not develop a tolerance to high LCFA concentrations even after repeated exposure to elevated concentrations (Ahring et al., 1992; Rinzema et al., 1994).

The concentrations at which 50% of the acetoclastic methanogen activity was inhibited were estimated at 860 mg/l for lauric acid (12:0), 1231 mg/l for oleic acid (18:1), and over 1440 mg/l for caprylic acid (8:0) (Koster and Cramer, 1987). Inhibition did not seem to be a function of chain length or degree of saturation. In addition, a mixture of fatty acids was more inhibitive to the biomass than individual LCFAs suggesting a synergistic effect among the different fatty acids. Ahring et al. (1992) reported total inhibition of the methanogenic process at 500 mg/l of oleic acid. Large variations in results from different inhibition studies may stem from differences in calcium and magnesium concentrations (Koster and Cramer, 1987). Various research showed that the addition of calcium chloride with the feed helps precipitate LCFAs adsorbed on the biomass and thus reduces toxicity (Angelidaki et al., 1990; McInerney, 1988; Roy et al., 1985).

Rinzema et al. (1994) suggested that LCFA adsorption on the solid phase supported the theory which related LCFA inhibition to physical interactions with the cell membrane (Galbraith et al., 1971). Inhibition would thus be a function of the biomass to LCFA ratio. However, their experiment showed that the acetotrophic methanogens were totally inhibited at caproic acid (10:0) concentrations ranging from 7.9 to 9.0 mol/m^3 (1359 to 1548 mg/l), irrespective of biomass concentration. They concluded that inhibition was a function of absolute LCFA concentration in the reactor instead of the LCFA:biomass ratio. Rinzema et al. (1994) also estimated that only 0.2% of the acetotrophic methanogens survived the lethal caproic acid dose.

Controversy also exists concerning the effect of co-digestion of LCFAs with a more readily degradable substrate. Hanaki et al. (1981) reported that the degradation of glucose

was not inhibited by LCFA additions ranging from 250 to 2000 mg/l. On the other hand, Beccari et al. (1999) observed that 350 mg/l of oleic acid delayed methane production from glucose by almost 40 days. The thermophilic digestion of cattle manure was also delayed by the addition of oil to the digester (Angelidaki et al., 1990). The presence of glucose, however, activated oleic acid digestion (Beccari et al., 1996).

Experiments on the inhibitory effect of LCFAs on anaerobic biomass were generally conducted with liquid mixtures of fatty acid salts (Rinzema et al., 1994), esters of fatty acids in a liquid form (Angelidaki et al., 1990; Hanaki et al., 1981), or with a fatty acid emulsion under continuous stirring (Beccari et al., 1996). Fatty acids were thus readily available for hydrolysis and/or oxidation. The problem may be less serious with wastewaters containing fat in particulate form. Only the surface of fat particles is available for hydrolysis and the free LCFA concentration to which the bacteria are exposed is less than the total concentration of fat on a mass basis. Glycerol trioleate was found to be less inhibitive than oleate, which suggested that toxicity depended on the rate at which the free LCFAs are delivered (Ahring et al, 1992; Angelidaki et al. 1990).

2.3.7 Fat particles in slaughterhouse wastewater

Saxena et al. (1986) treated slaughterhouse wastewater containing from 265 to 1020 mg/l of undissolved solids, of which over half were fats, in a packed-bed anaerobic reactor. Fat and solids deposition caused a rapid deterioration of the biomass at organic loading rates exceeding 2 kg COD/m^3/d. Low VFA concentrations in the effluent throughout the experiment indicated that methanogenesis was not rate limiting. The authors suggested that the limiting step was the slow degradation rate of fat particles.

Sayed et al. (1984; 1987; 1988a; 1988b;1993) investigated the effect of high solids concentrations on the anaerobic digestion of slaughterhouse wastewater in upflow anaerobic sludge blanket (UASB) reactors operated at 20°C and 30°C. The coarse and colloidal solids were 67% lipids, while dissolved solids were mostly proteins. Early results indicated that a portion of removed COD was not converted into methane but was eliminated by other means such as flocculation and adsorption of the colloidal matter on the sludge particles and entrapment of the SS in the sludge blanket. Feed interruptions of the UASB reactors during weekends allowed partial digestion of retained solids, but when the organic loading rate (OLR) was increased to 10-20 kg/m^3/d over 57 days of continuous loading, sludge flotation led to complete biomass washout. Process failure was attributed to biomass dilution by excessive accumulation of substrate material, mainly lipids, in the reactor.

Solids removal mechanisms were further studied by feeding membrane-filtered (soluble compounds), paper-filtered (soluble and colloidal material) and unfiltered wastewater (coarse, colloidal and dissolved solids) to UASB reactors operated at 20°C and 30°C. Results indicated that (1) acidification was the limiting phase in the conversion of the soluble fraction into methane, (2) precipitation. flocculation, and adsorption on the biomass were important means of elimination of the colloidal solids, (3) the overall controlling factor of the digestion process was the liquefaction rate of adsorbed insoluble materials and entrapped coarse suspended solids, thus the use of higher temperatures was critical, (4) lipids represented the "limiting fraction" in the treatment of colloidal and suspended solids.

Fat particle degradation rate was however never evaluated during slaughterhouse wastewater digestion. In fact, no work has been published on the characterisation and quantification of anaerobic degradation of fat particles in slaughterhouse wastewater.

2.4 Particle hydrolysis in an anaerobic environment

The hydrolysis process was described in Section 2.2 for anaerobic digestion in general and in Sections 2.3.1 and 2.3.2 for fat particles. The rate of hydrolysis depends on several factors, namely the remaining concentration of particulate organics, temperature, pH, substrate composition, biomass concentration, particle size, and mass transfer. The effect of substrate composition on hydrolysis has been discussed in Section 2.2.3. The following sections will review available information on how the remaining factors affect hydrolysis in an anaerobic environment.

2.4.1 Remaining concentration of particulates

From their study on acid phase reactors, Eastman and Ferguson (1981) concluded that the rate of hydrolysis (r_h) was first-order with respect to the remaining concentration of degradable particulates:

$$r_h = k_h X_s \qquad (2.7)$$

where, r_h is hydrolysis rate (mg/l/d); k_h is the first-order hydrolysis constant (d^{-1}); and X_s is the remaining concentration of degradable particulates (mg/l). The first-order hydrolysis model was described as an 'empirical expression' regrouping various factors, such as particle size and composition, and valid for constant operating conditions such as pH and temperature.

Ray et al. (1989) observed that the first-order model underpredicted the initial degradation rate of WAS in mesophilic reactors. The data were better represented by dividing particulate organics into rapidly and slowly degradable particles. Each fraction had its own hydrolysis rate that was first-order with respect to the remaining concentration of particles:

$$r_{arh} = k_{hr}A_r \tag{2.8}$$

and

$$r_{ash} = k_{hs}A_s \tag{2.9}$$

where, A_r and A_s are the concentrations of rapidly and slowly degradable particles, respectively (mg/l); r_{arh} and r_{ash} are the hydrolysis rates of the rapidly and slowly degradable particles, respectively (d^{-1}); k_{hr} and k_{hs} are first-order hydrolysis constants for particulate fractions A_r and A_s, respectively (d^{-1}). The concentrations of the two particulate fractions was not experimentally determined, but the authors assumed that A_r represented 5% of the total.

2.4.2 Temperature

Henze and Mladenovski (1991) reported that WAS hydrolysis rate was increased by a factor of 5 as reactor operating temperature went from 12°C to 20°C. They also observed a stronger dependence of anaerobic than aerobic hydrolysis on temperature. Kaijun et al. (1995) observed that the hydrolysis rate of raw domestic sewage sludge increased from 0.007 to 0.024 d^{-1} between 10°C and 20°C (a factor of 3.4), and from 0.024 to 0.105 d^{-1} between 20°C and 30°C (a factor of 4.4). The effect of temperature on hydrolysis is thus less than expected using the van't Hoff equation, which stipulates that the rate of chemical reaction will double with each 10°C rise in temperature. Switzenbaum and

Jewell (1980) also found that the effect of temperature on anaerobic processes was less than predicted by the van't Hoff equation.

2.4.3 pH

Most research on pH effect investigated the extent as opposed to the rate of particulate hydrolysis as pH was changed. The effect of pH appeared to be dependent on waste composition, but contradictory results have been reported. Eastman and Ferguson (1981) observed a higher degree of protein and carbohydrate solubilization at pH 6.67 than 5.15 during pretreatment of WAS for 36 h in acid phase reactors. Lipid was not hydrolysed at any pH, but lipid hydrolysis may have been overlooked because of free LCFA adsorption on the solid phase. Elefsiniotis and Oldham (1994) also observed an increase in carbohydrate degradability as pH was increased from 4.3 to 6.1 during the acid phase of primary sludge digestion. However, protein degradability decreased with an increase in pH, while lipid hydrolysis proceeding at a similar rate at all pH. Chyi and Dague (1992) observed an increase in cellulose solubilization as pH was decreased from 6.8 to 5.6 in acid phase reactors operated at an hydraulic retention time (HRT) of 48 h.

All the above studies were conducted in acid phase reactors where pH can be as low as 4.5 without affecting acidogenic bacteria (Marty, 1986). In reactors combining the acidogenic and methanogenic phases of anaerobic digestion, pH may be limited to the optimum range for methanogen growth, i.e. to values between 6.7 and 7.4 (Grady et al., 1999), and pH effect may be marginal.

2.4.4 Biomass concentration

Grady et al. (1999) argued that the first-order model was an oversimplification of the hydrolysis process. Since hydrolysis depends on the presence of extracellular enzymes, it

should be controlled by the ratio of particulate substrate to heterotrophic biomass concentration, as well as being first-order with respect to the heterotrophic biomass concentration, as described by the equation developed by Dold et al. (1980):

$$r_h = -k_h \left[\frac{X_s / X_{B,H}}{K_s + (X_s / X_{B,H})} \right] X_{B,H} \tag{2.10}$$

where, $X_{B,H}$ is the heterotrophic biomass concentration; and K_s is the half-saturation concentration. If biomass concentration is in excess, hydrolysis is first-order with respect to degradable particles, and equation 2.10 is equivalent to Eastman and Ferguson's first-order model. If substrate concentration is much larger than biomass concentration and K_s, then the reaction is first-order with respect to $X_{B,H}$ only.

However, excess biomass concentration remains to be defined for different waste compositions. Lee and Fan (1982) reported that increasing cellulase concentrations over 0.98 g/l had marginal effects on the hydrolysis of a 50-g/l cellulose substrate (substrate to biomass ratio of 50:1 on a weight basis). San Pedro et al. (1994) found that varying mixed-liquor volatile suspended solids (VSS) concentration between 164 and 400 mg/l had no significant effect on the hydrolysis of a substrate containing between 350 and 800 mg/l of starch and treated in aerobic-anaerobic sequencing batch reactors (substrate to biomass ratio ranging from 1.1:1 to 3.8:1).

Valentini et al. (1997) developed a mathematical model attenuating the effect of biomass concentration as the latter increased. They suggested that hydrolysis is between zero and first-order with respect to biomass concentration:

$$r_h = k_{h,A} \, X_s \, X_B^A \tag{2.11}$$

where, $k_{h,A}$ is a constant whose dimension varies according to the value of A; X_B is biomass concentration (mg/l); and A is a constant ranging from 0 to 1. With A equal to 0, the process is first-order with respect to substrate concentration only, and with A equal to 1, it is first-order with respect to both substrate and biomass concentrations. During cellulose hydrolysis, the value of A was estimated at 0.42. Valentini et al. (1997) argued that the physical meaning of the exponent A is that when enzyme concentration is low, all the enzyme molecules reach the substrate, whereas only a fraction of the enzyme is active at high concentration.

2.4.5 Mass transfer

Hobson (1983) reported that the colonisation of fibre particulates by hydrolytic bacteria occurred rapidly in the rumen and bacterial mass transfer was not rate limiting. Lee and Fan (1982) observed similar rates of enzyme adsorption on cellulose in stationary and vigorously agitated jar tests. They concluded that enzyme migration to the particle surface was not enhanced by mixing and thus cellulose hydrolysis was not controlled by enzyme mass transfer. Jain et al. (1992), on the other hand, estimated that mass transfer was the rate-controlling resistance of cellulose hydrolysis in fixed-film reactors. The role of enzyme or bacteria mass transfer during hydrolysis may depend on reactor configuration. It may not be an important factor in mixed reactors.

2.4.6 Particle size

Hydrolysis is a surface phenomenon: bacteria attach themselves to solids, erode the surface around them and, in the process, reduce particle size (Hobson. 1985). Consequently, the rate of hydrolysis depends on the number of sites available for colonisation and reaction, as opposed to total particle mass. Based on a spherical

representation of waste particles, smaller size particles will offer a greater surface area for hydrolysis reaction to occur than larger particles. Hills and Nakano (1984) observed a threefold increase in gas production when tomato peel size was reduced from 20 to 1.3 mm and the substrate was fed to mesophilic digesters at an HRT of 18 d. Reducing cellulose particle size from 50 to 20 μm increased solubilization by 1.6 times in a mesophilic acid phase reactor at HRTs ranging from 36 to 72 hr (Chyi and Dague, 1992). Over a 300-d digestion period in reactors operated at 17°C, gas production was increased 4.4 times when solid waste (37% paper) particle size was reduced by a factor of 10 (DeWalle et al., 1978). However, methane was only produced at the larger particle size, and the authors suggested that rapid hydrolysis of the smaller particles resulted in high VFA concentrations, which were inhibitive to the methanogens.

Sanders et al. (1999) related hydrolysis rate to the size reduction of spherical particles:

$$\eta = \sum_{0}^{n} 1 - \frac{(R_0 - k_h t)^3}{R_0^3} \frac{M_0^p}{M_0} \tag{2.12}$$

where, η is the fraction of hydrolysed starch mass; R_0 is the radius of particles within a range n (m); M_0 is total mass of particles (kg); and $M^p{}_0$ is the mass of particles within size range n (kg). The model fitted the hydrolysis of spherical starch particles with initial diameters ranging from 18 to 47 μm relatively well. The model could also predict the distribution of starch particle sizes ranging from 10 to 75 μm during the initial 24 hours of digestion.

For most waste, however, particles are not well approximated by a spherical model. Electron microscope pictures of pig slurry showed that solid spheres were the exception (Hobson, 1985). Pig slurry particles were better represented by forms like cylinders and

plates, which do not offer substantially more surface area when size is reduced. The hydrolysis model developed by Vavilin et al. (1996) considered particle shape:

$$r_h = k_h X_F^{1-n} X^n \tag{2.13}$$

where, X_F is the concentration of influent biodegradable organic matter (mg/l); X is the concentration of biodegradable suspended solids (mg/l); and n is the degree coefficient. The degree coefficient depends on particle shape. It is equal to 2/3 and 1/2 for spherical and cylindrical particles, respectively. The hydrolysis constant, k_h, is a function of particle diameter, particle density, biomass density and depth of bacterial layer on the particles, which makes it a highly variable number. Equation 2.13 and the first-order model were used to predict VSS degradation and biogas production rate during the anaerobic digestion of swine waste, sewage sludge, cattle manure and cellulose. Both equations fitted the experimental data equally well, except at low solids retention time, because the first-order model could not describe hydrolytic bacteria washout.

Negri et al. (1993) included hydrolytic biomass concentration as well as reaction surface in their first-order hydrolysis model:

$$r_h = k_h A_{hrs} X_h \tag{2.14}$$

where A_{hrs} is hydrolytic reaction surface (m), X_h is the concentration of hydrolytic enzymes (mg/l) and k_h is in m^2/d. Both the shape and size of particles must be known to determine the hydrolytic reaction surface. The model was used to simulate the hydrolysis of spheres of uniform diameters in plug-flow reactors, but was not validated with real data.

Hills and Nakano (1984) suggested that particle shape changed with size. They observed that tomato peel particles were less spherical and more plate-like as their size

increased. Their model included a factor attenuating the effect of particle diameter as the latter increased:

$$\frac{dG}{dt} = k_h \frac{1}{\Phi_s D_p} \tag{2.15}$$

where, G is methane production; D_p is particle diameter; Φ_s is a sphericity factor. Variable units were not specified. The value of Φ_s ranges from 0.28 for mica flakes to 1.0 for spheres, cubes and short cylinders. For tomato peels, the sphericity factor decreased from 1.0 to 0.3 as particle size increased from 1.3 to 20 mm. Although the model and measurement of D_p and Φ_s needed refinement, the authors concluded that during tomato peel digestion, methane production rate varies inversely to the product of the diameter and the sphericity factor.

As this review suggests, the effect of particle size on hydrolysis will depend on waste composition. Moreover, waste will often be composed of particles having a variety of shapes. Particle shape may also change as hydrolysis proceeds. More investigation will be needed to fully understand particle size effect on anaerobic hydrolysis.

2.5 Hydrolysis pretreatments

Hydrolysis is generally considered the rate-limiting step in the anaerobic digestion of wastes containing a sizeable amount of particulates (Pavlostathis and Giraldo-Gomez, 1991). For high solids waste, the maximum substrate concentration available to the biomass is the product of hydrolysis, not the raw waste fed to the digester (Gujer and Zehnder, 1983). Therefore, increasing hydrolysis and waste liquefaction has been the objective of many studies. The three main methods used to increase waste liquefaction prior to or during anaerobic digestion were heat, chemicals and biocatalysts.

2.5.1 Thermal pretreatment

Heating waste to 175-200°C under high pressure for 30 to 60 minutes was found to increase VS degradation in WAS (Anon. 1996; Haug et al., 1983; Stuckey and McCarty, 1984) and municipal refuse (Gossett et al., 1982), but it did not affect the treatment of primary sludge (Haug et al., 1978). Below 80°C, improvement in organic matter destruction was minimal (Hiraoka et al., 1984); above 200°C, the solubilized waste became toxic to the anaerobic biomass (Gossett et al., 1982; Stuckey and McCarty, 1984). The main beneficial effect of thermal pretreatment on sludge appeared to be the decomposition of triglycerides into VFAs (Hiraoka et al., 1984).

Haug (1977) argued that thermal treatment of WAS and sewage sludge was an economically sound practice because of increased gas production during thermophilic digestion. However, heat treatment could only apply to waste containing a very high solids content. For mostly soluble waste, the high-energy input may not compensate for the increase in treatability or in methane production (van Velsen and Lettinga, 1980). In addition, thermal pretreatment necessitates extensive infrastructure installation and, in certain cases, equipment with high-pressure tolerance.

2.5.2 Chemical pretreatment

Chemical treatment usually refers to the addition of an alkali or an acid to the feed, although the former is preferred since residual alkalinity is more compatible with anaerobic digestion than acidity (Gossett et al., 1982; Rajan et al., 1989). Alkali were first used in conjunction with thermal pretreatment to increase solubilization and control the inhibitory effect of some components. especially acidic compounds, released by heating (Gossett et al., 1982; Stuckey and McCarty, 1984; van Velsen and Lettinga, 1980). Large

doses of alkali were generally used (185 to 300 meq/L), and chemical costs combined with the energy required for thermal pretreatment were not worth the increase in treatment and the concomitant increase in biogas production (van Velsen and Lettinga, 1980).

Rajan et al. (1989) tested the effect of small doses (5 to 80 meq/l) of NaOH on WAS solubilization at ambient temperature. Mixing a 1% solids WAS for 12 hrs with 10 to 40 meq NaOH/l increased the ratio of soluble to total COD (SCOD:TCOD) by 13 to 42%. Higher NaOH doses did not significantly increase solubilization. Increasing pretreatment temperature from 20°C to 38°C resulted in a higher SCOD:TCOD ratio at NaOH doses below 12 to 16 g/100 g total solids, but temperature effect was marginal at higher doses. A sufficient drop in pH occurred during WAS pretreatment to avoid the need for waste neutralisation prior to anaerobic digestion (Huang et al., 1989; Rajan et al., 1989). Pretreating WAS with 20 meq NaOH/L for 24 hours at room temperature significantly improved VS reduction during anaerobic digestion and allowed a decrease in HRT from 20 to 7.5 days (Ray et al., 1990). Similar results were reported by Knezevic et al. (1995) and Lin et al. (1997) when pretreating WAS with 15 to 40 meq NaOH/L under ambient temperature and anoxic mixing for 5 to 24 hours.

Rajan et al. (1989) observed that NaOH increased WAS hydrolysis during the initial 30 minutes of pretreatment. Between 0.5 and 12 hrs of pretreatment, however, the first-order hydrolysis constant for the reaction was in the same order of magnitude as that estimated by Eastman and Ferguson (1981) for WAS hydrolysis in an acid phase reactor. The authors concluded that chemical pretreatment only boosted the liquefaction of the rapidly degradable particulates in WAS.

Karlsson (1990) tested various chemical (HCl, NaOH and $Ca(OH)_2$) pretreatments on WAS, and compared the results to biological hydrolysis during the acid phase of anaerobic treatment. Experimental conditions and procedures were not specified, but pretreatment efficiency was evaluated by a decrease in the carbohydrate, protein and lipid fractions in the "sludge phase". Biological and NaOH pretreatments decreased the amount of carbohydrate in the sludge phase by 40% and 26%, respectively. Biological, hydrochloric acid and NaOH pretreatments reduced protein in the sludge phase by 20%, 40% and 73%, respectively. For lipids, biological pretreatment showed no measurable effect, while acid and alkaline pretreatments both reduced lipid in the sludge phase by 28%.

2.5.3 Biocatalysts

Biocatalysts or bioaugmentation can be broadly defined as substances 'that activate or stimulate a biochemical reaction' (McKee et al., 1954). They usually consist of enzymes, preserved microorganisms and/or trace minerals. In the 1950's, a variety of biocatalysts were put on the market for use in the field of waste treatment. These products, it was claimed, helped reduce odours, scum formation, hydrogen sulfide concentration, VSS, and biological oxygen demand (BOD), as well as improved liquefaction, oxidation and biogas production (McKee et al., 1954). Some waste treatment plant operators reported positive results from the use of biocatalysts in anaerobic reactors (Anon., 1952; Corder, 1955). When tested under controlled laboratory conditions, however, the biocatalysts did not significantly improve VS reduction, grease liquefaction, pH, alkalinity, or biogas production (Grune and Sload, 1954; Heukelekian and Berger, 1953; McKinney, 1953; Pearson et al., 1957; Wells and McKinney, 1955). Heukelekian and Berger (1953) argued

that in a well-operated digester, biomass composition adjusted to the substrate and needed bacterial population developed naturally without the help of additives. Additionally, hydrolysing bacteria attach themselves to the particulates and secrete the enzymes *in situ*, thus creating around the particle an enzyme concentration that is much higher than that obtained with an enzyme supplement diluted in the mixed-liquor.

More recent studies, however, have reported better results from the use of biocatalysts. An enzymatic mixture containing protease, amylase, cellulase and lipase as well as *Bacillus subtilis* spores was tested on wastewaters high in lipid content (Bell and Oxham, 1971; Cail et al., 1986). The wastewater was pretreated for 24 h with the biocatalyst and digested for 2.8 d in mesophilic reactors. COD reduction increased from 59% in the control to 78% in reactors with pretreatment, grease removal increased from 47% to over 70%, and VS reduction from 34% to over 70% (Cail et al., 1986). Lagerkwist and Chen (1993) added a cellulolytic enzyme during the acidogenic phase of municipal solid waste digestion. The biocatalyst enhanced cellulose degradation during the acidogenic phase from 29% to 42%. Aoki and Kawase (1991) tested the effect of a protolytic enzyme pretreatment on municipal sewage sludge hydrolysis. The sludge was first heated to 90°C for one hour and enzyme was added immediately before the substrate was fed to a thermophilic acid-phase reactor. The pretreatment increased VS reduction from 49% in control to 58% with pretreatment. However, the biocatalyst was not tested without the thermal conditioning such that enzymatic pretreatment effect could not be isolated.

Some reports on the testing of biocatalysts remain negative. Adding enzyme supplements (lipase, cellulase and protease) to an anaerobic reactor digesting household solid waste did not increase degradation compared to the control (Rintala and Ahring,

1994). Koe and Ang (1989) tested the effect of a biocatalyst on the anaerobic digestion of domestic sewage sludge spiked with high doses of fatty acids and did not observed any beneficial effects. The enzymatic composition of the product was not reported but the biocatalyst was mainly composed of facultative bacteria that ferment substrate into acids (Koe and Ang, 1992). The authors argued that the biocatalyst would not improve a well functioning reactor since all the bacteria it contained were already present in the digester in proportion suitable for treating the substrate.

It seems that biocatalysts need to be better characterised in terms of their effects on waste solubilization, microorganism lysis, particle size reduction, and waste degradability. Their impact on the diverse components of waste – protein, carbohydrate and lipids – also needs to be documented.

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CHAPTER 3

TESTING OF ALKALINE AND ENZYMATIC HYDROLYSIS PRETREATMENTS FOR FAT PARTICLES IN SLAUGHTERHOUSE WASTEWATER

Abstract

Four pretreatments to hydrolyse and/or reduce the size of beef or pork fat particles in slaughterhouse wastewater (SHW) were tested: sodium hydroxide and three lipases of plant, bacterial and animal (pancreatic) origin. Hydrolysing agents and SHW containing between 2.5 and 3 g/l of fat particles were mixed at room temperature for 4 h. Additions of 5 to 400 meq NaOH/l did not increase soluble COD (SCOD) in SHW, but the average pork fat particle size was reduced to 73% ± 7% of the initial average particle size (D_{in}) at NaOH concentrations ranging from 150 to 300 meq/l. Pretreatment with pancreatic lipase PL-250 reduced the average pork fat particle size to a maximum of 60% ± 3% of D_{in}. As pork fat D_{in} was decreased from 359 to 68 μm, the enzyme concentration required to obtain the maximum particle size reduction increased from 200 to 1000 mg/l. A 4-h pretreatment with PL-250 also increased the free long-chain fatty acid (LCFA) concentration in solution to a maximum of 15.5 mg/l, indicating some solubilization of the pork fat particles in SHW. Soluble COD was not significantly increased by the pretreatment, but SCOD is not a good indicator of enzymatic lipolysis because of enzyme and LCFA adsorption on the fat particle surface. Pancreatic lipase appeared more efficient with beef fat than pork fat, possibly because beef fat contains less

polyunsaturated fatty acids than pork fat. The bacterial lipase LG-1000 was also efficient in reducing average fat particle size, but high doses (> 1000 mg/l) were required to obtain a significant reduction after 4 h of pretreatment. Soluble COD was not increased by pretreatment with LG-1000. No particle size reduction or changes in SCOD were noted after 4 h of pretreatment with the plant lipase EcoSystem Plus. It was concluded that PL-250 was the best pretreatment to hydrolyse fat particles in SHW. However, its impact on the efficiency of a downstream anaerobic digestion process remains to be tested.

Keywords: Lipid, fat, long-chain fatty acid, hydrolysis pretreatment, lipase, sodium hydroxide, alkaline treatment, particle size

3.1 Introduction

Undissolved solids represent between 30% and 75% of the polluting charge in slaughterhouse wastewater (SHW) (Massé and Masse, 2000; Moodie and Greenfield, 1978). The anaerobic treatment of SHW is often slowed or impaired because of high concentrations of suspended solids (SS). Saxena et al. (1986) treated SHW containing approximately 1000 mg SS/l in a packed-bed anaerobic reactor. Fat and SS deposition caused a rapid deterioration of the biomass at organic loading rates exceeding 2 kg COD/m^3/d. The first few cells of an anaerobic baffled reactor treating meat packing plant wastewater eventually became filled with solids (Martínez et al., 1995). The system basically acted as a solids separator and operated marginally as a biological treatment process. Sayed et al. (1984; 1987; 1988a; 1988b) investigated the effect of high SS concentrations on the anaerobic treatment of SHW. Their results suggested that the process-controlling factor was the liquefaction of colloids adsorbed on the bacteria and

the hydrolysis of coarse SS entrapped within the biomass bed. They concluded that lipids represented the limiting fraction in the treatment of solids.

The application of a pretreatment to hydrolyse SS, especially fats, may improve the anaerobic digestion of unsettled SHW. Rajan et al. (1989) pretreated waste activated sludge (WAS) containing 1% of undissolved solids with 10 to 40 meq NaOH/l. The ratio of soluble to total COD was increased by 13 to 42%. Pretreating WAS with 20 meq NaOH/l for 24 h significantly increased volatile solids reduction during anaerobic digestion and permitted a decrease in the hydraulic retention time from 20 to 7.5 d (Ray et al., 1990). Similar results were reported by Knezevic et al. (1995) and Lin et al. (1997).

Karlsson (1990) tested various chemical (HCl, NaOH and $Ca(OH)_2$) and biological (acid fermentation) hydrolysis pretreatments on WAS. Experimental conditions and procedures were not specified, only that pretreatment efficiency was evaluated by a decrease in the carbohydrate, protein and lipid fractions in the 'sludge phase'. Biological and NaOH pretreatments decreased the amount of carbohydrate in the sludge phase by 40% and 26%, respectively. Biological, hydrochloric acid and NaOH pretreatments reduced protein in the sludge phase by 20%, 40% and 73%, respectively. For lipids, biological pretreatment showed no measurable effect, while acid and alkaline pretreatments both reduced lipid in the sludge phase by 28%. This experiment suggested that the lipid portion of WAS is the most difficult organic fraction to hydrolyse using pretreatments.

Enzyme supplements have also been used to increase hydrolysis during or prior to anaerobic digestion and mixed results were obtained (Aoki and Kawase, 1991; Bell and Oxham, 1971; Cail et al., 1986; Lagerkwist and Chen, 1993; Rintala and Ahring, 1994).

An enzymatic mixture containing protease, amylase, cellulase and lipase as well as *Bacillus subtilis* spores was tested on wastewaters high in lipid content (Bell and Oxham, 1971; Cail et al., 1986). Pretreating the wastewater for 24 h with the biocatalyst increased COD reduction from 59% in the control to 78% in reactors with pretreatment, grease removal from 47% to over 70%, and solids reduction from 34% to over 70% (Cail et al., 1986). On the other hand, when an enzymatic supplement (lipase, cellulase and protease) was injected directly into an anaerobic reactor treating household solid waste, the digester did not perform differently from the control (Rintala and Ahring, 1994).

Hydrolysis pretreatments mostly have been tested on WAS or municipal waste, which contain at least ten times more SS than SHW. Moreover, experiments usually have dealt with complex waste, and pretreatment effects on the different organic fractions (protein, lipid, carbohydrate) have seldom been assessed. The fate of lipids is particularly important for the stability of anaerobic processes. During lipolysis, long-chain fatty acids (LCFAs) are released into solution, and high free LCFA concentrations can have an irreversible inhibitory effect on anaerobic bacteria (Ahring et al., 1992; Angelidaki et al., 1990; Hanaki et al., 1981; Koster and Cramer, 1987; Rinzema et al., 1994). Finally, pretreatments have been evaluated solely by their ability to increase soluble COD and not by their efficiency in decreasing the size of particles in solution. Smaller particles have a higher surface to volume ratio, and thus offer a larger surface area for bacterial colonisation and reaction. Particle size reduction has been shown to increase the rate and extent of anaerobic treatment (Chyi and Dague, 1992; DeWalle et al., 1978; Hills and Nakano, 1984; Levine et al., 1991). Thus, there is a need for research on hydrolysis pretreatments dealing with the lipid fraction of relatively dilute organic wastewaters, such

as those found in the food industry, and with particle size reduction as well as solids solubilization.

The objective of this research was to evaluate the effect of alkaline and enzymatic pretreatments on solubilization and size reduction of pork or beef fat particles in SHW. Slaughterhouse wastewater was chosen as the liquid substrate because this experiment was part of a larger study evaluating effects of pretreatments on anaerobic digestion of SHW. Sodium hydroxide was selected as the alkaline pretreatment since it already had produced positive results with sludges. Three commercial lipases of plant, microbial and animal origin also were tested because these products are becoming more available commercially. The first enzyme was a pork pancreatic lipase called Pancreatic Lipase 250 (PL-250, Genencor International, Rochester, N.Y.). Pancreatic Lipase 250 is claimed to be efficient for hydrolysing triglycerides containing LCFAs with more than 12 carbons, such as those in animal fat. The second enzyme was a bacterial lipase extracted from *Rhizomucor miehei* called Lipase G-1000 (LG-1000, Genencor International, Rochester, N.Y). Lipase G-1000 is reported to hydrolyse natural fats, such as oils, beef tallow, butter fats and lard oil, with a preference for shorter chain fatty acids (< 12 carbons). The third enzyme was a plant lipase called EcoSystem Plus (ESP, Neozyme International Inc., Newport Beach, Ca.). Neozyme claims that ESP effectively breaks down fat particles in aerobic or anaerobic environments.

3.2 Material and Methods

3.2.1 Substrate

Pork or beef fat in distilled water was cut into small particles with a rotating knife in a food processor. The solution was filtered through a metal sieve to remove particles greater than 1410 µm and stored at 4°C for a maximum of 4 d. Different fat particle size distributions were obtained by filtering out particles using metal sieves with pore sizes of 100, 250 500 and 1000 µm. The SHW was collected from a beef and hog abattoir in Embrun, Ontario, and stored at −10°C. The wastewater contained SS, including pieces of meat and hair, which were removed by filtering through a 38 µm metal sieve. Solids distribution in filtered SHW is shown in Table 3.1. The experimental substrate consisted of a 50:50 mixture of SHW and distilled water containing pork or beef fat particles.

Table 3.1 Solids distribution in filtered slaughterhouse wastewater

Solids	Concentration (mg/l)
Total	5600
Total volatile	1500
Dissolved	5150
Between 1 and 10 µm	400
Larger than 10 µm	50

3.2.2 Experimental conditions

Tests were conducted by adding various enzyme and NaOH concentrations to 800 ml of substrate in 1000-ml beakers. An experiment usually included three to five product concentrations and two controls. Beakers were gently mixed (60 rpm) at room temperature (23 ± 2°C) with paddle type mixers to prevent fat particle flotation. The

amount of fat sticking onto the paddle mixer or the glass beaker surface was minimal. Controls received no hydrolysing product but were also mixed at room temperature for the duration of the experiment. Most tests lasted 4 h, but some experiments were extended to 24 h, and during one experiment with NaOH, samples were collected after 15, 40, 60 and 120 minutes of pretreatment. However, the main purpose of the experiment was to determine which pretreatment scenario best hydrolysed fat particles or reduced their size after a fixed period of time (4 h), not to calculate hydrolysis rates.

The effect of NaOH was tested on SHW containing approximately 3 g/l of pork fat particles. Initial average particle size (D_{in}) and NaOH doses are given in Table 3.2. Experiments with PL-250 are presented in Table 3.3. They included varying D_{in}, fat particle concentration, and type (beef or pork) of fat. The effect of the bacterial lipase LG-1000 was tested on two solutions of SHW containing approximately 2.5 g/l of pork fat particles. The first solution had a D_{in} of 166 ± 15 μm and received seven LG-1000 doses ranging from 50 to 5000 mg/l. The second solution had a D_{in} of 271 ± 11 μm and received 500, 2000 and 3500 mg/l of LG-1000. Four ESP concentrations (50, 100, 500 and 5000 μl/l) were tested on SHW containing approximately 3 g/l of pork fat particles with a D_{in} of 290 ± 19 μm.

Table 3.2 Effect of a 4-h NaOH pretreatment on the average size of pork fat particles, SCOD and pH in slaughterhouse wastewater

D_{in} (µm)	NaOH (meq/l)	pH	Ratio $D_{treated}$: D_{in}	Ratio $SCOD_{treated}$: $SCOD_{control}$
161 ± 4	60	11.0	1.02	1.05
	120	12.6	0.90	0.96
218 ± 8	250	13.2	0.67	1.01
	250	13.2	0.79	0.93
255 ± 7	50	11.4	0.89	0.82
	100	12.9	0.81	0.92
	150	13.1	0.72	0.99
	250	13.3	0.80	1.02
	300	13.3	0.81	1.12
	350	13.4	0.83	1.07
315 ± 15	200	13.2	0.77	0.96
	250	13.2	0.68	0.99
	300	13.3	0.67	0.99
	400	13.4	0.81	0.97

D_{in} = initial average particle size
$D_{treated}$ = average particle size after 4 h of pretreatment
$SCOD_{control}$ = SCOD in control samples after 4 h of mixing
$SCOD_{treated}$ = SCOD after 4 h of pretreatment minus SCOD from the enzyme

Table 3.3 Experiments conducted with Pancreatic Lipase 250 on slaughterhouse wastewater containing fat particles

Fat origin	Fat particle conc. (g/l)	Initial particle size (µm)	PL-250 conc. (mg/l)
Pork	2.5	68 ± 5	30, 100, 250 , 500, 1000, 3000
		109 ± 7	5, 20, 60, 80*, 125*, 250**, 350, 500**, 3000
		147 ± 14	10, 25, 50, 100*, 150*, 200, 250*, 300*, 400*, 500, 1000
		254 ± 8	1000*
		359 ± 26	25, 80, 150** , 250, 500*, 1000*, 3000
	8	126 ± 2	50, 100, 150, 200, 275, 300, 400, 450, 500
	0.8	75 ± 13	50*, 275, 400, 500
Beef	2.5	225 ± 19	25, 50, 100, 250*, 350, 500*, 1500, 5000

* Dose tested in duplicate (2X)
** Dose tested in triplicate (3X)

3.2.3 Particle size analysis

Particle size distribution was measured by serial filtration of a 100 ml sample through nylon and polyethylene mesh-filters with openings of 925, 526, 292, 114, 53 and 10 μm. Between each filtration, funnel and flask were thoroughly rinsed with distilled water to ensure that all fat particles with a diameter less than the filter pore size passed through the filter. A portion of the filtrate from the 10-μm filter, representing about 15 ml of treated substrate, was passed through a 1-μm isopore polycarbonate membrane filter. All filters were dried at 105°C for 24 h prior to being weighted.

Serial filtration has been shown to be a reliable method to determine particle size distribution in wastewater (Levine et al., 1985; Neis and Tiehm, 1997). However, Faisst (1980) reported that accumulated solids sometimes formed a cake on the filter, thus decreasing the effective filter opening size. In this experiment, error due to cake formation was estimated by filtering 50 and 100 ml samples from eight fat particle substrates. Results were similar in both filtered volumes. Coefficients of variation (CVs; standard deviation over average) were generally below 20% for weight of particles retained on individual filters, and below 7% for average particle size. There was no systematic bias due to sample volume. All controls as well as samples pretreated with ESP were also analysed in duplicate using the same sample volume (100 ml). Coefficients of variation between replicate analyses were less than 10% for 72% of the filters, and above 20% for 9% of the filters. High CVs were associated with filters with larger pore sizes (526 and 925 μm). However, CVs for average particle size were usually well below 10%, because the averaging process attenuated differences in individual size ranges. Preliminary experiments with serial filtration of fat particles in SHW thus

indicated that the method was reliable, especially for determination of average particle size.

3.2.4 Chemical analyses

Soluble COD was determined in filtered samples (0.45 μm filter) by the closed reflux titrimetric method (APHA, 1992).

Free LCFA concentrations were analysed according to the method outlined in Chou et al. (1996; 1998). Briefly, 10 ml of filtered (0.45 μm filter) sample was acidified with 2 drops of 50% H_2SO_4 and extracted three times with 2.5 ml of hexane. The organic layer was concentrated to about 0.5 ml under nitrogen flow, mixed with a cocktail of methanol, toluene and H_2SO_4, and heated to 80°C for 16 h. The cocktail was then extracted three times with hexane. The hexane layer was mixed with an equal amount of pH 11 phosphate buffer, transferred to v-bottomed vials, and evaporated to dryness under nitrogen. Vials were then washed with a known quantity of hexane, between 200 and 500 μl, which was transferred to chromatographic vials and stored at –20°C. Samples were analysed with a model 3700 gas chromatograph (Varian, Sunnyvale, Ca.) equipped with a flame ionisation detector. The identification and quantification of the eluted LCFAs were accomplished by comparing their retention time and area with those of known standards on a non-polar capillary DB-5 column. All samples were extracted and analysed in duplicate. Injecting increasingly diluted standards showed that the area under the peak was proportional to LCFA concentration. Wastewater samples were also spiked with LCFAs and recovery averaged 91.9% ± 10.0%.

LCFAs were measured on approximately half of the samples from the most efficient pretreatment (PL-250). Analysed samples included all those at the largest and smallest

D_{in} (68 and 357 μm) at a pork fat particle concentration of 2.5 g/l. At a D_{in} of 110 μm, only the six replicate samples, at PL-250 concentrations of 125, 250 and 500 mg/l, were analysed for LCFAs. Samples containing beef fat were also analysed for LCFA concentration, except the three samples at a D_{in} of 272 μm.

3.2.5 Data and statistical analyses

Average particle size. On a mass basis, particle size distribution in a fluid tends to be log-normal and skewed toward lower particle sizes (Cooper and Alley, 1994). Average particle size can be calculated using (Levine et al., 1991):

$$\ln D = \frac{\sum_{i=1}^{n} m_i \ln D_i}{\sum_{i=1}^{n} m_i} \tag{3.1}$$

where, D is the geometric mass mean size (μm); D_i is nominal size of particles in size range i, set to size between two consecutive filters (μm); and m_i is mass of solids in size range i (g).

In the experimental substrate, fat particle size distribution was found to be log-normal, but the skew shifted to larger particle sizes as D_{in} was increased. Equation 3.1 yielded an average particle size equivalent to the 'median size' on a mass basis, i.e. half the weight of particles was above average size and half was below it.

Data analysis. Particle size and SCOD data collected during the experiments with NaOH, LG-1000 and ESP were analysed as ratios of treated to control values ($D_{treated}$:D_{in} and $SCOD_{treated}$:$SCOD_{control}$). Since the analysis showed that average particle size was not altered by mixing the substrate at room temperature, average particle size in control

samples was taken as D_{in}. Soluble COD, on the other hand, was lower in controls subjected to 4 h of mixing at room temperature than in the initial substrate. Consequently, $SCOD_{control}$ was taken as the SCOD concentration in the control samples after 4 h of mixing. The value of $SCOD_{treated}$ was equivalent to the SCOD measured in pretreated samples minus the SCOD added with the enzyme. Ratios were fitted to first-order, linear and quadratic equations with respect to product concentration. Parameters were estimated using SAS 6.08. The program also provided analyses of variance (F-test) for parameters and models, as well as 95% confidence intervals.

For the PL-250 experiments, sufficient particle size data were collected to analyse results from each D_{in} separately. Data from each D_{in} were fitted to a first-order equation of the form:

$$D - D_{min} = (D_{in} - D_{min})e^{-bC_{PL-250}} \quad (3.2)$$

where, D is the average size of particles in pretreated substrate (μm); D_{min} is the minimum average particle size (μm) that can be obtained by a 4-h treatment with PL-250; D_{in} is the initial average particle size (μm); C_{PL-250} is PL-250 concentration (mg/l); and b is the first-order coefficient (l/mg).

SAS 6.08 provided standard errors for the parameters and the model, the asymptotic 95% confidence interval for each parameter, and the coefficient of correlation between the two parameters. The statistical significance of all non-linear models was verified by comparing pure error (error between true replicates at each D_{in}) to model error (Borowiak, 1989). The significance model had the form:

$$G_F = \frac{\left[\dfrac{SS_{res} - \gamma_{pe}S_{pe}^2}{n_s - p - \gamma_{pe}}\right]}{S_{pe}^2} \quad (3.3)$$

where, SS_{res} is sum of square of residuals for the model; S^2_{pe} is the pooled estimate of variance of replicates (pure error); γ_{pe} is the degree of freedom of S^2_{pe}; n_s is the total number of data points for the model; and p is the number of parameters in the model. The model is considered significant when the value of G_F was smaller than that of $F_{(n-p-\gamma_{pe},\ \gamma_{pe},\ 0.05)}$.

The relation between the parameters of Equation 3.2 (D_{min} and b) and D_{in} was examined using the linear regression function in Excel 97. Results from this analysis were used to modify Eq. 3.2 in accordance with the empirical model building technique of Scott and Sylvestre (1979). The technique consists of fitting sets of parameter estimates (D_{min} and b) to experimental conditions (D_{in}). It generates fewer parameters than other techniques such as straight polynomial fitting. Although essentially empirical, it allows some insight into the process mechanisms.

3.3 Results and Discussion

3.3.1 Sodium hydroxide

Sodium hydroxide concentrations ranging from 50 to 400 meq/l were tested on SHW containing 3 g/l of pork fat particles. The SCOD concentration of the substrate was not significantly affected by 4 h of pretreatment with NaOH. Except for one sample (50 meq/l), in which an 18% decrease in SCOD was measured following pretreatment, the ratio $SCOD_{treated}$:$SCOD_{control}$ ranged from 0.92 to 1.12, for an overall average of 1.00 (Table 3.2). Animal fats are mainly composed of saponifiable lipids, i.e. they form water-soluble products or soaps when subjected to alkaline hydrolysis (Gaudy and Gaudy,

1980). However, under the conditions of this experiment, water-soluble products could not be produced in measurable quantities. Karlsson (1990) reported that NaOH was much more efficient in hydrolysing proteins than lipids. It is thus possible that the SCOD increase reported during WAS pretreatment with NaOH (Knezevic et al., 1995; Lin et al., 1997; Rajan et al., 1989) mainly resulted from the hydrolysis of proteins as opposed to lipids.

The alkaline pretreatment, however, reduced the average size of pork fat particles in all treated samples but one (60 meq/l). After 4 h of mixing at room temperature, the ratio $D_{treated}:D_{in}$ ranged from 1.02 at 60 meq/l to 0.67 at 250 and 300 meq/l (Table 3.2). However, increasing NaOH concentration above 100 meq/l, or pH above 13, did not seem to improve particle size reduction significantly. Results also indicated that most of the particle size reduction occurred within the first 15 minutes of pretreatment. In a sample receiving 250 meq NaOH/l, the average particle size was reduced from 315 ± 15 µm to 201, 228, 238, 207 and 214 µm after 15, 40, 60, 120 and 240 min of pretreatment, respectively. Pretreating WAS with NaOH also showed that most solubilization occurred within the first 15 minutes of pretreatment (Rajan et al., 1989).

Variability was too high and sample size too small to determine the effect of D_{in} on particle size reduction. All $D_{treated}:D_{in}$ ratios were thus fitted to first-order, linear and quadratic models. The best fit was obtained with the quadratic equation:

$$\frac{D_{treated}}{D_{in}} = 1.05 - 2.45\, C_{NaOH} + 5.17\, C_{NaOH}^2 \quad (3.4)$$

where, C_{NaOH} is sodium hydroxide concentration (meq/l) and $D_{treated}$ is average particle size after pretreatment (µm). Model and parameter estimates were significant at $P < 0.05$. Observed and calculated values as well as the 95% confidence interval of the mean are

presented in Figure 3.1. Based on Eq. 3.4, the average particle size will be reduced to a maximum of 73% of D_{in} at a NaOH concentration of 250 meq/l. At this alkaline concentration, the substrate is highly basic, pH of 13.2, and must be neutralised prior to further biological treatment. Moreover, high NaOH concentrations could hamper bacterial flocculation by making the cell surface more negative (Gaudy and Gaudy, 1980), resulting in a negative effect on processes, such as the upflow anaerobic sludge blanket reactor (UASB), that depend on granular biomass settleability.

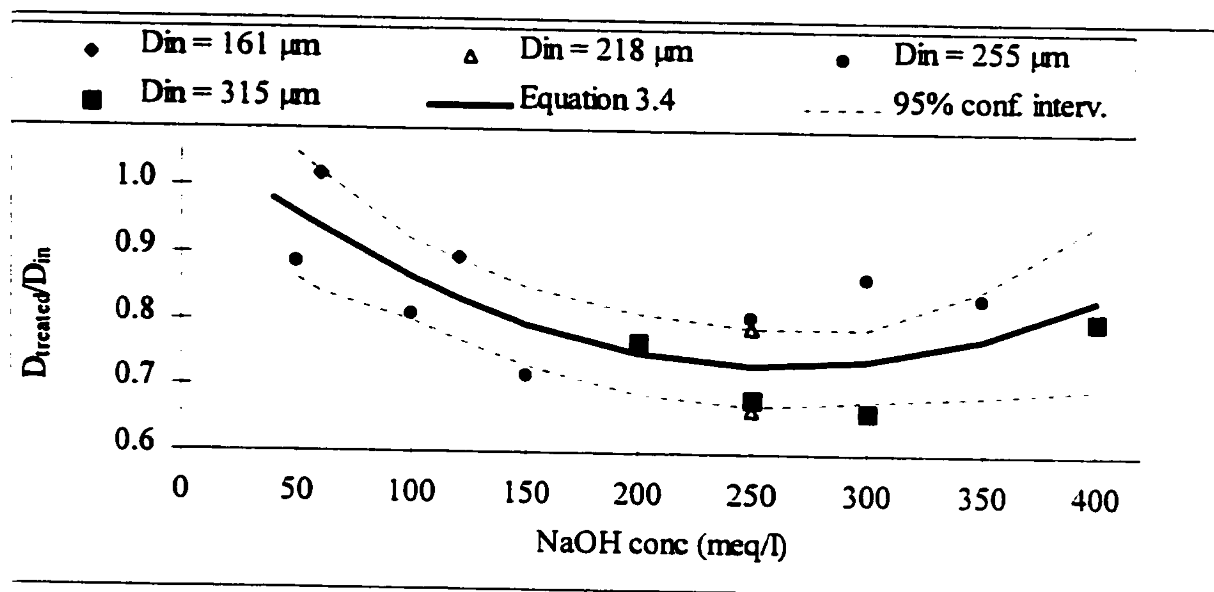

Figure 3.1 Reduction in the average size of pork fat particles in slaughterhouse wastewater after a 4-h pretreatment with NaOH; observed and calculated (Eq. 3.4) values.

Data from individual filters suggested that alkaline pretreatment essentially reduced the concentration of particles larger than 500 μm, while increasing that of smaller particles. It was observed that larger pork fat particles were more filamentous and less

spherical than smaller ones, and probably were severed at weaker points into two or more smaller particles under the action of the alkali.

3.3.2 Pancreatic Lipase 250

Size reduction of pork fat particles. The average particle size in SHW containing 2.5 g/l of pork fat was consistently reduced by a 4-h pretreatment with PL-250 (Fig. 3.2). The ratio $D_{treated}:D_{in}$ ranged from 0.96 to 0.48 and tended to decrease with increasing PL-250 concentration. To characterise the effect of D_{in} and enzyme concentration on particle size reduction, particle size data were grouped by D_{in} and each group was fitted to Eq 3.2. The parameter estimates (D_{min} and b) were found to be linearly correlated ($p < 0.01$) to D_{in}, with a y-intercept not significantly different from 0 (Fig. 3.3). Equation 3.2 was thus rewritten by setting D_{min} and b as linear functions of D_{in}, and a new set of parameters was estimated using all data points:

$$D - 0.6D_{in} = (0.4D_{in})\, e^{-(4.9E-5D_{in})C_{PL-250}} \quad (3.5)$$

The model was significant at $P < 0.05$ and fitted the observed data relatively well (Fig. 3.2).

Equation 3.5 predicts that 4 h of pretreatment with PL-250 will reduce the average pork fat particle size to a maximum of 60% ± 0.03% of D_{in}. Pretreatment will produce a smaller final average particle size in substrates with low D_{in}, but size reduction in absolute term ($D_{treated}$ - D_{in}) will be greater as D_{in} is increased. As was already observed, substrates with high D_{in} contained a higher concentration of larger particles, which were more filamentous and easily broken down at weak points than smaller particles.

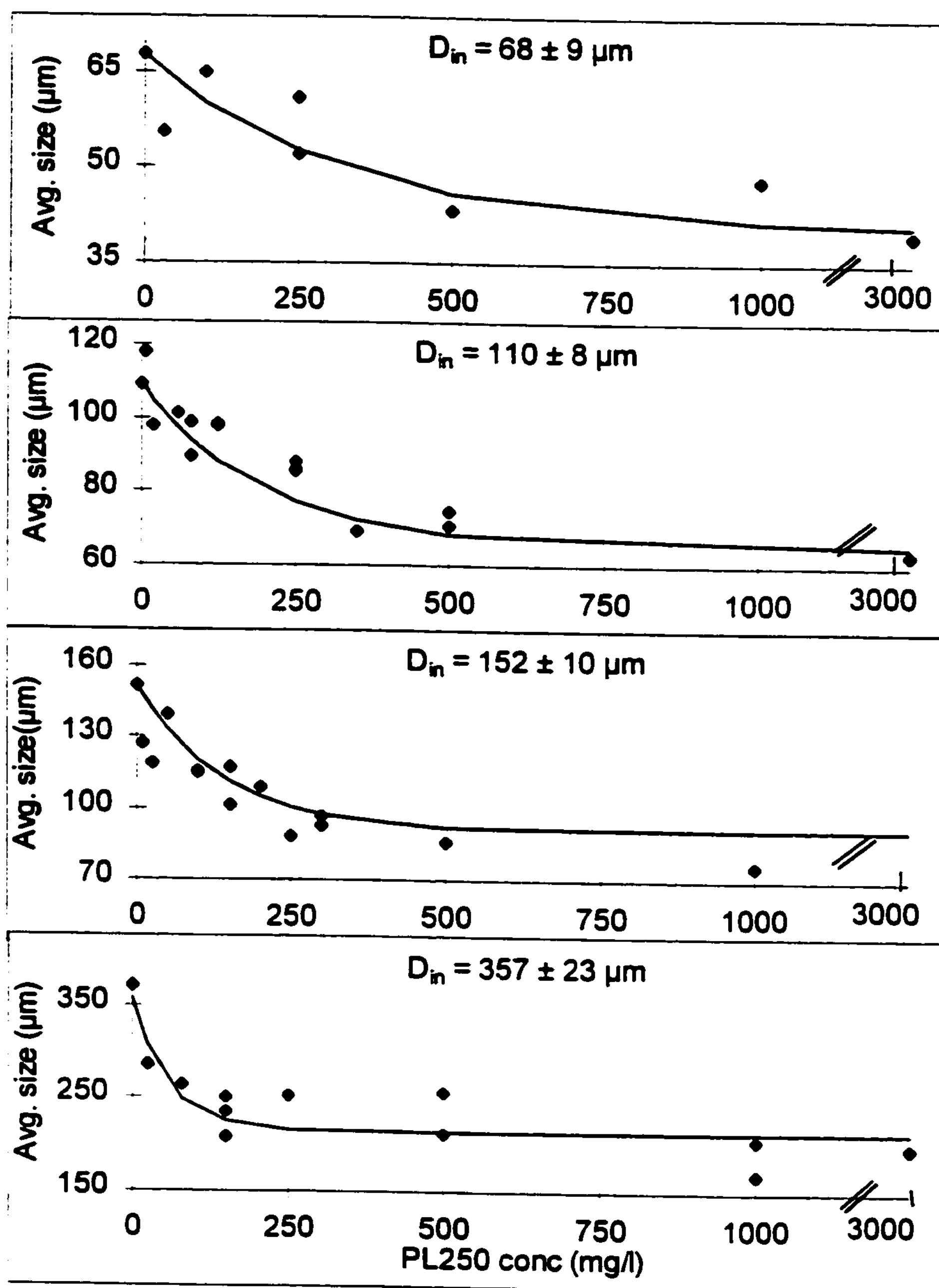

Figure 3. 2 Average size of particles in slaughterhouse wastewater containing 2.5 g/l of pork fat particles after a 4-h pretreatment with the pancreatic lipase PL-250. Observed (◆) and calculated (Eq. 3.5) values at 4 initial average particle sizes (D_{in}).

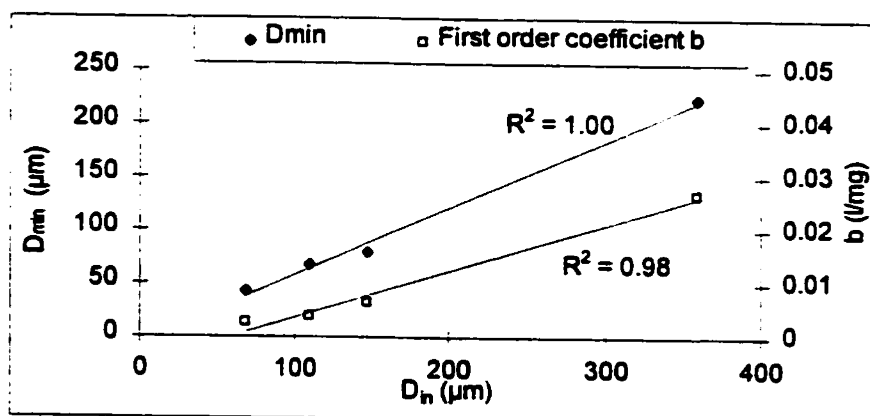

Figure 3. 3 Variation in parameters D_{min} and b (Eq. 3.2) with respect to initial average particle size (D_{in}).

Equation 3.5 also suggests that higher doses of enzyme will be needed for maximum size reduction at low D_{in}. Smaller particles should offer a greater total surface area, and thus more attachment and reaction sites for the lipase, than larger particles. More biocatalyst may thus be needed to fill all the adsorption sites and produce maximum effect. However, the lesser number of weak points for cleavage in smaller fat particles might actually be the limiting factor.

Individual filtration data indicated that the concentration of pork fat particles larger than 500 µm was consistently reduced during the 4-h pretreatment with PL-250. The reduction ranged from 11% to 80% and tended to increase with enzyme concentration. In most samples, an increase in the concentration of pork fat particles smaller than 50 µm also was observed. Pretreatment effect on particles of intermediate size depended on PL-250 dose. The concentration of pork fat particles between 50 and 500 µm increased in most samples receiving less than 250 mg PL-250/l, and decreased in 62% of the samples treated with 250 mg/l or more. This pattern suggests that at lower enzyme concentrations, large pork fat particles are mainly reduced to the next measured size. As biocatalyst

concentration is increased, pork fat particles are further broken down into increasingly smaller sizes. It would also tend to support the first-order model that indicated that higher doses of enzyme will be needed for maximum size reduction at low D_{in}.

Long-chain fatty acid production. The experimental SHW substrate initially contained 0.44 ± 0.11 mg/l of free LCFAs, representing approximately 0.1% of initial SCOD (2.94 g COD per g of oleic acid). In control samples, free LCFA concentration was not changed by 4 h of mixing at room temperature. In treated samples, free LCFA concentration increased at all PL-250 doses but one (Fig. 3.4). The maximum LCFA concentration, 15.5 mg/l, was detected at a PL-250 dose of 250 mg/l and a D_{in} of 357 μm. This LCFA concentration represented a liquefaction of about 0.7% of the fat particles in SHW. However, some of the hydrolysed LCFAs probably remained adsorbed on the fat particles, since only the free LCFAs in solution were measured. Results were too variable to determine the effect of D_{in} on triglyceride hydrolysis. However it would seem that the free LCFA concentration in solution reached a plateau of 9.3 ± 3.2 mg/l at PL-250 concentration of 250 mg/l.

Oleic acid (18:1) accounted for 47% of total LCFAs, while palmitic (16:0) and linoleic (18:2) acids represented 19% each. This corresponds well to the LCFA distribution reported for pork fat (Grinstone et al., 1986). Palmitoleic (16:1), stearic (18:0), and myristic (14:0) acids also were detected in most samples and represented between 2% and 6% of total LCFA concentration.

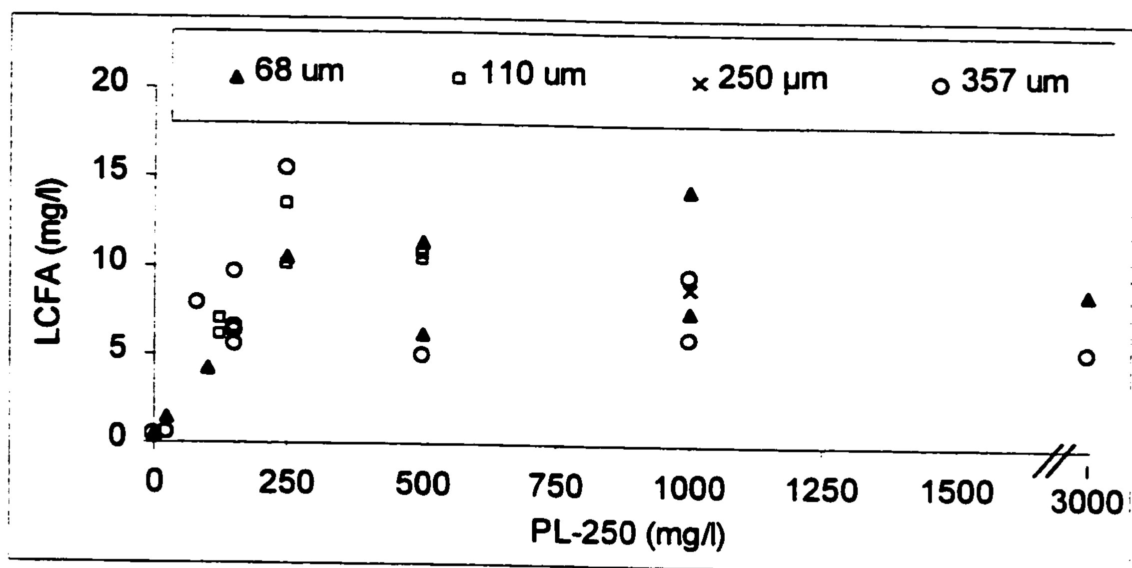

Figure 3. 4 Free long-chain fatty acid concentration in slaughterhouse wastewater containing 2.5 g/l of pork fat particles after a 4-h pretreatment with the pancreatic lipase PL-250.

Soluble COD. The ratio $SCOD_{treated}$:$SCOD_{control}$ in SHW containing 2.5 g/l of pork fat particles ranged from 0.82 to 1.21, with no apparent trend with respect to D_{in} or PL-250 concentration. These results, however, may not represent adequately enzymatic pretreatment effect on the SCOD of SHW. Lipase is a soluble enzyme that must adsorb on insoluble matter (lipid) for the hydrolysis reaction to occur (Brockman, 1984). Added PL-250 thus partitioned itself between the liquid and the solid phase, which made it difficult to measure which fraction of the SCOD increase was attributable to added enzyme and which fraction was due to hydrolysed triglycerides. To gain a better understanding into this phenomenon, changes in SCOD were monitored as PL-250 was added to pure distilled water and to distilled water containing 0.8 and 2.5 g/l of pork fat particles. In pure distilled water, PL-250 produced 1.05 mg of COD per mg of enzyme. The COD was entirely soluble and remained stable during 4 h of mixing at room

temperature. When 200 and 400 mg/l of PL-250 were added to distilled water containing fat particles, the initial SCOD concentration decreased by about 9% and 14%, respectively, within one minute of mixing (Table 3.4). The initial rate of lipase adsorption appeared to be a function of enzyme concentration only and not of fat particle concentration or available adsorption sites. After 4 h of mixing, SCOD concentrations in treated samples were similar to those evaluated at time 0 (Table 3.4). Increase in SCOD between one minute and 4 h of mixing may have been the result of fat particle hydrolysis and the release of the LCFAs in solution. However, a fraction of the SCOD increase may also have been caused by enzyme desorption from the fat particle surface. The experiment thus suggested that a decrease or no apparent changes in SCOD does not necessarily show inefficiency of the lipase in hydrolysing fat particles. However, during this experiment, increase in SCOD due to maximum free LCFAs released in solution during PL-250 pretreatment (15.5 mg/l) would only represent approximately 3% of initial SCOD.

Table 3.4 Soluble COD concentration in distilled water containing pork fat particles and Pancreatic Lipase 250

Fat particle conc. (g/l) PL-250 concentration (mg/l)	0.8 200	0.8 400	2.5 200	2.5 400
SCOD time 0[†] (mg/l)	217	427	240	450
SCOD after 1 min (mg/l)	199	368	216	387
% decrease 1 min	8.5	13.9	10.0	14.0
SCOD after 4 h (mg/l)	216	417	250	439
% decrease 4 h	0.7	2.4	-4.2	2.4

[†] Sum of substrate SCOD before PL-250 addition and SCOD value of PL-250 (1.05 g/g)

Pork fat particle concentration. The effect of a 4-h pretreatment with PL-250 was also tested on SHW containing 0.8 and 8 g/l of pork fat particles. The average particle size

data obtained during these two experiments were fitted to Eq. 3.2 (Figs. 3.5a and 3.5b). The first-order coefficient, b, was estimated at $(5.6 \times 10^{-5})D_{in}$, $(4.9 \times 10^{-5})D_{in}$, and $(1.4 \times 10^{-4})D_{in}$ l/mg for fat particle concentrations of 8.0, 2.5 and 0.8 g/l, respectively. However, the confidence interval was wide, and b was not significantly different at the three fat particle concentrations tested. The value of D_{min} was estimated at 0.67 ± 0.08, 0.60 ± 0.03 and 0.49 ± 0.09 of D_{in} for 8, 2.5 and 0.8 g/l of fat particles, respectively, but again differences were not statistically significant. The trend in parameter estimates with fat particle concentration however suggests that, since increasing particle concentration is equivalent to increasing surface area, more time and/or product may be needed to attain maximum effect.

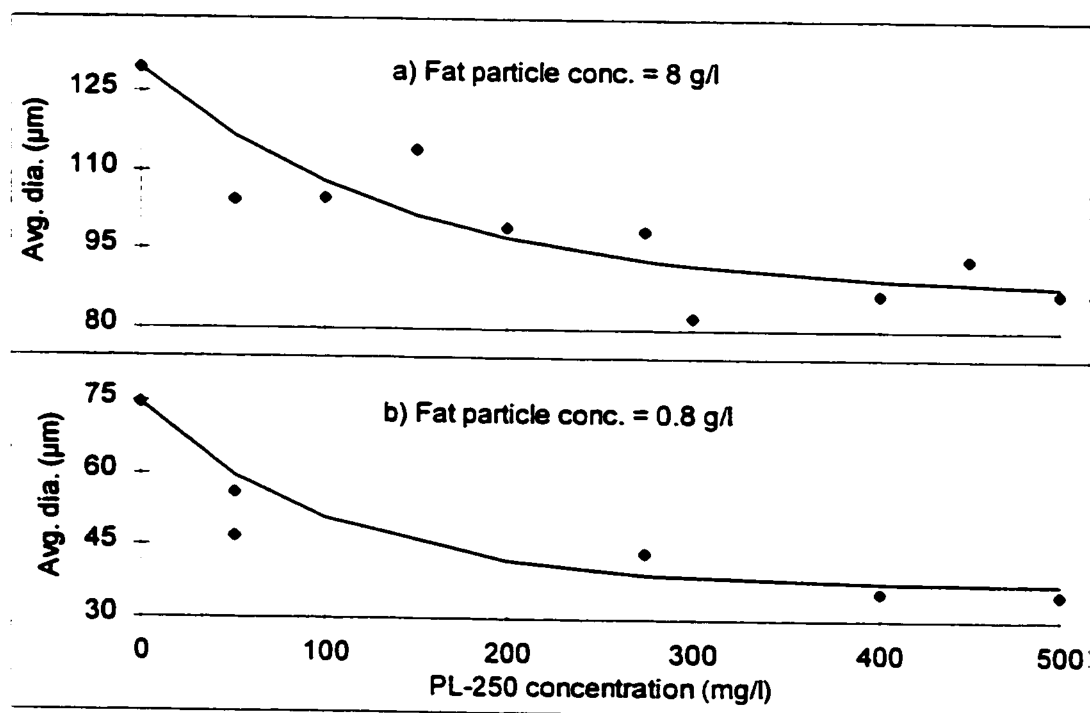

Figure 3. 5 Average particle size in slaughterhouse wastewater containing a) 8.0 g/l and b) 0.8 g/l of pork fat particles after a 4-h pretreatment with the pancreatic lipase PL-250: observed (◆) and calculated (Eq. 3.2) values.

The ratio $SCOD_{treated}$:$SCOD_{control}$ ranged from 0.92 to 1.13 at 8.0 g/l, and from 0.88 to 1.03 at 0.8 g/l. There was no pattern of SCOD changes with particle concentration or PL-250 dose.

Beef fat particles. In SHW containing 2.5 g/l of beef fat particles, the ratio $D_{treated}$:D_{in} ranged from 0.71 at 25 mg PL-250/l to 0.32 at 250 mg PL-250/l. Pancreatic lipase 250 thus appeared more efficient in reducing beef than pork fat particles (Fig. 3.6a). Using Eq. 3.2, D_{min} was estimated at 0.41 ± 0.05 of D_{in}, i.e. about 30% less than the value obtained with pork fat. Results for free LCFA concentrations were highly variable, but levels also seemed slightly higher in beef than pork fat substrates. Free LCFA concentrations were over 10 mg/l at all tested PL-250 doses, except one (Fig. 3.6b). The maximum LCFA concentration was 17.8 mg/l at a PL-250 concentration of 1500 mg/l.

Better results with beef than pork fat may be due to the different triglyceride compositions. Brockerhoff and Jensen (1974) reported that pancreatic lipase hydrolyses polyunsaturated fatty acids at a slower rate than monounsaturated or saturated fatty acids. In beef, 18.4% of the triglycerides are composed of saturated fatty acids, while 45.1% have only one unsaturated fatty acid; pork fat has 6.6% and 29.7% of its triglycerides with zero and one unsaturated fatty acid, respectively (Grinstone et al., 1986). On the other hand, the polyunsaturated linoleic (18:2) and linolenic (18:3) acids make up from 3% to 7.5% of beef fat and from 4.5% to 17.5% of pork fat (Grinstone et al., 1986). In the samples treated with PL-250, saturated fatty acids averaged 35% and 28% of total free LCFAs released in beef and pork fat substrates, respectively, and linoleic acid represented 9% and 19% of total LCFAs in beef and pork fat samples, respectively.

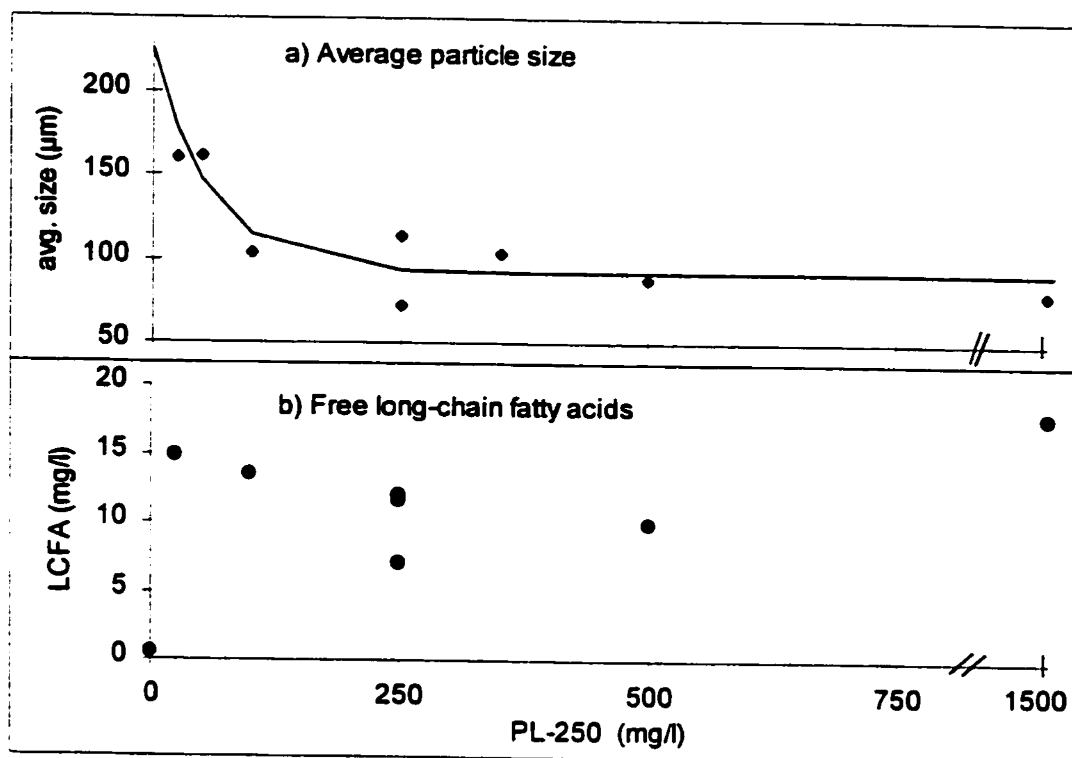

Figure 3. 6 The effect of the pancreatic lipase PL-250 on 2.5 g/l of beef fat particles in slaughterhouse wastewater: a) Average particle size: observed (◆) and calculated (Eq. 3.2) values, and b) Free long-chain fatty acid concentration.

Sodium chloride addition. During lipolysis, the pH could be lower at the fat-lipase interface than in the bulk solution, due to negatively charged LCFAs on solids surface attracting hydrogen ions (Brockerhoff and Jensen, 1974). A negative pH gradient at the solid-liquid interface, where the enzyme-substrate complex is formed. could inhibit lipase activity, and the addition of NaCl is generally recommended to neutralise the negative charges at the interface (Brockerhoff and Jensen, 1974). The effect of supplementing 1 g/l NaCl to SHW was thus tested on 8 pork fat samples receiving between 100 and 500 mg/l of PL-250.

Results indicated no apparent beneficial effect of NaCl addition on particle size reduction or SCOD changes (Table 3.5). There may have been sufficient residual NaCl or other salts in the SHW to mask the benefit of adding NaCl. In the wastewater from six slaughterhouses, sodium concentration was estimated at 272 ± 85 mg/l (Massé and Masse, 2000). The wastewaters also contained approximately 50 mg/l of calcium and 20 mg/l of magnesium ions, which bind inhibitory LCFAs and remove them by forming calcium or magnesium salts (Brockerhoff and Jensen, 1974).

Table 3.5 Effect of NaCl addition on Pancreatic Lipase 250 efficiency

PL-250 (mg/l)	NaCl (g/l)	Initial average diameter (μm)	Ratio $D_{treated}$: D_{in}	Ratio $SCOD_{treated}$: $SCOD_{control}$
100	1	146	0.78	1.21
100	0	146	0.79	1.07
300	1	146	0.66	1.05
300	0	146	0.63	0.98
400	1	146	0.62	
400	0	146	0.49	0.91
500	1	219	0.40	0.84
500	0	219	0.41	0.97

D_{in} = initial average particle size
$D_{treated}$ = average particle size after 4 h of pretreatment
$SCOD_{control}$ = SCOD in control samples after 4 h of mixing
$SCOD_{treated}$ = SCOD after 4 h of pretreatment minus SCOD from the enzyme

3.3.3 Lipase G-1000

A decrease in the average size of pork fat particles was observed after 4 h of pretreatment with LG-1000, but the pretreatment effect was minimal below 1000 mg/l of LG-1000 and near maximum above this concentration (Fig. 3.7). The ratios $D_{treated}$:D_{in}

were fitted to first-order, linear and quadratic equations and the best fit was obtained with the linear equation (Fig. 3.7):

$$\frac{D_{treated}}{D_{in}} = 0.90 - 0.13\, C_{LG-1000} \qquad (3.6)$$

where, $C_{LG-1000}$ is the concentration of LG-1000 (g/l).

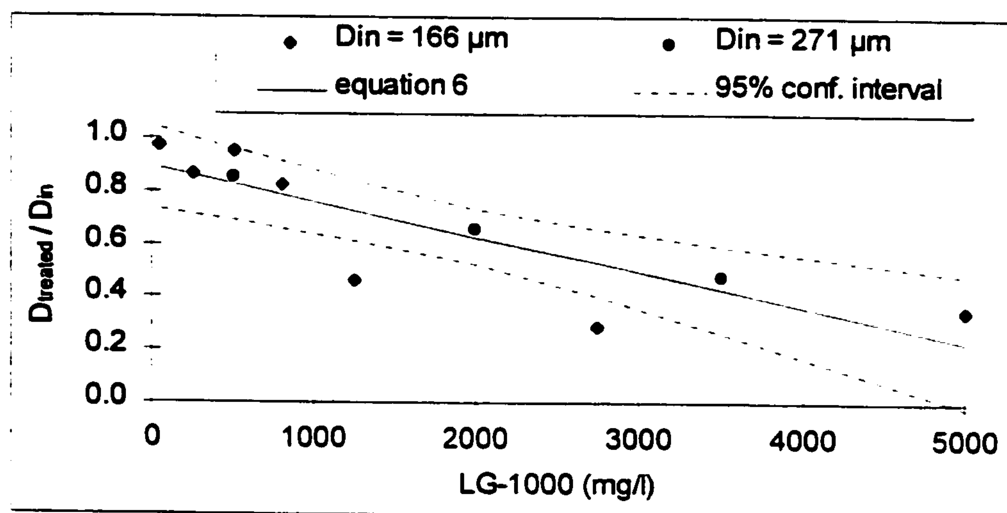

Figure 3. 7 Reduction in the average size of fat particles in slaughterhouse wastewater after a 4-h pretreatment with the bacterial lipase LG-1000: observed (◆) and calculated (Eq. 3.6) values.

The model and both parameters were statistically significant ($P < 0.01$). Based on Eq. 3.6, a LG-1000 concentration of 1500 mg/l would be needed to reduce the average pork fat particle size to 60% of D_{in} after 4 h of pretreatment. By comparison, 300 and 500 mg/l of PL-250 would be needed to achieve the same reduction at the two pork fat particle sizes tested with LG-1000. Lipase G-1000 was thus less efficient than PL-250 for a 4-h pretreatment. However, prolonging pretreatment time to 24 h greatly increased LG-1000 efficiency. In samples receiving 500, 2000 and 3500 mg LG-1000/l, the ratios $D_{treated}$:D_{in}

were 0.85, 0.66 and 0.48, respectively, after 4 h of pretreatment, and 0.41, 0.28 and 0.19, respectively, after 24 h of pretreatment. The particle size reduction obtained after 4 h of pretreatment represented 20 to 60% of that measured after 24 h. Lipase G-1000 may thus require more time than PL-250 to efficiently hydrolyse pork fat particles.

After 4 h of pretreatment, the ratio $SCOD_{treated}:SCOD_{control}$ ranged from 0.77 to 1.07. High enzyme concentrations were usually correlated to low ratios, maybe indicating larger adsorption of enzyme. In samples receiving 500 and 3500 mg/l of LG-1000, the SCOD increased by 6% and 27%, respectively, between 4 and 24 h of pretreatment. As mentioned previously, changes in SCOD may not be a precise indicator of lipolysis. However, in view of the large particle size reduction obtained after 24 h of pretreatment, the SCOD increase with time may suggest liquefaction of lipid material.

3.3.4 EcoSystem Plus

There was no noticeable change in average pork fat particle size after 4 and 24 h of pretreatment at ESP concentrations of 50, 100, 500 and 5000 μl/l (Table 3.6). After 4 h of pretreatment, the SCOD was lower in treated than control samples, except at 50 μl/l, probably indicating enzyme adsorption to the solid phase (Table 3.6). After 24 h, the SCOD was approximately 10% higher in treated than control samples. Some solubilization and/or enzyme desorption may have occurred. However, the fact that average particle size was not reduced by enzymatic pretreatment, regardless of biocatalyst concentration or pretreatment time, may suggest that ESP had little effect on the hydrolysis of pork fat particles in SHW.

Table 3.6 Changes in SCOD and average particle size following pretreatment with EcoSystem Plus (ESP)

Treatment time (h)	ESP concentration (μl/l)	Ratio $SCOD_{treated}$: $SCOD_{control}$	Ratio $D_{treated}$:D_{in}
4	50	1.27	1.04
	100	0.94	1.04
	500	0.90	0.91
	5000	0.78	1.00
24	50	1.07	1.08
	100	1.12	1.05
	500	1.09	1.08

D_{in} = initial average particle size
$D_{treated}$ = average particle size after 4 h of pretreatment
$SCOD_{control}$ = SCOD in control samples after 4 h of mixing
$SCOD_{treated}$ = SCOD after 4 h of pretreatment minus SCOD from the enzyme

Plant lipase is considered highly active, but optimum conditions and preferred substrate vary greatly among different plants (Huang, 1984). Neozyme did not specify the exact origin of the lipase contained in ESP, only that the mixture also includes trace minerals and enzyme enhancing agents, and that it effectively breaks down fat particles in aerobic or anaerobic environments. Product literature suggested that the rate of reaction of ESP depended on the presence of an indigenous bacterial population in the raw wastewater. A BOD test was thus performed on the SHW, and approximately 8 mg/l of dissolved oxygen was consumed after 4 h at 25°C. After 24 h, all dissolved oxygen had disappeared from a mixture of 290 ml of saturated distilled water containing 10 ml of substrate. These results confirmed the presence of active aerobic microorganisms in the raw wastewater. However, bacterial presence did not appear to stimulate the ESP reaction.

3.4 Conclusion

A 4-h pretreatment with 50 to 400 meq/l of NaOH did not produce a significant increase in the SCOD concentration of SHW containing approximately 3 g/l of pork fat particles. However, particle size was reduced to 73% ± 7% of D_{in} at NaOH concentrations ranging from 150 to 300 meq/l. Given the high doses of NaOH required and the resulting increase in pH, alkaline pretreatment would not be a recommended hydrolysis pretreatment for pork fat particles in SHW. Four hours of pretreatment with PL-250 reduced the size of pork fat particles in solution to about 60% of D_{in}. At low D_{in}, higher doses of enzyme were needed to obtain maximum particle size reduction. Pancreatic Lipase 250 was more efficient with beef than pork fat, probably because the former contains less polyunsaturated fatty acids than the latter. Release of LCFAs into solution also indicated slight solubilization of lipids with PL-250. Soluble COD was not significantly increased by the PL-250 pretreatment, but SCOD is not a good indicator of enzymatic lipolysis, because of enzyme and LCFA adsorption on the fat particle surface. The bacterial lipase LG-1000 was also efficient in reducing fat particle size but high doses (> 1000 mg/l) or long pretreatment time (24 h) were needed to obtain an appreciable reduction. The plant lipase Ecosystem Plus had no effect on fat particle size. Since PL-250 appeared to be the best hydrolysing pretreatment, especially for a relatively short pretreatment time (4 h), further experiments will be conducted with PL-250 to characterise the hydrolysis rate and pretreatment effect on a downstream digestion process.

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CHAPTER 4

THE EFFECT OF AN ENZYMATIC PRETREATMENT ON THE HYDROLYSIS AND SIZE REDUCTION OF FAT PARTICLES IN SLAUGHTERHOUSE WASTEWATER

Abstract

The effect of an enzymatic pretreatment, Pancreatic Lipase 250 (PL-250), on the hydrolysis and size reduction of fat particles in slaughterhouse wastewater was characterised for enzyme doses ranging from 125 to 1000 mg/l and initial particle sizes (D_{in}) varying between 53 and 383 μm. Treatment with PL-250 significantly reduced the size of pork fat particles in slaughterhouse wastewater. Particle size reduction increased with D_{in}, possibly due to the more filamentous and plate-like configuration of the larger fat particles, which could be easily broken at weak points. Size reduction also increased with enzyme concentration, but the benefit of adding more enzyme diminished greatly as enzyme dose was increased. The maximum long-chain fatty acid (LCFA) concentration in filtered samples was detected after four to six hours of treatment and ranged from 8.2 to 34.9 mg/l. The linear rate of LCFA released in solution during enzymatic pretreatment ranged from 39.4 to 169.9 mg/l/d, and increased with enzyme concentration up to 500 mg/l. At a PL-250 concentration of 1000 mg/l, the LCFA release rate decreased, maybe due to excessive layering of adsorbed enzyme on the fat particles which prevented LCFA desorption from the particles or increased degradation of released LCFAs. There was no significant D_{in} effect on the linear constant. The pretreatment appeared to be more

efficient with beef than pork fat particles. However, the effect of an enzymatic pretreatment on a downstream anaerobic treatment of slaughterhouse wastewater containing fat particles remains to be tested.

Keywords: anaerobic digestion, enzyme, hydrolysis, lipase, lipid, pretreatment, slaughterhouse wastewater

4.1 Introduction

Particle hydrolysis is considered the rate-limiting step during anaerobic degradation of industrial wastewaters containing both dissolved and insoluble solids. The soluble organics are usually methanized long before the suspended solids (SS) have been hydrolysed, and the complete degradation of the particles requires an extended hydraulic retention time.

Some studies have reported a slower hydrolysis rate for lipid particles than other fractions of organic waste. During the anaerobic degradation of municipal solid waste, the first-order hydrolysis constant varied between 0.005 and 0.010 day^{-1} for lipids, while it ranged from 0.015 to 0.075 day^{-1} for proteins, and from 0.025 to 0.200 day^{-1} for carbohydrates (Christ et al., 1999). The first-order hydrolysis constants for lysed waste-activated sludge were estimated at 1.3, 1.2, and 0.76 day^{-1} for proteins, carbohydrates and lipids, respectively (Shimizu et al, 1993). During the anaerobic treatment of slaughterhouse wastewater containing high concentrations of SS, the accumulation of solids within the biomass bed, especially fats, seriously impaired the performance of anaerobic reactors operated at high organic loading rates (Martinez et al, 1995; Saxena et al, 1986; Sayed and de Zeeuw, 1988). If fat liquefaction is the rate-limiting factor, the

application of a pretreatment to hydrolyse particles, particularly fats, may accelerate and stabilise the anaerobic digestion of slaughterhouse and meat packing plant wastewaters.

Most studies on hydrolysis pretreatments have dealt with complex waste, and pretreatment effects on different organic fractions (protein, lipid, and carbohydrate) have not been assessed. The fate of lipids during such pretreatments is particularly important for the stability of the anaerobic process. During lipolysis, long-chain fatty acids (LCFAs) are released into solution, and high free LCFA concentrations can have an irreversibly inhibitive effect on the anaerobic biomass (Hanaki et al., 1981; Koster and Cramer, 1987; Rinzema et al., 1994). Masse et al. (2001) tested the effect of four hydrolysis pretreatments on fat particles in slaughterhouse wastewater: sodium hydroxide (NaOH) and three lipases of plant, bacterial and pancreatic origin. The most efficient treatment was the pancreatic lipase PL-250 that could reduce the initial average particle size (D_{in}) of pork fat by up to 40% after four hours of mixing at room temperature. Particle size reduction has been shown to increase the rate and extent of anaerobic treatment (Chyi and Dague, 1992; DeWalle et al., 1978; Hills et Nakano, 1984; Levine et al., 1991). Pretreatment with PL-250 also increased free LCFA concentration in filtered samples, indicating some hydrolysis of the lipid fraction. The objective of this study was to evaluate the rates of LCFA release in solution and particle size reduction of pork and beef fat particles in slaughterhouse wastewater during an enzymatic pretreatment with PL-250.

4.2 Materials and Method

4.2.1 Pancreatic Lipase 250

Pancreatic Lipase 250 (PL-250; Genencor International, Rochester, N.Y.) is a lipolytic enzyme isolated from pig pancreas. It contains mainly lipase, but includes a small quantity of amylase and protease for carbohydrate and protein hydrolysis. The enzymatic mixture was designed to hydrolyse neutral fats in septic tanks, and is supposed to be especially efficient with LCFAs containing more than 12 carbons (Genencor, 1998). The optimum operating temperature is 40-50°C, but the enzyme is reported to be still effective at lower temperatures. Optimum pH is between 7.0 and 8.0, but the product is stable at pH ranging from 6.0 to 9.5. Enzymatic activity is reported as 250 lipase units/g, one lipase unit being the 'activity which liberates one milliequivalent of fatty acid in two hours under the condition of the assay' (Genencor, 1998)

4.2.2 Substrate

Pieces of pork or beef fat were immersed in distilled water and cut into small particles with a rotating knife. The solution was filtered through a metal sieve to remove particles greater than 1410 μm and stored at 4°C for a maximum of four days. Various particle size distributions were obtained by filtering out smaller or larger particles.

Slaughterhouse wastewater was chosen as the liquid substrate because this experiment was part of a larger study evaluating effects of pretreatments on anaerobic digestion of slaughterhouse wastewater. The wastewater was collected from a plant in Embrun, Ontario, that slaughters beef and hog. It was filtered through a 38-μm metal sieve to remove most indigenous SS. The filtered wastewater contained 92% w/w of its solids in

dissolved form (< 0.45 μm), and less than 1% w/w of its solids had a diameter greater than 10 μm. The wastewater was stored at -10°C until used.

The experimental substrate consisted of a 50:50 mixture of filtered slaughterhouse wastewater and distilled water containing fat particles. The substrate had an average soluble chemical oxygen demand of 1465 ± 72 mg/l, which is representative of a low strength slaughterhouse wastewater (Massé et Masse, 2000). The fat particle concentration averaged 2.0 ± 0.1 g/l, which is high since slaughterhouse wastewater is reported to have a fat content ranging from 200 to 1700 mg/l, with a typical value of 500 mg/l (Hammer and Jacobson 1970; Martinez et al., 1995; Rands and Cooper, 1966). The high fat content was selected to obtain a more accurate measurement of fat particle distribution during treatment.

4.2.3 Experimental conditions

Two replicate test jars were filled with 1750 ml of substrate. At time 0, a 125-ml sample was collected from each jar and analysed for particle size distribution and free LCFA concentration in solution. After PL-250 addition, the substrate was gently mixed with a palette type mixer (60 rpm) at room temperature (21 to 24°C) for 24 hours. Samples were drawn at different time periods, ranging from 5 min to 26 hours, from one or both jars.

The effect of PL-250 on pork fat particles in slaughterhouse wastewater was monitored at enzyme concentrations ranging from 125 to 1000 mg/l and initial average particle sizes (D_{in}) varying between 53 and 383 μm. For the experiment with beef fat, PL-250 concentrations and D_{in} were 250 mg/l and 210 μm, respectively.

4.2.4 Physical and chemical analyses

Long-chain fatty acids were extracted and analysed according to the method described in Chou et al. (1996; 1998) and Masse et al. (2001). Particle size distribution was measured by serial filtration as outlined in Masse et al. (2001).

4.2.5 Data and statistical analysis

Average particle size calculations were described in Masse et al. (2001). Parameters for all empirical models were estimated using the NLIN (non-linear) procedure in the SAS statistical treatment software 6.12 (SAS Institute Inc., 1988). The program provided standard errors for the parameters and the model, the approximate 95% confidence limits for each parameter, and the coefficient of correlation between the two parameters. The statistical significance of the non-linear models was verified by comparing pure error (error between true replicates at each D_{in}) to model error as explained in Borowiak (1989) and Masse et al. (2001).

4.3 Results and discussion

4.3.1 Control samples

Control samples (no PL-250 addition) were analysed for pork fat particle size distribution after 0 to 27 hours of mixing at room temperature (Fig. 4.1). Particle size distribution and average particle size remained relatively constant in the initial seven hours of mixing. However, in the 27-h sample, the mass of particles retained on the 925-μm filter and average particle size tended to be slightly higher than in samples collected earlier (Fig. 4.1). Agglomeration of pork fat particles, in the form of long filaments, was

sometimes observed in control jars after 24 hours of mixing. These larger particles may have been oversampled in some of the jars.

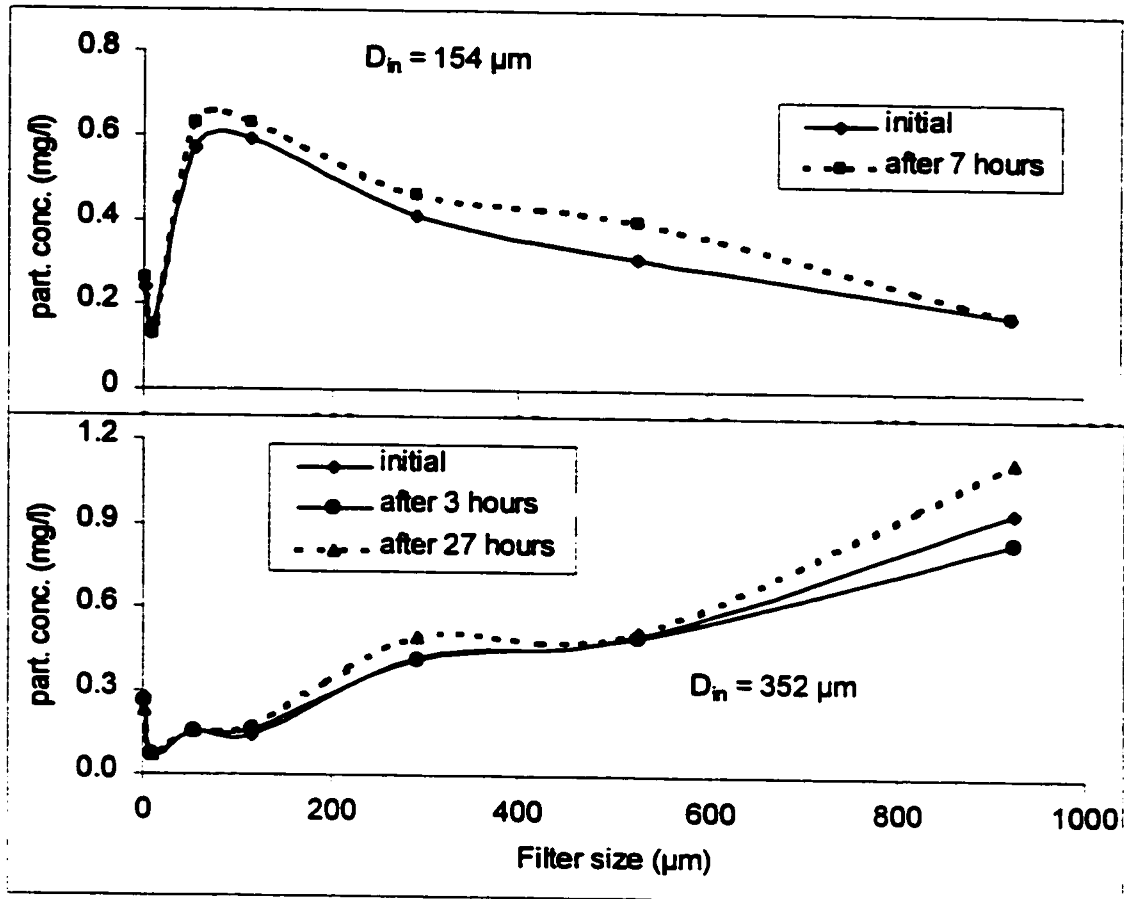

Figure 4. 1 Pork fat particle size distribution in two control samples after different periods of mixing at room temperature. D_{in} is initial average particle size.

Free LCFAs in solution were analysed in one control sample after 0, 3, and 27 hours of mixing. Long-chain fatty acid concentration was 0.45, 0.52 and 0.09 mg/l at the three times, respectively. The reduction in LCFA concentration in the 27-hour sample, which was also observed in treated samples, may be partly due to bacterial degradation during mixing. A BOD test performed on the wastewater revealed the presence of an active

bacterial population in the raw slaughterhouse wastewater, which could have degraded part of the LCFAs in solution.

Results from control samples indicated that hydrolysis or size reduction of pork fat particles did not occur by simply mixing the wastewater at room temperature. This is consistent with the results of Batstone et al. (1997), who reported no hydrolysis of fat particles in slaughterhouse wastewater after a 50-hour residence time in an acidification/equalisation tank. Some active treatment is necessary for lipolysis to occur at a measurable rate.

4.3.2 The reduction of pork fat particle size

Pretreatment with the enzymatic product PL-250, in concentrations ranging from 125 to 1000 mg/l, significantly decreased the average size of pork fat particles in solution (Fig. 4.2). As shown in Figure 4.3, the enzymatic treatment reduced the concentration of larger particles while increasing the concentration of smaller particles.

The reduction in average particle size appeared to follow a first-order reaction with respect to average particle size, as follows:

$$D - D_{min} = (D_{in} - D_{min})e^{-b_1 t} \qquad (4.1)$$

where D is the average particle size following treatment (μm), D_{min} is the minimum average particle size (μm) that could be reached during treatment; t is time (day); and b_1 is the first-order coefficient (day^{-1}). Further analysis of data indicated that the first-order coefficient b_1 was linearly correlated to D_{in} ($p < 0.01$), with a y-intercept not significantly different from 0, but did not present any discernible pattern with respect to enzyme

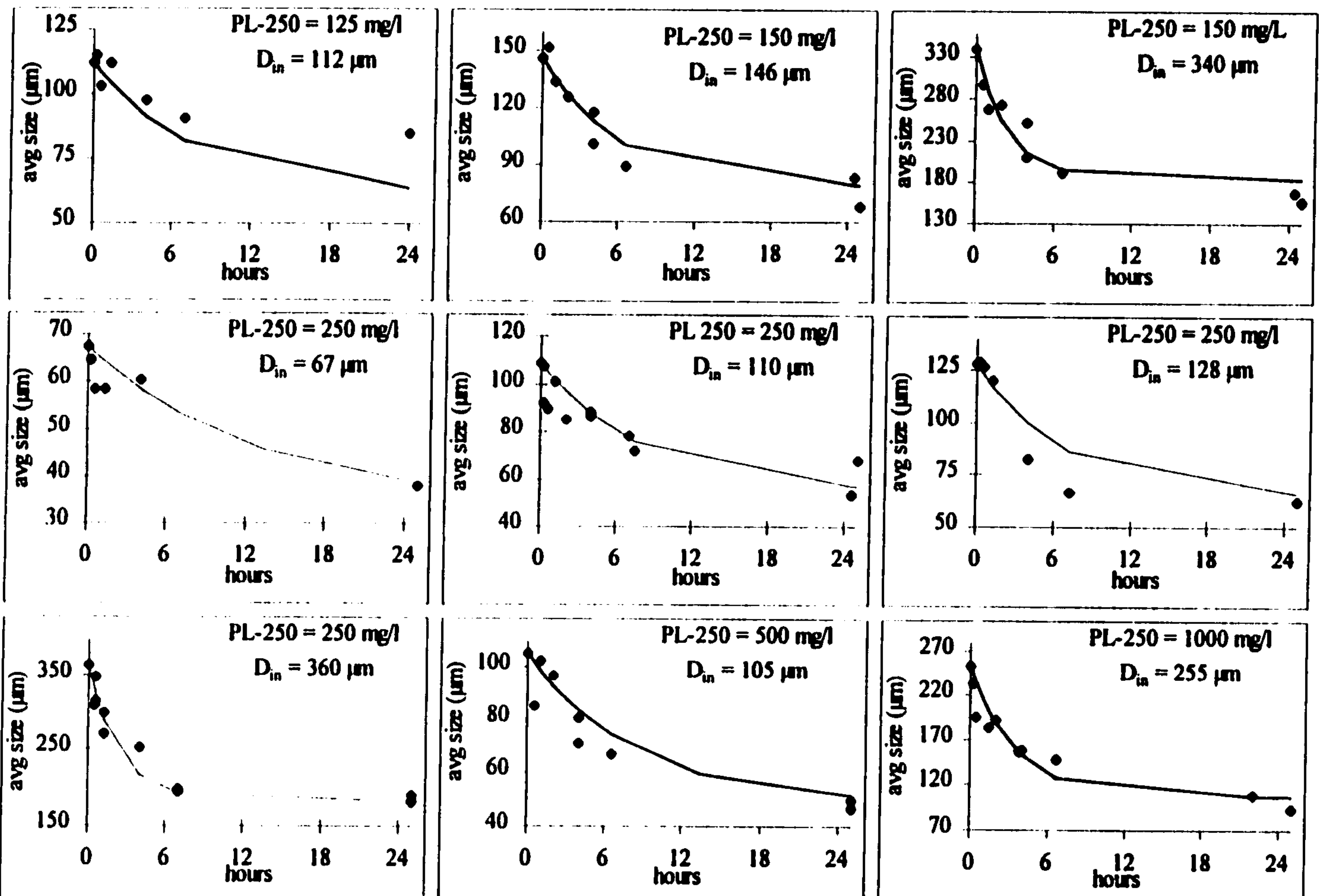

Figure 4.2 Average size of pork fat particles during treatment with Pancreatic Lipase 250 (PL-250): observed data (diamonds) and Eq. 4.3 (solid lines). D_{in} is initial average particle size.

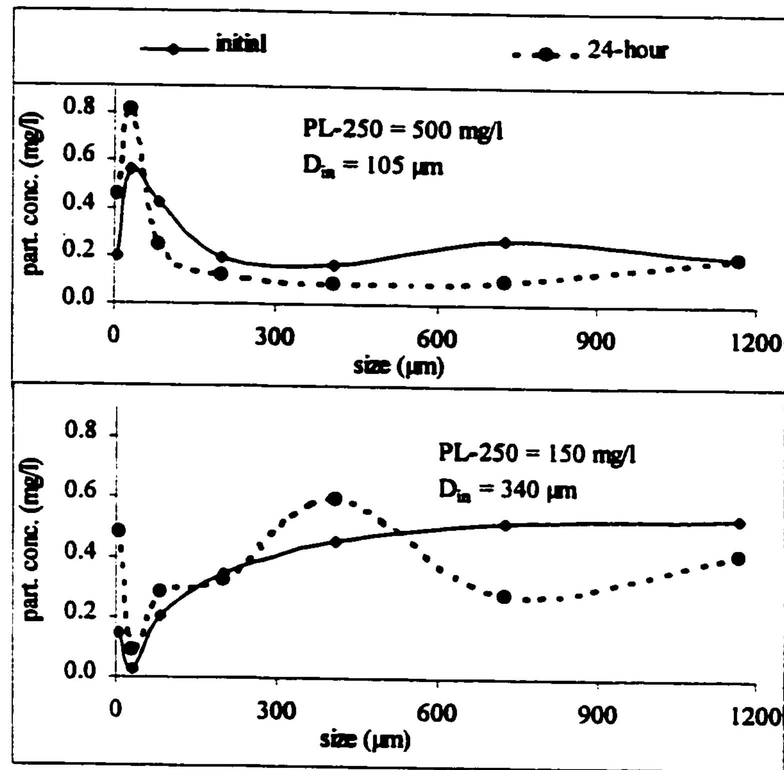

Figure 4.3 Particle size distribution in two samples at time 0 and after 24 hours of treatment with Pancreatic Lipase 250 (PL-250). D_{in} is initial average particle size.

concentration. Parameter D_{min} was related to both D_{in} and PL-250 concentration through the following equation:

$$D_{min} = D_{in}C^{-z} \qquad (4.2)$$

where, C is PL-250 concentration in mg/l and z is the exponent of C.The relation between the parameters (b_1, D_{min}) and the experimental conditions (D_{in}, PL-250 concentration) was incorporated into equation 4.1 as follows:

$$D - D_{in}\, C^{-0.124} = (D_{in} - D_{in}\, C^{-0.124})\, e^{-(0.028)(D_{in}} \qquad (4.3)$$

Modelled and observed data are presented in Figure 4.2. The empirical model was significant at $P < 0.05$. The approximate 95% confidence interval ranged from 0.022 to

0.033 μm^{-1} day $^{-1}$ for the first-order coefficient (average of 0.028 um^{-1} day^{-1}), and from 0.119 to 0.129 for the exponent of C (average of 0.124). The two parameters were only slightly correlated (correlation coefficient of 0.66), and residuals were well distributed on either side of the zero line when plotted against time, D_{in}, and PL-250 concentration.

Equation 4.3 suggests that pork fat particle size reduction increases with PL-250 concentration. However, the benefit of augmenting the dose of enzyme decreased substantially at higher enzyme concentrations: quadrupling PL-250 concentration from 250 to 1000 mg/l only increased maximum particle size reduction by 8%.

Equation 4.3 also suggests that average pork fat particle size is reduced to a fixed fraction of D_{in} equal to $C^{-0.124}$. Final average particle size will be smaller for substrate with low D_{in}, but size reduction in absolute term ($D_{treated}$ - D_{in}) will be greater as D_{in} is increased. Maximum particle size reduction was also achieved at a faster rate in substrate with larger D_{in}. The larger pork fat particles were observed to be filamentous and plate-like, while the smaller particles were more spherical and denser. The facility with which the large filamentous particles could be broken at weak points probably increased the fat particle size reduction rate. This phenomenon would support the model representing particle reduction as being first-order with respect to average particle size, that is particle size reduction rate slowed down as particles were becoming smaller.

During every run, average particle size reached a minimum value after 4 to 24 hours of treatment (Fig. 4.2), suggesting that the enzyme was deactivated or inhibited with treatment time. Enzyme concentration did not seem to be an important factor in the deactivation or inhibition rate: the time at which maximum particle size reduction was achieved was similar at all tested PL-250 doses. Causes of lipase deactivation include

inhibition by LCFA accumulation near the particle surface (Martinelle and Hult, 1994) and a decrease in the surface concentration of triglycerides as particle lipolysis proceeds (Brockman, 1984).

4.3.3 LCFA concentration in solution

In the initial substrate, total free LCFA concentration in solution averaged 0.42 ± 0.15 mg/l. Dissolved LCFA concentration increased in all samples treated with PL-250, indicating triglyceride hydrolysis under the enzymatic pretreatment. At PL-250 concentrations ranging from 125 to 500 mg/l, maximum free LCFA concentration was detected six to seven hours after enzyme addition (Fig. 4.4), and ranged from 8.2 to 34.9 mg/l. The highest LCFA concentration was obtained at a D_{in} of 105 μm and a PL-250 concentration of 500 mg/l (Fig. 4.4). Maximum LCFA concentration tended to increase with PL-250 doses up to 500 mg/l. At a PL-250 dose of 1000 mg/l, the maximum free LCFA concentration was lower and was detected earlier than at any other enzyme concentration (Fig. 4.4). One possible explanation is that high PL-250 enzyme (soluble protein) concentrations stimulated the indigenous bacteria in slaughterhouse wastewater, and the free LCFAs were oxidised at a faster rate. The decrease in LCFA concentration observed in all samples between 8 and 24 hours of treatment (Fig. 4.4) suggested degradation of the released LCFAs. A second explanation is that excess enzyme accumulated in multiple layers on the fat particles and blocked LCFA release from the particles (Lee and Fan, 1982). The hydrolysed LCFAs would have remained adsorbed on the fat particles, under the enzyme layers, instead of being mixed in solution under the action of the palette mixer, as they were at lower doses.

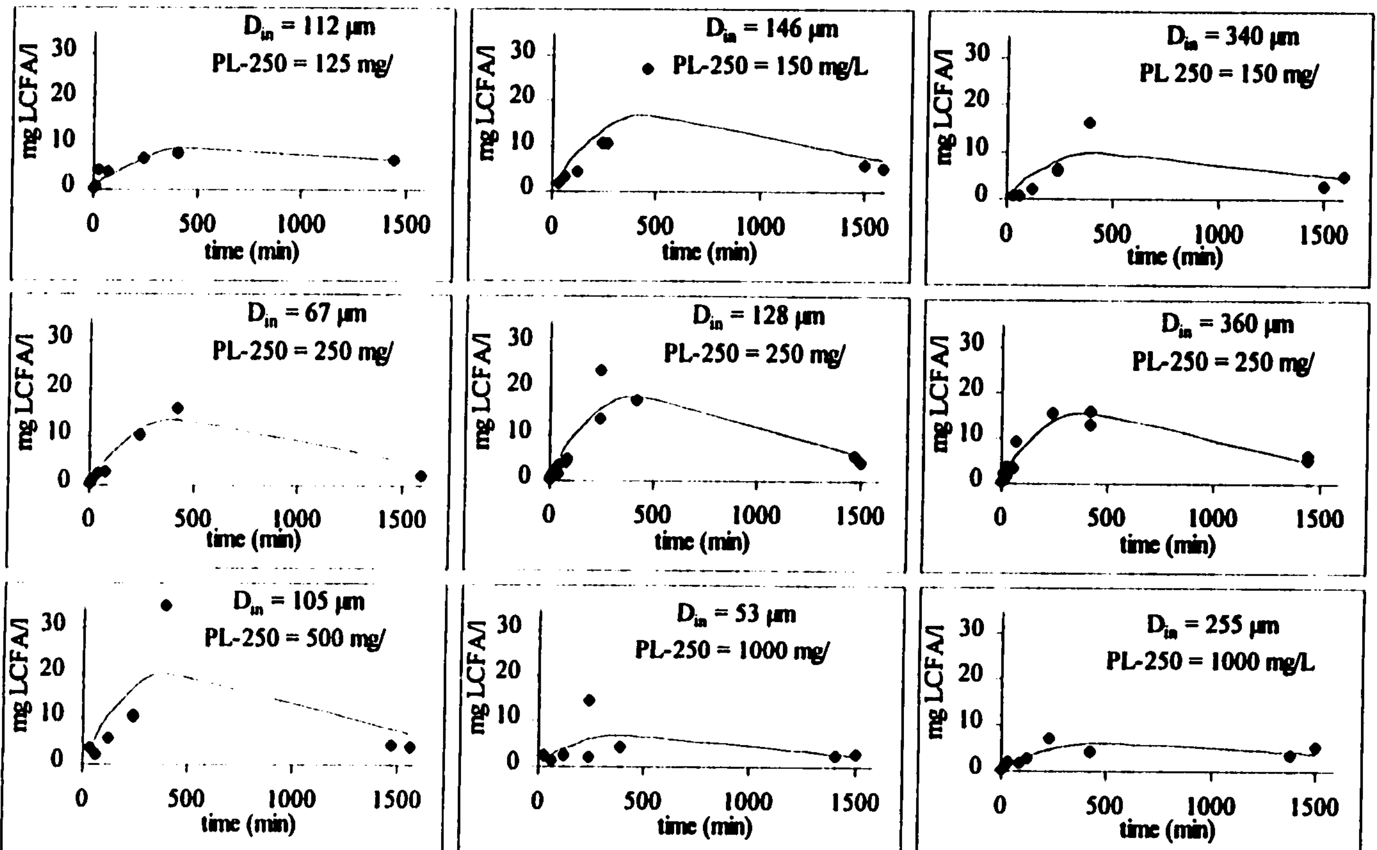

Figure 4.4 Long-chain fatty acid production for various initial average diameters (D_{in}) and Pancreatic Lipase (PL-250) concentrations: observed data (diamonds) and Eq. 4.5 (solid lines).

Oleic acid (18:1) represented 45% ± 8% w/w of the LCFAs detected in solution. Linoleic (18:2) and palmitic (16:0) acids accounted for 18% w/w each, while stearic (18:0) and palmitoleic (16:1) represented approximately 7% w/w each. There was no effect of PL-250 concentration or D_{in} on the type of LCFAs detected in solution.

The equation describing the release of LCFA during enzymatic pretreatment was based on three observed phenomena. First, there was a linear (zero-order) increase in free LCFAs in solution during the first few hours of treatment (Fig. 4.4). Secondly, the decrease in LCFA concentration after 24 h of treatment suggested the disappearance (degradation or adsorption) of a fraction of the LCFAs with treatment time. Finally, particle size reduction data indicated enzyme deactivation or inhibition during treatment. An equation was thus written to include all processes:

$$\frac{dL}{dt} = k_h' e^{-at} - k_x L \tag{4.4}$$

where, L is LCFA concentration in mg/l; k_h' is linear LCFA release in solution in mg/l/d; a is deactivation or inhibition rate constant in day^{-1} and t is time in day; and k_x is the LCFA 'disappearance rate' in day^{-1}. The integrated form of equation 4.4 was:

$$L = \frac{k_h'}{k_x - a}(e^{-at} - e^{-k_x t}) + L_0 e^{-k_x t} \tag{4.5}$$

where, L_0 is the LCFA concentration in solution at time 0.

Parameters (k_h', k_x, a) were estimated for each of the nine tests (corresponding to nine enzyme concentration/D_{in} combinations). Given the high number of parameters, the model was unstable and the procedure converged for only 4 of the 9 data sets. However, parameter a did not present any pattern with respect to D_{in} or enzyme concentration. It averaged 2.72 ± 0.84 day^{-1}, indicating a 50% reduction in enzyme activity after 6 h of

treatment. Parameter *a* was thus set as a constant, and sets of parameters (k_h', k_x) were estimated for each curve. The model fitted the observed data relatively well, except that it tended to underestimate the maximum LCFA concentration in some of the tests (Fig. 4.4).

The rate of LCFA production, k_h', ranged from 39.4 to 169.9 mg/l/d. It formed a quadratic relation ($r^2 = 0.81$) with enzyme concentration, i.e. it increased with PL-250 dose up to 500 mg/L, but decreased significantly thereafter. There was also a slight tendency for k_h' to decrease with increasing D_{in}, but the trend was not significant

4.3.4 Beef fat

Average particle size reduction rate and LCFA production were also evaluated for beef fat during two tests at a PL-250 dose of 250 mg/l and a D_{in} of 210 μm. Figure 4.5a presents average particle size during treatment, alongside Eq. 4.1 with D_{min} and b_1 equal to $0.34D_{in}$ and $0.062D_{in}$ day^{-1}, respectively. The enzymatic treatment was more efficient in reducing the size of beef than pork fat particles. After seven hours of treatment, an average particle size reduction of 70% was observed with beef fat, compared to 40% for pork fat particles at the same PL-250 concentration/D_{in} combination.

The greater particle size reduction obtained with beef than pork fat may be due to the different triglyceride distribution in both fats. Brockerhoff and Jensen (1974) reported that pancreatic lipase hydrolyses polyunsaturated fatty acids at a slower rate than monounsaturated or saturated fatty acids. Pork fat has been found to contain more polyunsaturated LCFAs than beef fat (Grinstone et al., 1986). In this experiment, the polyunsaturated linoleic acid represented 11% and 18% of total LCFAs in beef and pork fat samples, respectively. However, total LCFAs released in solution was not

significantly higher in beef than pork fat substrate. In Figure 4.5b, observed LCFA data are plotted against Eq. 4.5, using the parameters obtained for pork fat at that D_{in}/PL-250 combination. The fit was at least as good as that obtained with pork particles.

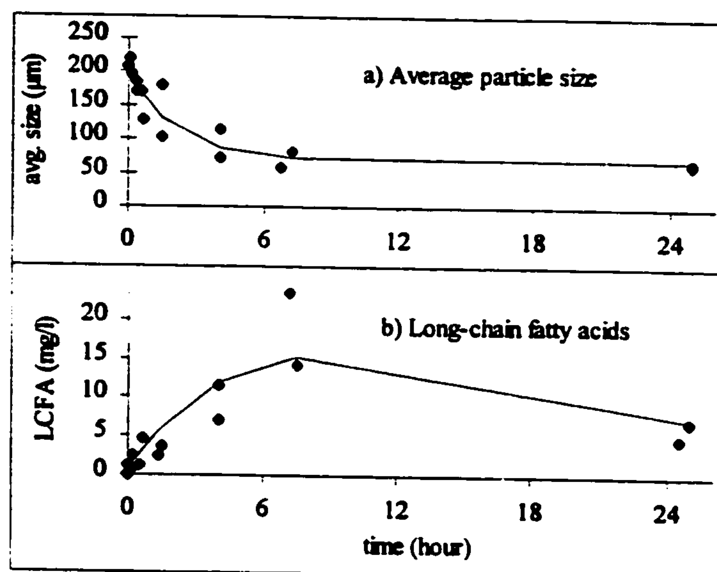

Figure 4.5 The effect of 250 mg/l of Pancreatic Lipase 250 on beef fat particles (D_{in} of 210 µm) in slaughterhouse wastewater on: a) Average particle size, observed data (diamonds) and Eq. 4.1 (solid line); and b) LCFA production, observed data (diamonds) and Eq. 4.5 (solid line)

4.4 Conclusion

The pancreatic lipase PL-250 effectively reduced the size of pork fat particles and hydrolysed some triglycerides in slaughterhouse wastewater. The increase rate of particle size reduction as D_{in} was increased may be due to the more filamentous and plate-like configuration of larger fat particles, while smaller particles were denser and more spherical. The linear rate of LCFA release in solution during enzymatic pretreatment ranged from 39.4 to 169.9 mg/l d^{-1}, and increased with enzyme concentration up to 500

mg/l. There was no significant relation between k_h' and D_{in}. The pretreatment was more efficient with beef than pork fat. However, the effect of an enzymatic pretreatment on the rate of anaerobic digestion remains to be tested.

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CHAPTER 5

NEUTRAL FAT HYDROLYSIS AND LONG-CHAIN FATTY ACID OXIDATION DURING ANAEROBIC DIGESTION OF SLAUGHTERHOUSE WASTEWATER

Abstract

Neutral fat hydrolysis and long-chain-fatty acid (LCFA) oxidation rates during anaerobic digestion of pork slaughterhouse wastewater were determined. Control substrate (filtered slaughterhouse wastewater) and experimental substrate (filtered slaughterhouse wastewater and pork fat particles with average initial size (D_{in}) ranging from 60 to 450 μm were fed to anaerobic sequencing batch reactors operated at 25°C. At the D_{in} tested, there was no significant particle size effect on neutral fat hydrolysis rate. The first-order neutral fat hydrolysis rate averaged 0.63 ± 0.07 d^{-1}. LCFA oxidation rate was modelled using a Monod-type equation. The maximum substrate degradation rate (k_{max}) and the half-saturation concentration (K_s) averaged 164 ± 37 mg LCFA/l/d and 35 ± 31 mg LCFA/l, respectively. Pork fat particle degradation was mainly controlled by LCFA oxidation rate and, to a lesser extent, by neutral fat hydrolysis rate. However, anaerobic degradation of substrates containing highly hydrolysed lipids, such as sewage sludge, should be largely controlled by LCFA oxidation. Soluble COD could not be used to determine the rate of lipid hydrolysis due to LCFA adsorption on the biomass.

Keywords: anaerobic, hydrolysis, fat, long-chain fatty acids, slaughterhouse, particulate

5.1 Introduction

Animal fat is mainly composed of triglycerides (neutral fats) made of esters of fatty acids and glycerol (Grinstone et al., 1986). During anaerobic digestion, neutral fats are hydrolysed (lipolyzed) into free long-chain fatty acids (LCFAs) and glycerol. The hydrolysis process is catalysed by extracellular lipases released by acidogenic bacteria. The free LCFAs are subsequently oxidised to shorter chain fatty acids by acetogenic bacteria. The oxidation process becomes thermodynamically unfavourable unless the hydrogen partial pressure is maintained at extremely low levels (Hanaki et al., 1981). The process thus relies on the ability of the hydrogenotrophic methanogens to utilise the molecular hydrogen produced during oxidation.

Based on various studies on anaerobic digestion of sewage sludge in mesophilic reactors, Gujer and Zhender (1983) estimated that the apparent first-order hydrolysis rate for lipids ranged from 0.08 to 1.7 d^{-1}. However, the apparent first-order hydrolysis rate included both neutral fat hydrolysis and LCFA oxidation, since Gujer and Zhender's model was based on the assumption that hydrolysis controlled the degradation process and gas production was proportional to lipid particle solubilization. In fact, it has not been clearly established which, of hydrolysis or LCFA oxidation, is the rate-limiting step of lipid anaerobic degradation. Heukelekian and Mueller (1958) monitored the distribution of different grease fractions during anaerobic digestion of sewage sludge at 20°C. During the first week of treatment, 50% of the fatty acid esters were hydrolysed while the free LCFA concentration remained relatively constant. The authors concluded that LCFA oxidation was not rate limiting, since the free LCFAs released by hydrolysis were oxidised as they were produced. O'Rourke (1968) also reported rapid hydrolysis of

the fatty acid esters during anaerobic digestion of sewage sludge at temperatures ranging from 20°C to 35°C. However, hydrolysis was followed by a significant accumulation of free LCFAs in the reactor. The author concluded that hydrolysis was rapid and LCFA oxidation was the rate-controlling process of lipid anaerobic degradation at temperatures between 20°C and 35°C.

O'Rourke (1968) used a Monod-type equation to model LCFA oxidation. The maximum LCFA utilisation rate (k_{max}) increased from 3.85 d^{-1} at 20°C to 6.67 d^{-1} at 35°C, while the half-saturation concentration (K_s) decreased from 4620 mg/l at 20°C to 2000 mg/l at 35°C. Thus, as temperature was increased, both the degradation rate and the affinity of the biomass for the LCFAs increased. Novak and Carlson (1970) fed individual LCFAs to mesophilic (37°C) anaerobic reactors. The maximum LCFA utilisation rate averaged 1 d^{-1} for the saturated myristic (14:0), palmitic (16:0) and stearic (18:0) acids, while it was estimated at 4.0 and 5.0 d^{-1} for the unsaturated oleic (18:1) and linoleic (18:2) acids, respectively. The half-saturation concentration was evaluated at 105, 150 and 417 mg/l for myristic, palmitic and stearic acids, respectively, while it averaged 3180 and 1816 mg/l for oleic and linoleic acids, respectively. Degradation rate was thus higher for the unsaturated LCFAs, but the affinity of the biomass for the LCFAs was lower than for saturated acids. The Monod parameters reported by O'Rourke (1968) and Novak and Carlson (1970) were estimated using limited data sets of four to six values, and the error associated with the estimates was not provided. Angelidaki et al. (1999) reported much lower parameter values for the modelling of LCFA oxidation during anaerobic digestion of manure and glycerol triolate in thermophilic (55°C) digester. The maximum LCFA utilisation rate and the half-saturation concentration were set at 0.55 d^{-1}

and 20 mg/l, respectively. However, the method used for parameter estimation and the error associated with the estimates were not reported.

Lipid hydrolysis rate estimates reported in the literature also present large variations. During thermophilic digestion of municipal solid waste in small batch reactors, the first-order hydrolysis constant for lipid particles ranged from 0.005 to 0.010 d^{-1} (Christ et al., 1999). However, the hydrolysis rate may have been underestimated, since calculations were based on ratios of soluble to particulate compounds. In batch-type system, free LCFAs will readily adsorb onto solid surfaces, especially cell membranes, and will not be entirely recovered in filtered or centrifuged samples (Gujer and Zhender, 1983). During the mesophilic digestion of mechanically lysed waste-activated sludge (WAS), the hydrolysis constant was estimated at 0.76 d^{-1} (Shimizu et al., 1993). Hydrolysis also appeared to be measured from the release of soluble compounds, but the digester was operated as a continuously-fed, completely-mixed system. Solids surface may have been saturated with LCFAs or other adsorbable compounds, such that hydrolysed LCFAs were released into solution. Variations in hydrolysis rates may also be due to differences in the type of lipids (triglycerides, phospholipids, sterols) in the substrate or in lipid particle size. Food waste particle size will probably be much larger than biopolymers released from lysed WAS. This may partly explain the lower hydrolysis rates obtained under thermophilic than mesophilic conditions.

There are no studies presenting estimates for both neutral fat hydrolysis and LCFA oxidation rates during anaerobic digestion of a fat-containing substrate. Additionally, there is no quantitative information on the anaerobic digestion of fat particles in wastewaters from the meat processing industry. Most experimental work was performed

on sewage sludge, which has a high fat content, especially in the form of free LCFAs. O'Rourke (1968) used a sludge containing 4.3 g/l of total fat, of which about 1.9 g/l were free fatty acids (C_5 and above) and 0.9 g/l were esters of fatty acids. Heukelekian and Mueller (1958) measured 1.6 and 6.2 g/l of fatty acid esters and free LCFAs, respectively, in the initial sewage sludge samples. Comparatively, total fat content of slaughterhouse and meat packing wastewater ranges from 0.2 to 1.7 g/l, with a typical value of 0.5 g/l (Hammer and Jacobson, 1970; Martínez et al., 1995; Rands and Cooper, 1966).

The objective of this experiment was thus to determine the hydrolysis and oxidation rates of pork fat particles of various average initial sizes (D_{in}) during anaerobic digestion of slaughterhouse wastewater. The experiment was conducted in anaerobic sequencing batch reactors (ASBRs) operated at 25°C.

5.2 Materials and methods

5.2.1 Substrate

Pork fat (lard) was immersed in distilled water and cut into small particles with a rotating knife. The fat solution was filtered through a metal sieve to remove particles greater than 2000 μm and stored at 4°C for a maximum of one week. Different fat particle size distributions were obtained by filtering out particles using metal sieves with pore sizes of 75, 106, 250, 500 and 1000 μm. The five fat particle size distributions used in the study are shown in Figure 5.1. Wastewater was collected from a hog slaughterhouse in St-Valérien, Québec. It was filtered through a 75 μm metal sieve to remove part of the indigenous suspended solids (SS) and stored at 2°C. It was heated to room temperature

18 h prior to feeding. The experimental substrate was composed of filtered slaughterhouse wastewater and fat particles in distilled water (FSHW+F). The control substrate consisted of filtered slaughterhouse wastewater and distilled water (FSHW). Substrate characteristics are given in Table 5.1.

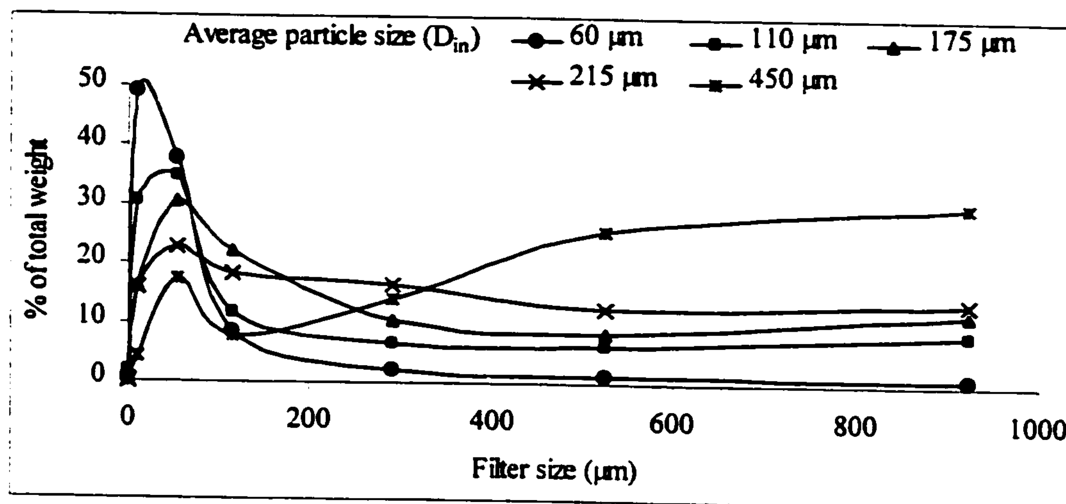


Figure 5.1 Pork fat particle distribution used for the five experiments

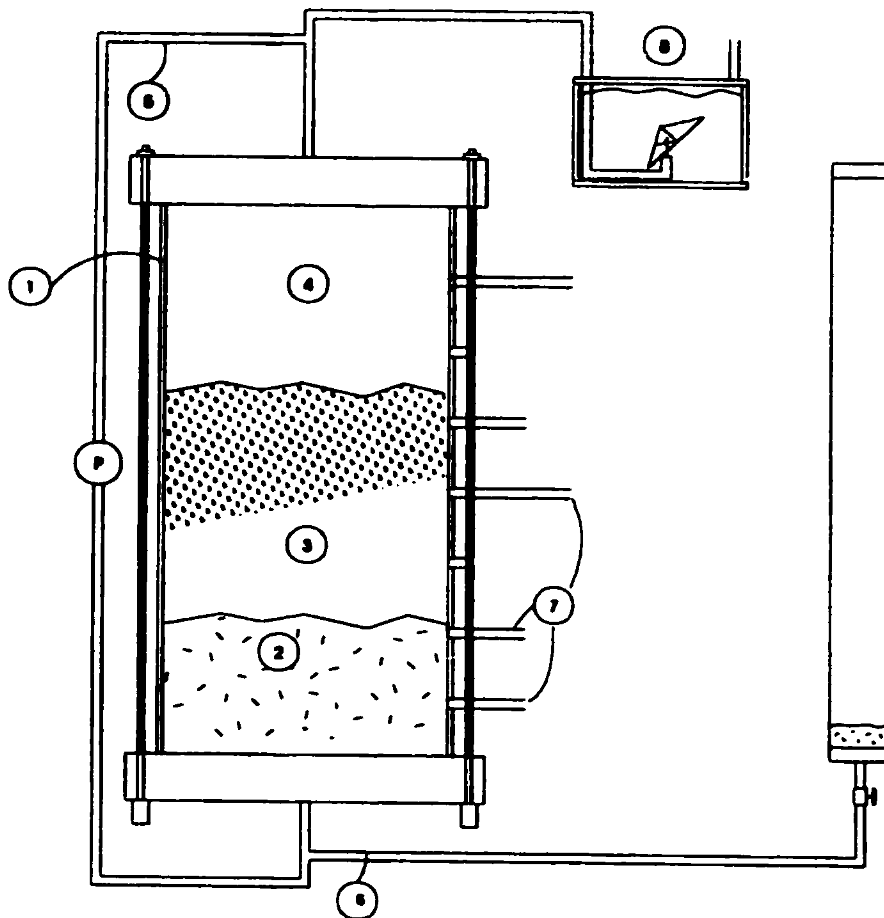
Table 5.1 Characteristics of control (FSHW, filtered slaughterhouse wastewater) and experimental (FSHW+F, filtered slaughterhouse wastewater and pork fat particles) substrates

Exp. no.	D_{in} (µm)	Total lipid (mg/l)		Soluble COD (mg/l)		Total COD (mg/l)	
		FSHW	FSHW+F	FSHW	FSHW+F	FSHW	FSHW+F
1	110	169 ± 56	739 ± 17	2405 ± 203	2434 ± 215	3419 ± 157	5181 ± 68
2	175	153 ± 19	766 ± 61	2283 ± 167	2160 ± 85	3464 ± 215	4899 ± 171
3	215	146 ± 32	746 ± 82	2231 ± 113	2208 ± 98	3081 ± 237	4201 ± 22
4	450	195 ± 83	664 ± 86	2086 ± 109	2288 ± 313	2799 ± 33	3987 ± 632
5	60	143 ± 43	675 ± 21	2484 ± 255	2520 ± 293	3255 ± 252	4534 ± 209
avg.		160 ± 43	724 ± 61	2322 ± 212	2332 ± 221	3230 ± 295	4560 ± 516

5.2.2 Reactor operation

One of the four plexiglass 42-l ASBRs used for the experiment is illustrated in Figure 5.2. Before the initiation of this study, all of the ASBRs had been treating unfiltered slaughterhouse wastewater for more than 9 months at 20°C or 25°C. The wastewater had an average fat content of 249 ± 101 mg/l. One month prior to the experiment, the anaerobic sludge was removed from all reactors, mixed in Nalgene bottles flushed with nitrogen, and redistributed to the ASBRs. The reactors were placed in a room maintained at 25°C and fed hog slaughterhouse wastewater containing 410 ± 105 mg/l of total fat.

Five experiments, corresponding to five initial average fat particle sizes (D_{in}), were conducted (Table 5.1). The ASBRs with total operating liquid volume of 21 l were operated for three 3-d cycles at each D_{in}. During each experiment, two of the ASBRs chosen at random received 12 l of FSHW (duplicate controls) and two reactors received 12 l of FSHW+F at the desired D_{in} (duplicate sample runs). FSHW and FSHW+F substrates were introduced into the ASBRs over a 1-h period. The react and settle periods lasted for approximately 69 h. During the react phase, the ASBR content was mixed one minute every 15 minutes by recirculating the biogas with a dual-head air pump with a maximum capacity of 22.5 l/min. The supernatant was withdrawn over a 1 h period. During the idle period (about 1 h), 9 l of sludge was left in each ASBR to start the next cycle. The sludge bed (determined during the idle phase) contained 14.5 ± 1.8 g volatile SS (VSS)/l. At the end of each experiment, the react phase of the last (third) cycle was extended by one to four days to allow complete degradation of undigested particles. The sludge was then removed from all reactors, mixed under anaerobic conditions and redistributed among the ASBRs. This ensured that initial conditions were uniform in all



1 300 mm diameter plexiglas digester
2 Sludge bed zone
3 Variable volume zone
4 Head space zone
5 Gas recirculation and outlet line
6 Influent line
7 Effluent and sampling ports
8 Gas meter

Figure 5.2 Schematic of the 42-l anaerobic sequencing batch reactor (Massé, 1995)

reactors at the onset of each of the five experiments. The reactors were then fed unfiltered slaughterhouse wastewater for three ASBR cycles before another experiment was conducted.

5.2.3 Sample collection and analysis

During the second ASBR cycle of each experiment, five to six samples were collected at different times from each reactor. These samples were analysed for soluble chemical oxygen demand (SCOD) and/or volatile fatty acid (VFA) concentrations. SCOD was determined by the closed reflux colorimetric method (APHA, 1992) using the filtrate from a 0.45 μm Millipore filter. VFAs were analysed using a Perkin Elmer Autosystem gas chromatograph (Perkin-Elmer, Norwalk, CT) equipped with a high-resolution megabore column maintained at 145°C and connected to a flame ionization detector.

During the third cycle of each experiment, four to seven samples were collected at different times from each of the two reactors fed FSHW+F. During experiments 4 and 5, samples were also collected from the reactors fed FSHW. These samples were analysed for neutral fat, LCFA, SS, and VSS. Neutral fat and LCFA concentrations were determined by a method adapted from that described in Kaluzny et al. (1985). Briefly, total fat was first extracted with ether according to the Roese-Gottlieb method (AOAC, 1984). Efficiency of the method was estimated by extracting water and FSHW samples supplemented with 123 to 846 mg/l of fat particles. Total fat recovery averaged 100.4% ± 14.2%. High variation was mainly due to samples containing less than 150 mg/l of fat. Total fat extracts were evaporated dry, resuspended in hexane and passed through aminopropyl bonded phase (Bond Elut) columns attached to a vacuum apparatus. Total fat in the sample was retained on the column. The efficiency of the Bond Elut

columns in retaining total fat was verified by collecting the hexane eluate from 14 samples. Residues averaged 0.11 ± 0.20 mg and represented 1.52% ± 2.85% of the neutral fat in the samples. The Bond Elut columns were then washed with 4 ml of a chloroform-isopropanol solvent, which eluted the neutral lipids. This eluate was evaporated dry for 24 h at 105°C and weighed. The columns were again washed with a 2% acetic acid in ethyl ether solvent to elute the free LCFAs. The LCFA extracts were methylated and analysed by gas chromatography as described in Masse et al. (2001). Three solutions containing a mixture of myristic, palmitic and stearic acids were also passed through the Bond Elut columns and methylated. LCFA recovery averaged 97% for two of the standard solutions and 87% for the third one.

Before each feeding, the fat solution was analysed for particle size distribution by serial filtration as described in Masse et al. (2001). Feed samples (FSHW and FSHW+F) were analysed for SCOD, total COD (TCOD), SS, VSS and total fat. Feed samples from the first and last experiments were also analysed for neutral fat, LCFA and VFA concentrations. At the beginning of each experiment and at the end of most cycles, sludge samples were collected and analysed for SS and VSS. Some of the sludge samples were also analysed for total fat, neutral fat and LCFA.

5.2.4 Data analysis

Lipids represent between 3% and 20% of microorganism dry cell weight (Mackie et al., 1991). These lipids are mainly phospholipids but also include neutral fats. Thus, the neutral fat extracted from the ASBR mixed-liquor samples originated from the pork fat particles added with the substrate as well as from the microorganisms. To calculate pork

fat hydrolysis rate, the amount of residual neutral fat from the pork fat particles had to be isolated from microorganism neutral fat as follows:

$$N = N_T - VSS\,(f_{ns}) \tag{5.1}$$

where, N is the concentration of neutral fat originating from the pork particles in the substrate (mg/l); N_T is the neutral fat concentration measured in the mixed-liquor sample (mg/l); VSS is the VSS concentration in the mixed-liquor sample (mg/l); and f_{ns} is the fraction of neutral fat in the sludge bed at the beginning of each experiment (mg neutral fat/mg VSS). The value of f_{ns} was determined by measuring sludge VSS and neutral fat content at the beginning of each experiment (before the first cycle at each D_{in}).

Fat particle hydrolysis was calculated using a first-order equation as follows:

$$N = N_0 e^{-k_h t} \tag{5.2}$$

where N_0 is the pork particle neutral fat concentration in the mixed-liquor after feeding (time 0) (mg/l); t is time (d); and k_h is the first-order hydrolysis constant (d^{-1}).

Oxidised LCFAs at time t in each replicate reactor were calculated as follows:

$$L_{ox(t=t)} = L_0 + L_{p(t=t)} - L_{r(t=t)} \tag{5.3}$$

where, $L_{ox\,(t=t)}$ is the amount of free LCFAs oxidised between time 0 and time t (mg/l); L_0 is the average free LCFA concentration at time 0 (mg/l); L_p is free LCFA production (mg/l), equivalent to the neutral fat hydrolysed between times 0 and t, as calculated using Eq. 5.2; and L_r is the average free LCFA concentration remaining in the mixed-liquor at time t (mg/l).

Free LCFA results were fitted to a Monod-type equation, as follows:

$$r_{ox} = \frac{L_{ox(t=2)} - L_{ox(t=1)}}{t_2 - t_1} = \frac{k_{max} L_{avg}}{K_s + L_{avg}} \tag{5.4}$$

where r_{ox} is the LCFA oxidation rate (mg LCFA/l/d); k_{max} is the maximum LCFA degradation (oxidation) rate (mg LCFA/l/d); L_{avg} is the average LCFA concentration at times 1 and 2 (mg/l); and K_s is the half-saturation concentration (mg LCFA/l). Biomass concentration was not included in Eq. 5.4 because it was kept as a constant in all experiments and was much in excess of substrate concentration. Parameter values were converted from weight of LCFA to weight of COD by using the theoretical value of 2.91 g of COD per g LCFA for a mixture of 70% 18-carbon and 30% 16-carbon LCFAs.

Parameters for all models were estimated using SAS, version 8. The statistical significance of the non-linear models was verified by comparing pure error (error between true replicates at each D_{in}) to model error as described in Masse et al. (2001). Significant differences between parameters were estimated using ANOVA or Scheffe subroutines in SPSS 9.0 for Windows. In the text and figures, average values are provided with their standard errors. Values given as percent are all weight by weight (w/w).

5.3 Results and Discussion

5.3.1 Substrate

Total fat concentration averaged 160 ± 43 and 724 ± 61 mg/l in FSHW and FSHW+F, respectively (Table 5.1). Free LCFA concentration was similar in both substrates. In Experiments 1 and 5, free LCFA concentrations averaged 81 ± 19 and 82 ± 27 mg/l in FSHW and FSHW+F, respectively. Lipids other than neutral fat and free LCFAs accounted for approximately 20% of total fat in all samples.

5.3.2 Fat content in the sludge bed

Figure 5.3 presents lipid distribution in the sludge bed at the beginning of each experiment (prior to the first cycle). Total lipids ranged from 0.115 ± 0.015 g/g VSS in Experiment 1 to 0.153 ± 0.016 g/g VSS in Experiment 4. Although not statistically significant, there seemed to be a slight increase in total fat concentration in the sludge bed over the experimental period. The concentration of LCFAs averaged 0.006 ± 0.005 g/g VSS and represented 3.6% ± 3.5% of total lipids in the sludge bed. Neutral fat content in the sludge bed (f_{ns}; Eq. 5.1) averaged 0.068 ± 0.009 g/g VSS. Neutral fat represented 50.2% ± 9.7% of total fat, which was high compared to the maximum of 30% reported for the proportion of neutral fat in total lipid from archaebacteria (Mackie et al., 1991). However, there was no consistent trend of neutral fat accumulation in the sludge bed with each new experiment (Fig. 5.3). The additional reaction time given to the ASBRs at the end of each experiment probably allowed for the digestion of undegraded pork fat particles and prevented significant neutral fat accumulation in the reactors. Increase in total fat content might have been partly due to the synthesis of phospholipids, which predominate among cell membrane lipids (Mackie et al., 1991), or to the accumulation of fats other than neutral fats or LCFAs. Fats other than neutral fats and free LCFAs represented 46.2% ± 8.7% of total fat in the sludge bed, and tended to increase between experiments 1 and 4 (Fig. 5.3).

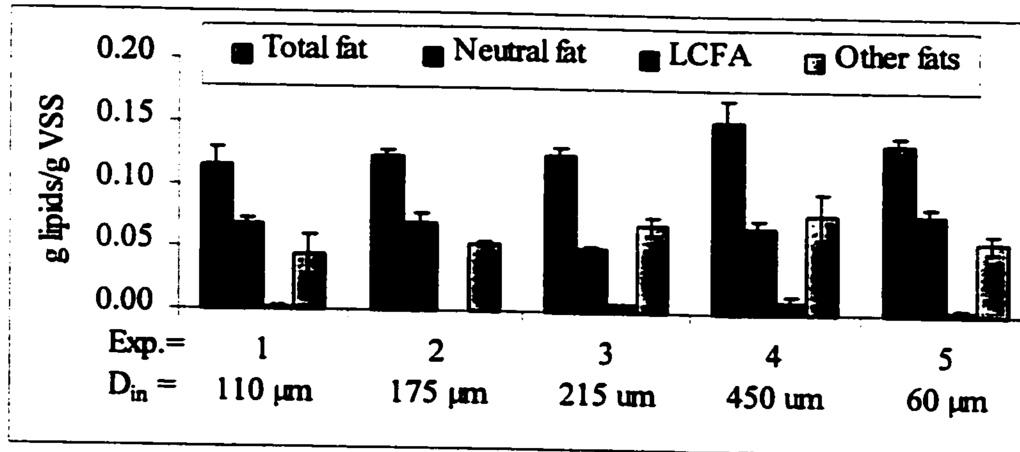


Figure 5.3 Fat distribution in the ASBR sludge bed at the beginning of each experiment (prior to the first ASBR cycle) at various average initial pork fat particle sizes (D_{in})

5.3.3 Neutral fat concentration and hydrolysis rate

Observed and calculated (Eq. 5.2) concentrations of pork neutral fat during the anaerobic digestion of FSHW+F in ASBRs are presented in Figure 5.4 for each D_{in} tested. The first order hydrolysis constants (k_h) ranged from 0.58 ± 0.26 d^{-1} at a D_{in} of 175 µm to 0.72 ± 0.16 d^{-1} at 215 µm (Fig. 5.5). First-order model fit was significant ($P < 0.05$) at all D_{in}, except 175 µm because of high variability in results at that D_{in}.

It has been suggested that the first-order model represents an oversimplification of the hydrolysis process and the following equation should be used (Grady et al., 1999):

$$r_h = -k_h\left[\frac{X_s / X_{B,H}}{K_s + (X_s / X_{B,H})}\right]X_{B,H} \qquad (5.5)$$

where, r_h is the hydrolysis rate ($time^{-1}$); $X_{B,H}$ is the heterotrophic biomass concentration (mg/l); and X_s is particulate substrate concentration (mg/l). However, if the heterotrophic biomass concentration is in excess of particulate concentration, Eq. 5.5 reduces to a first-

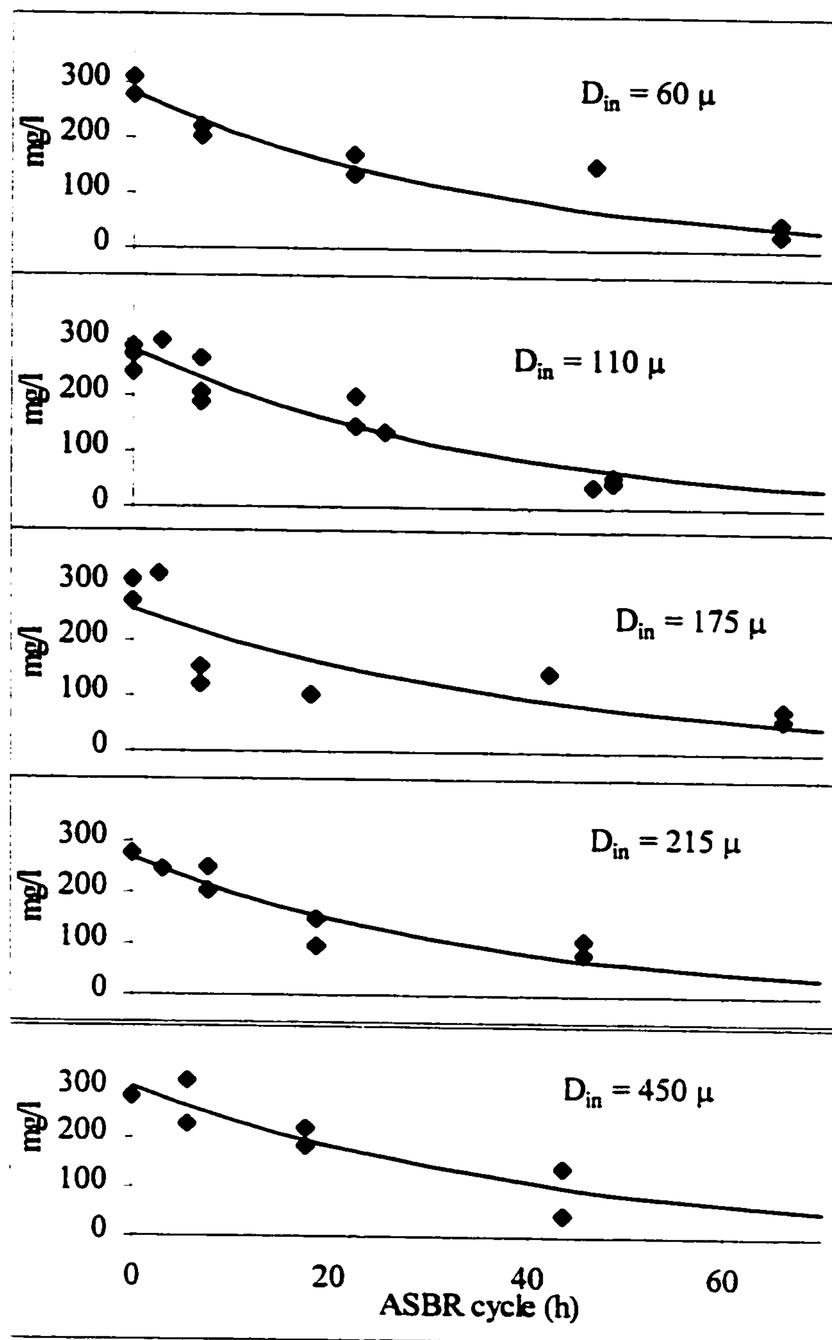


Figure 5.4 Observed and calculated (Eq.5.2) concentrations of pork neutral fat in the mixed-liquor during one ASBR cycle at each initial average pork fat particle size (D_{in}).

order equation. In the ASBRs used in this experiment, neutral fat at time 0 and VSS concentration in the mixed-liquor averaged 282 ± 21 and 6199 ± 763 mg/l, respectively. Heterotrophic biomass concentration was probably in excess of substrate concentration during the entire ASBR cycle, which would justify the use of the first-order equation.

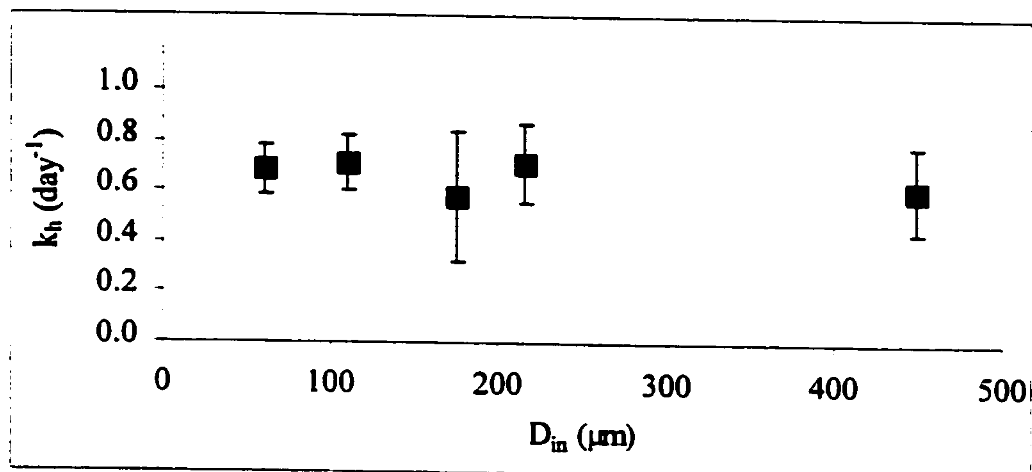


Figure 5. 5 First-order hydrolysis constant (k_h, Eq. 5.2) at each initial average particle size (D_{in})

For the D_{in} tested in this experiment, there was no significant correlation between fat particle size and the hydrolysis rate (Fig. 5.5). The lack of particle size effect on neutral fat hydrolysis rate may be due to a change in particle shape with size. As particle size increased, the fat particles tended to become more filamentous and plate-like, while smaller fat particles appeared denser and more spherical. Hydrolysing bacteria could probably colonise the inside as well as the outside of the large filamentous particles. The increase in specific surface area (m^2/m^3) with a decrease in particle size may not have been that significant.

Since neutral fat hydrolysis rate was not a function of particle size, data from all five experiments were pooled. The overall first-order constant (k_h) was estimated at 0.63 ± 0.07 d^{-1}. Model fit was significant at $P < 0.05$. This value for lipid hydrolysis rate is similar to that estimated by Shimizu et al. (1993) for fat in lysed WAS digested in a mesophilic reactor (0.76 d^{-1}).

5.3.4 LCFA distribution and oxidation rate

In the control ASBRs (FSHW), free LCFA concentration decreased from 48.0 ± 20.2 mg/l at time 0 to 14.9 ± 6.7 mg/l after 45 h of reaction. The free LCFA concentrations in the experimental ASBRs (FSHW+F) are presented in Figure 5.6 for each D_{in} tested. Fairly high variation between some replicate samples was partly due to the numerous analytical steps required in LCFA determination. Free LCFA concentration tended to increase in the first 20 hrs of the ASBR cycle, and decreased thereafter. However, free LCFA concentrations rarely exceeding 200 mg/l, and were always below the range of concentrations (500 to 1200 mg/l) reported for LCFA oxidisers and methanogens inhibition (Koster and Cramer, 1987; Ahring et al., 1992).

Saturated palmitic (16:0) and stearic (18:0) acids represented 28.3% ± 0.3% and 21.9% ± 0.6%, respectively, of the free LCFAs in the ASBRs mixed-liquor. Unsaturated oleic (18:1) and linoleic (18:2) acids accounted for another 48.0% ± 0.5% of the free LCFAs. Traces of myristic (14:0) and palmitoleic (16:1) were also detected in some of the samples. No significant changes in the proportion of the different free LCFAs during the ASBR cycle were detected.

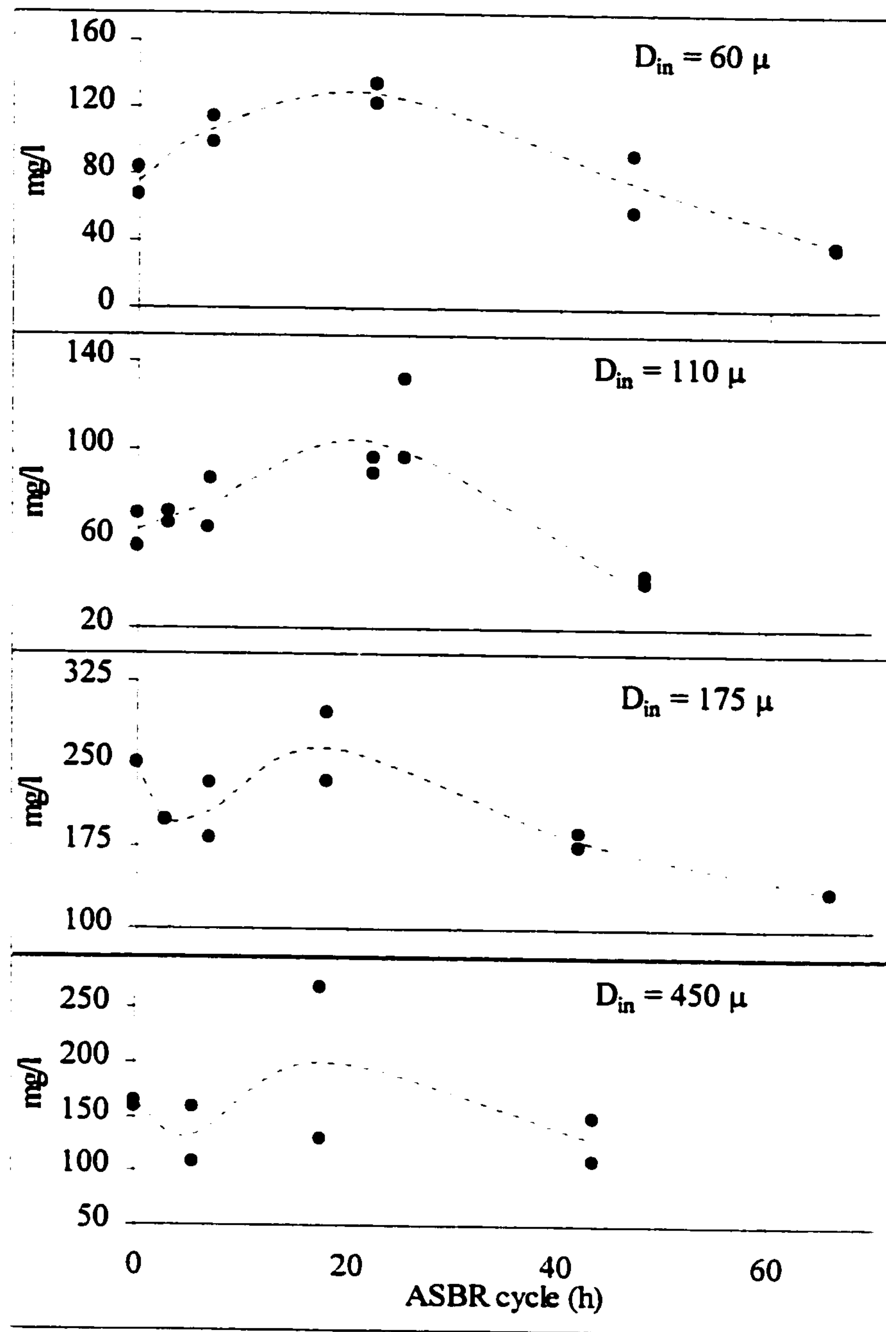


Figure 5.6 Long-chain fatty acid concentration in the mixed-liquor during one ASBR cycle at each initial average particle size (D_{in}). The broken line shows the average LCFA concentration.

The LCFA oxidation data (Eq. 5.3) were fitted to the Monod equation described above (Eq. 5.4). The maximum LCFA degradation (oxidation) rate (k_{max}) and half-saturation concentration (K_s) averaged 164 ± 37 mg LCFA/l/d (477 mg COD/l/d) and 35 ± 31 mg LCFA/l (102 mg COD/l), respectively. The model fit was significant at $P < 0.05$. Observed LCFA in the ASBRs at a D_{in} of 60 μm and results simulated using Monod equation are shown on Figure 5.7. (Note: observed and simulated data are also shown at all D_{in} on Figure 6.3).

The low half-saturation concentration (K_s) compared to values reported in the literature may denote a high affinity of the ASBR sludge for the LCFA substrate. In ASBRs and other high-rate anaerobic reactors, solid retention times (SRTs) are long and independent of hydraulic retention times (HRTs). A biomass fully acclimatised to the substrate is thus developed. Long SRTs and acclimatisation are particularly important for lipid-degrading microorganisms, since their growth rate is almost one order of magnitude slower than that of protein- and carbohydrate-degrading bacteria (Chynoweth and Mah, 1971; Pavlostathis and Giraldo-Gomez, 1991).

The low estimated K_s value may also reflect the low LCFA concentrations measured in this study compared with those reported in research on sewage sludge. Some models, such as the Contois model, consider K_s as a variable directly proportional to input substrate concentration:

$$K_s = aS_0 \tag{5.6}$$

where, a is a proportionality constant; and S_0 is input substrate concentration (mg/l) (Pavlostathis and Giraldo-Gomez, 1991). At 25°C, O'Rourke (1968) reported initial free LCFA and half-saturation concentrations of 5700 and 3720 mg COD/l, respectively. The

corresponding proportionality constant *a* would be 0.65. In the present study, LCFA concentration at time 0 averaged 88 mg/l. With a K_s of 164 mg/l, the proportionality constant was 0.40. A low K_s value of 20 mg/l was also reported by Angelidaki et al. (1999) for the digestion of trioleate, a substrate which probably initially contained very low free LCFA concentrations.

On a VSS basis, the maximum specific LCFA oxidation rate (specific k_{max}) averaged 0.077 mg COD/mg VSS/d. This estimate is lower than the specific k_{max} values reported in the literature (Angelidaki et al., 1999; O'Rourke, 1968; Novak and Carlson 1970), and may be the result of differences in substrate composition or to the high biomass to LCFA ratio in the ASBRs. Sanders et al. (1999) reported that in batch reactors, substrate became covered with bacteria within a few hours of treatment and enzymes were 'excessively present'. For slaughterhouse wastewaters treated by high-rate anaerobic reactors having excess biomass concentrations, a removal rate value given on a volumetric COD basis (mg COD/l/d) may be more representative of pork fat particle degradation than estimates given on a specific biomass basis.

5.3.5 Simulation of fat particle degradation

Figure 5.7a presents observed and calculated pork fat particle degradation during anaerobic treatment of slaughterhouse wastewater in ASBRs operated at 25°C. Neutral fat hydrolysis was calculated using the first-order equation (Eq. 5.2) with average k_h value of 0.63 d^{-1}, while LCFA oxidation was simulated using Eq. 5.4 with K_s and k_{max} values of 35 mg/l and 164 mg/l/d, respectively. Observed data were those measured at a D_{in} of 60 μm (Experiment 5). Observed and calculated results shows that the LCFA concentration increased slightly during the first 14 h of digestion, as rapid neutral fat

hydrolysis occurs. The LCFA concentration then steadily decreased until the end of the cycle. This degradation pattern, which is similar to that reported by Heukelekian and Mueller (1958), suggests that pork fat degradation rate is primarily a function of LCFA oxidation and, to a lesser extent, neutral fat hydrolysis.

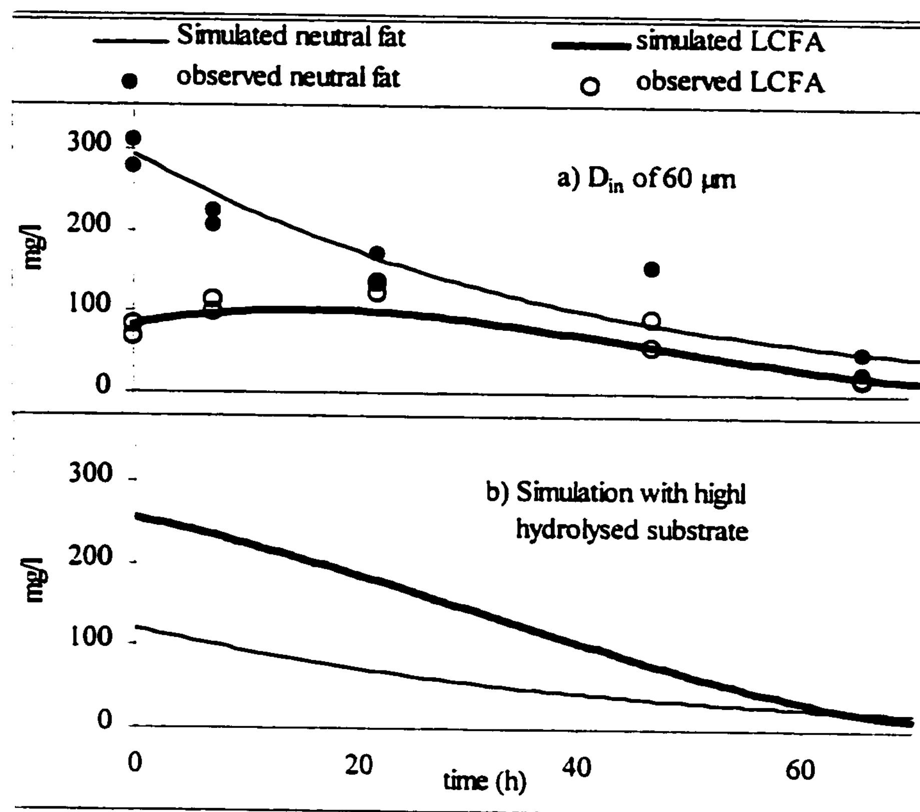


Figure 5.7 Simulated and observed free LCFA and neutral fat concentrations in ASBR mixed-liquor a) using average parameter estimates (Eqs. 5.2 and 5.4) and data collected at an initial average particle size (D_{in}) of 60 µm; and b) using average parameter estimates and a substrate with 68% free LCFAs and 32% neutral fat

Fat degradation was also simulated using a highly hydrolysed substrate mixture containing a free LCFA to neutral fat ratio similar to that reported by O'Rourke (1968), that is 68% LCFA and 32% neutral fat (Fig. 5.7b). Total fat concentration and parameter

estimates were the same as those used for Fig. 5.7a. With a highly hydrolysed substrate, the degradation process appears to be essentially limited by the rate of LCFA oxidation. Given the low K_s value estimated in this experiment, the maximum LCFA oxidation rate is rapidly attained and the rate at which additional free LCFAs are produced (hydrolysis rate) does not substantially affect the LCFA oxidation rate. Substrate composition may thus partly explain the difference between the results presented in this study and those of O'Rourke (1968).

Total fat degradation rate was slightly faster with the hydrolysed (Fig. 5.7a) than the FSHW+F (Fig. 5.7b) substrates. Free LCFA and neutral fat concentrations were reduced by 83% after 59 and 69 h of anaerobic treatment with hydrolysed and FSHW+F substrates, respectively. It was estimated that treatment time would be reduced by 14.5% by using a highly hydrolysed substrate.

5.3.6 Soluble COD and VFAs

Initial soluble COD concentrations averaged 2322 ± 212 and 2332 ± 221 mg/l in control and experimental substrates, respectively (Table 5.1). Figure 5.8a presents SCOD concentration during one ASBR treatment cycle (D_{in} of 60 µm). Similar results were obtained at the other D_{in}. At time 0, two SCOD values are shown on Figure 5.8a. The higher values represent the SCOD concentration that was expected based on the SCOD concentration inputted with the substrate. The lower points present the actual values measured in the mixed liquor at time 0. The difference between the two points indicates the amount of soluble organics that was adsorbed on the solids during the 1-h feeding period. Results from all experiments suggested that between 17% and 50% of substrate SCOD was adsorbed on the biomass during the reactor feeding phase.

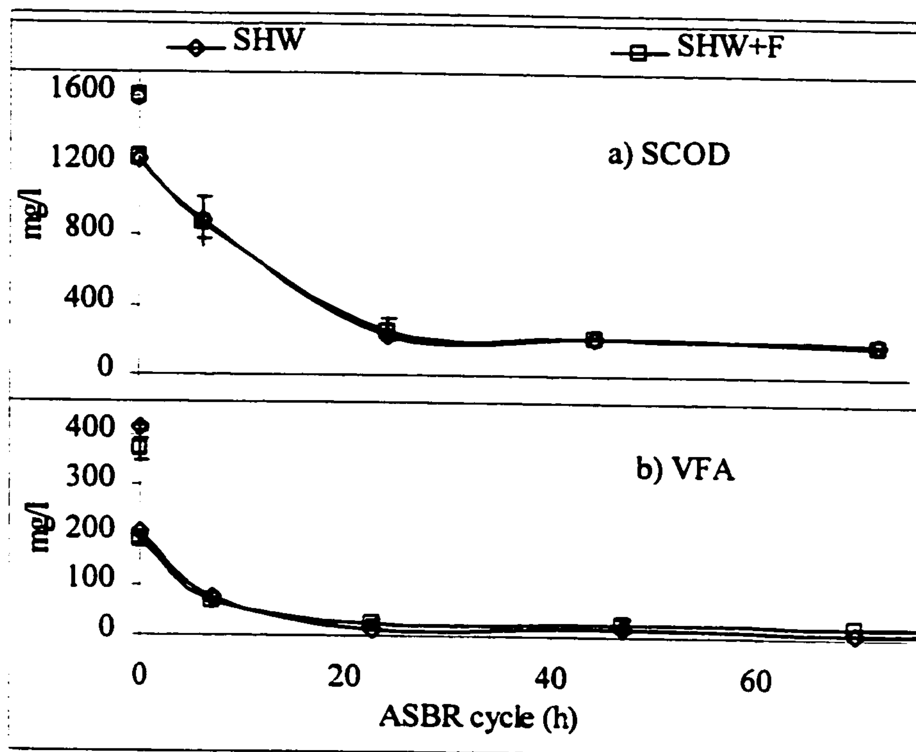


Figure 5.8 a) Soluble COD and b) Volatile fatty acids (VFA) concentrations in ASBRs fed control (FSHW) and experimental (FSHW+F) substrates at a D_{in} of 60 μm (Exp. 5)

After 18 to 24 h of anaerobic treatment, substrate SCOD had been decreased by 91% ± 2% and 90% ± 2% in FSHW and FSHW+F reactors, respectively (Fig. 5.8a). At the end of the 69-h treatment cycle, overall SCOD reduction averaged 94% ± 1% in all reactors. Similar pattern of SCOD reduction during anaerobic digestion of FSHW and FSHW+F suggested that SCOD analysis could not be used to measure differences in LCFA concentration between control and experimental reactors. In Experiment 5, for example, SCOD averaged 231 ± 3 and 253 ± 10 mg/l in FSHW and FSHW+F, respectively, after 24 h of anaerobic treatment (Fig. 5.8a). At the same time period, the LCFA concentration averaged 78 ± 36 and 374 ± 26 mg COD/l in FSHW and FSHW+F reactors, respectively. The SCOD analysis was obviously unable to reflect differences in free LCFA

concentration. An analytical method that removes bound LCFAs from solids surfaces must be used to measure lipid hydrolysis.

Figure 5.8b presents VFA concentration in solution in FSHW and FSHW+F reactors during Experiment 5. A 50% difference between the VFAs fed with the substrate and VFA concentration in the mixed-liquor at time 0 also suggested adsorption of the VFAs during feeding. Within the first 24 h of reaction, VFA concentrations had dropped below 20 mg/l in all bioreactors and remained low for the rest of the cycle. VFA oxidation did not seem to be a limiting factor during treatment. However, the amount of VFAs adsorbed on the biomass was not measured.

5.4 Conclusion

The anaerobic digestion at 25°C of pork fat particles in slaughterhouse wastewater indicated that average particle sizes ranging from 60 to 450 μm had no significant effect on the rate of neutral fat hydrolysis. The hydrolysis rate of pork fat particles averaged 0.63 ± 0.07 d^{-1}. Modelling of LCFA oxidation with a Monod-type equation yielded a maximum substrate removal rate and a half-saturation concentration of 164 ± 37 mg LCFA/l/d and 35 ± 31 mg LCFA/l, respectively. Under the conditions of this experiment, that is approximately 75% neutral fat and 25% free LCFA and digestion at 25°C, pork fat particle degradation was mainly controlled by LCFA oxidation and, to a lesser extent, by neutral fat hydrolysis. However, the anaerobic degradation of a substrate containing highly hydrolysed fat, such as sewage sludge, should be limited by the LCFA oxidation rate. Soluble COD could not be used to determine the rate of lipid hydrolysis due to LCFA adsorption on the biomass.

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CHAPTER 6

EFFECT OF HYDROLYSIS PRETREATEMENT ON FAT DEGRADATION DURING ANAEROBIC DIGESTION OF SLAUGHTERHOUSE WASTEWATER

Abstract

Slaughterhouse wastewater containing pork fat particles with initial average particle sizes (D_{in}) ranging from 60 to 450 μm was pretreated for 5.5 h with 250 mg/l of Pancreatic Lipase 250 (PL-250). Approximately 35% of substrate neutral fat was hydrolysed during pretreatment. Enzyme pretreated and control substrates were fed to anaerobic sequencing batch reactors (ASBRs) operated at 25°C. During anaerobic digestion, the first-order neutral fat hydrolysis rate averaged 0.50 ± 0.13 and 0.63 ± 0.07 d^{-1} for pretreated and control substrates, respectively. There was no significant particle size or pretreatment effect on pork fat hydrolysis rate in the ASBRs. Subsequent long-chain fatty acid (LCFA) oxidation followed Monod-type kinetics. Higher free LCFA concentrations in enzyme pretreated feed than control feed resulted in slightly increased LCFA removal rate at the onset of anaerobic digestion with pretreated substrate. However, it was estimated that pretreating the substrate had only a small effect on fat particle digestion at 25°C, marked by a decrease of about 5% in digestion time to achieve 80% reduction in substrate neutral fat and LCFA concentration. The transformation of influent COD into methane after 69 h of anaerobic treatment averaged 82% and 78% with pretreated and control substrates, respectively. The difference was mainly due to lower methane production in reactors fed control than enzyme pretreated substrates in the initial

20 h of anaerobic treatment. However, pretreatment effect could be more important for digestion at higher temperatures, due to an increase in oxidation rate.

Keywords: anaerobic digestion, ASBR, fat particles, hydrolysis, lipid, long-chain fatty acids, pretreatment, enzyme, lipase

6.1 Introduction

Lipids represent an important fraction of the particulate organic charge in slaughterhouse wastewater (Sachon, 1986; Sayed et al., 1988). Anaerobic treatment of slaughterhouse wastewater at 20°C and 30°C has been reported to be slowed down or impaired because of high concentrations of suspended solids, particularly fats (Saxena et al., 1986; Martínez et al., 1995). These results suggested that hydrolysis pretreatment of fat particles could accelerate the anaerobic treatment of slaughterhouse wastewater.

Pretreating slaughterhouse wastewater with Pancreatic Lipase 250 (PL-250) significantly reduced the size of pork fat particles (Masse et al., 2001a; 2001b). Additionally, an increase in free LCFA concentration in solution indicated complete hydrolysis of a fraction of the fat particles during enzymatic pretreatment. However, pretreatment effect on downstream anaerobic treatment of slaughterhouse wastewater was not determined. The objective of this study was to evaluate the effect of hydrolysis pretreatment with PL-250 on the anaerobic digestion at 25°C of pork fat particles in slaughterhouse wastewater.

6.2 Materials and Method

Pancreatic Lipase 250 (PL-250, Genencor International, Rochester, N.Y.) is a commercial lipolytic enzyme isolated from pig pancreas. The experimental substrate (FSHW+FE) consisted of filtered slaughterhouse wastewater containing approximately 2000 mg/l of pork fat particles mixed with 250 mg/l of PL-250 enzyme for 5.5 h at 25°C. Two control substrates were also mixed for the duration of the pretreatment: (1) filtered slaughterhouse wastewater without pork fat particles or enzyme (FSHW) and (2) filtered slaughterhouse wastewater containing 2000 mg/l of pork fat particles but no enzyme (FSHW+F). Prior to reactor feeding, all substrates were diluted with filtered slaughterhouse wastewater in order to decrease the concentration of added pork fat particles to approximately 560 mg/l. This procedure was followed because optimum conditions for enzyme pretreatment had been determined at a fat particle concentration of 2000 mg/l, while lipid concentration in slaughterhouse wastewater is typically around 500 mg/l.

Four experiments, corresponding to initial average fat particle sizes (D_{in}) of 175, 215, 450, and 60 μm, respectively, were conducted. During each experiment, FSHW, FSHW+F and FSHW+FE substrates were fed to duplicate anaerobic sequencing batch reactors (ASBRs) for three cycles. Characteristics of all substrates during each experiment are presented in Table 6.1. The six ASBRs were operated at 25°C and a hydraulic retention time (HRT) of 3 days. Specific characteristics and operation of the ASBRs as well as mixed-liquor, sludge and substrate sample collection, analytical procedures and data analyses were described in Masse et al. (2001c). Biogas production

Table 6. 1 Average characteristics of the FSHW, FSHW+F and FSHW+FE substrates fed to anaerobic sequencing batch reactors operated at 25°C

Exp. no.	D_{in}[†] (μm)	Total lipid conc. (mg/l)			Soluble COD (mg/l)			Total COD (mg/l)		
		FSHW	FSHW+F	FSHW+FE	FSHW	FSHW+F	FSHW+FE	FSHW	FSHW+F	FSHW+FE
1	175	153 ± 125	766 ± 61	685 ± 27	2283 ± 167	2160 ± 85	2351 ± 144	3464 ± 215	4988 ± 171	4861 ± 22
2	215	146 ± 32	746 ± 82	732 ± 59	2231 ± 113	2208 ± 98	2393 ± 91	3081 ± 237	4201 ± 22	4531 ± 693
3	450	195 ± 83	664 ± 86	689 ± 121	2086 ± 109	2289 ± 313	2379 ± 165	2799 ± 33	3987 ± 632	4018 ± 134
4	60	143 ± 43	675 ± 21	714 ± 33	2484 ± 255	2520 ± 293	2907 ± 17	3255 ± 252	4534 ± 209	4738 ± 293
avg.		157 ± 42	719 ± 71	705 ± 57	2295 ± 219	2294 ± 225	2507 ± 263	3192 ± 306	4405 ± 451	4477 ± 426

[†] Initial average particle size.

(methane and carbon dioxide) was determined periodically by gas chromatography.

During experiment 1 (D_{in} of 175 μm), two additional reactors were fed FSHW pretreated with PL-250. Enzyme dose and pretreatment conditions were the same as those used with the experimental substrate (FSHW+FE). At the end of all four experiments, duplicate ASBRs were fed 5 l of tap water containing 0, 75, 250 and 1000 mg/l of PL-250 for three cycles.

6.3 Results and Discussion

6.3.1 Enzyme effect on biomass and FSHW degradation

Pancreatic Lipase 250 was entirely soluble and had a COD equivalent of 1.05 g/g. Methane production from bioreactors fed PL-250 dissolved in tap water was corrected for methane produced by control reactors operated simultaneously and fed tap water only. Corrected methane yield was always in excess of the COD inputted as enzyme (Table 6.2). Excess methane production ranged from 0.72 to 1.09 g CH_4-COD, and was probably due to additional degradation of decayed bacteria in reactors receiving enzyme. On average, 44%, 31% and 25% of methane production due to enzyme addition was recorded in the first, second and third days of the ASBR cycle, respectively.

In the ASBRs fed FSHW pretreated with PL-250. there was no significant methane production in excess of the COD added as enzyme (Table 6.2). Some enzymatic activity may have been lost during the 5.5 h of mixing with the substrate. Additionally, enzyme COD (0.88 g) represented only 2% of TCOD in FSHW. and small differences in methane volume were more difficult to detect. With FSHW, 50%. 21% and 29% of the methane

production due to enzyme addition was registered in the first, second and third days of the ASBR cycles, respectively.

Table 6. 2 Methane production from Pancreatic Lipase 250 in water and FSHW

Medium	PL-250 added		CH_4	CH_4	Excess CH_4
	mg/l	g COD	(l)[†]	(g COD)[‡]	(g COD)[††]
Water	75	0.39	0.43 ± 0.15	1.12 ± 0.39	0.72 ± 0.39
	250	1.31	0.81 ± 0.17	2.11 ± 0.45	0.80 ± 0.45
	1000	5.25	2.42 ± 0.25	6.34 ± 0.65	1.09 ± 0.65
FSHW	70	0.88	0.39 ± 0.05	1.02 ± 0.14	0.14 ± 0.14

† Corrected for methane produced by reactors fed the same medium without the enzyme
‡ Based on 0.38 l of methane per g COD at 25°C
†† Total g CH_4-COD produced minus g COD inputted as enzyme

Results from these experiments suggested that PL-250 was entirely degradable and, at the dose used in this experiment, the enzyme had minimal effect on biomass and filtered slaughterhouse wastewater degradation during anaerobic digestion. In the experimental substrate (FSHW+FE), enzyme COD amounted to 0.88 g and represented an average of 1.6% of TCOD. Methane production due to total enzyme degradation would represent approximately 2% of total methane produced by the experimental ASBR reactors (16.6 ± 1.3 l CH_4) during the 3-d cycles. Consequently, in this experiment, daily methane production rates from the experimental reactors were not corrected for enzyme COD because of the small additional biogas volume it generated and the difficulty of determining the amount to be subtracted in each time period.

6.3.2 Pretreatment effect on substrate and initial conditions in the ASBRs

Total fat concentration averaged 157 ± 42, 719 ± 71, and 705 ± 57 mg/l in FSHW, FSHW+F and FSHW+FE substrates, respectively (Table 6.1). Fat distribution in the

substrates used at a D_{in} of 60 μm is presented in Table 6.3. Similar free LCFA concentrations in FSHW and FSHW+F substrates suggested that pork fat particles were not hydrolysed during mixing at room temperature. On the other hand, approximately 35% of the neutral fat was hydrolysed during enzymatic pretreatment of FSHW+FE. In a previous experiment on PL-250 characterisation, an average free LCFA concentration of 14.6 ± 0.3 mg/l was measured in filtered samples of FSHW containing 2000 mg/l of pork fat particles and mixed for 4 to 6 h with 250 mg/l of PL-250 (Masse et al., 2001b). The substantially higher free LCFA concentration detected in the FSHW+FE substrate in this experiment (245 ± 2 mg/l), when bound free LCFAs as well as free LCFAs in solution are considered, suggests that most free LCFAs produced during lipolysis remain adsorbed on fat particle surface and can not be recovered in filtered samples.

Table 6. 3 Neutral fat and free LCFA concentrations in the substrates used at a D_{in} of 60 μm

Substrate	Neutral fat (mg/l)	Free LCFA (mg/l)	Other lipids (mg/l)
FSHW	89 ± 30	79 ± 16	32 ± 12
FSHW+F	483 ± 37	82 ± 38	173 ± 41
FSHW+FE	311 ± 44	245 ± 2	158 ± 9

Figure 6.1 presents pork neutral fat and free LCFA concentrations in the mixed-liquor samples collected immediately after ASBR feeding (time = 0) at each D_{in}. Initial neutral fat concentration was 38% lower in reactors fed FSHW+FE (177 ± 24 mg/l) than FSHW+F (287 ± 8 mg/l). On the other hand, initial neutral fat concentration in reactors fed FSHW+FE was similar at all D_{in}, suggesting that fat particle size did not have an effect on the amount of fat hydrolysed during pretreatment. Initial free LCFA

concentration varied considerably at the different D_{in} tested, but there was no consistent particle size effect on free LCFA concentration at time 0. Moreover, the average difference in free LCFA concentrations between pretreated and control reactors (93 mg/l) is consistent with the difference in neutral fat concentration (110 mg/l). This increase in free LCFA concentration following pretreatment corresponds to the neutral fat hydrolysed during during enzymatic pretreatment. The higher initial free LCFA concentrations observed at D_{in} of 175 and 450 μm were probably due to residual free LCFAs remaining in the reactors at the end of the previous cycle, and not to different degree of neutral fat hydrolysis during pretreatment.

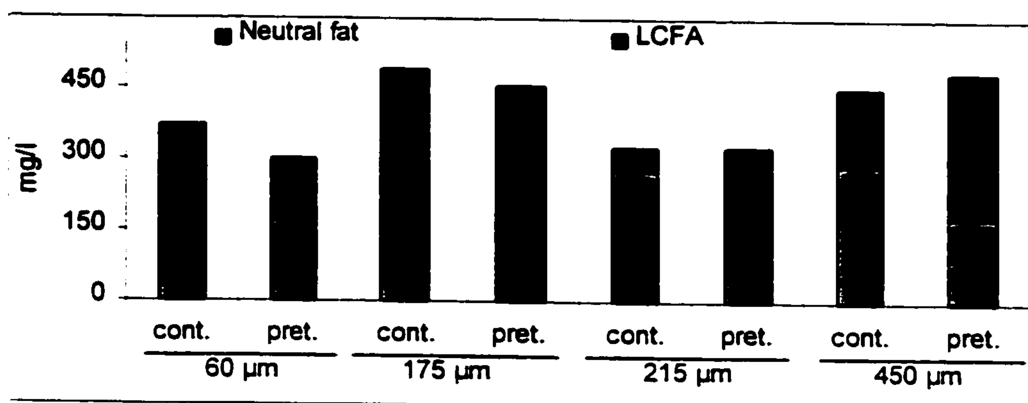

Figure 6. 1. Neutral fat and LCFA concentrations in the mixed-liquor immediately after ASBR feeding

6.3.3 Neutral fat hydrolysis rate

Neutral fat hydrolysis rate during anaerobic treatment in the ASBRs was calculated based on first-order kinetics using the method described in Masse et al. (2001c). The first-order neutral fat hydrolysis rate (k_h) ranged from 0.43 to 0.68 d^{-1} for FSHW+FE and

from 0.58 to 0.72 d^{-1} for FSHW+F (Fig. 6.2). There was no particle size effect on neutral fat hydrolysis rate with either substrate. It has been suggested previously that the specific surface area (m^2/m^3) available for hydrolysis of pork fat particles was not inversely proportional to size, and reducing particle size did not provide significantly more specific surface area for enzymatic reaction (Masse et al., 2001c).

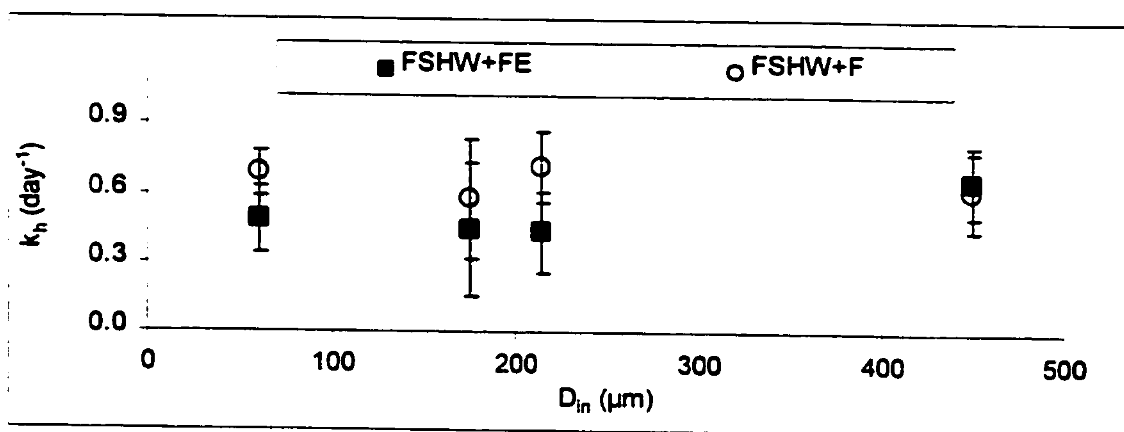

Figure 6. 2 First-order hydrolysis rate coefficient (k_h) at different initial average particle sizes (D_{in}) for FSHW+F and FSHW+FE substrates

Using data collected at all D_{in}, the first-order neutral fat hydrolysis rate coefficient was estimated at 0.50 ± 0.13 d^{-1} and 0.63 ± 0.07 d^{-1} for enzyme pretreated and control substrates, respectively. There was no significant pretreatment effect on the neutral fat hydrolysis rate during anaerobic digestion (Fig. 6.2). One of the main reported effects of PL-250 was to decrease the size of fat particles (Masse et al., 2001b). However, since fat hydrolysis rate is not a function of particle size, enzymatic pretreatment of the substrate showed no beneficial effect on the hydrolysis of the residual neutral fat once the substrate was fed to the ASBRs. Additionally, the dilution of the enzyme before ASBR feeding (to

bring fat particle concentration into typical slaughterhouse wastewater range) may have attenuated any positive effects of residual enzyme.

6.3.4 Pork fat anaerobic degradation

Measured and empirically modelled concentrations of pork neutral fat and free LCFAs during anaerobic treatment in the ASBRs are presented in Fig. 6.3 for FSHW+FE and FSHW+F at all D_{in}. Neutral fat and free LCFA concentrations were modelled using first-order hydrolysis (Eq. 5.2) and Monod-type oxidation kinetics (Eq. 5.4), respectively. Model parameters were estimated using data from the FSHW+F runs, as outlined in Masse et al. (2001c). The first-order neutral fat hydrolysis rate constant (k_h) was estimated at 0.63 d^{-1}, while the maximum LCFA oxidation rate (k_{max}) and the half-saturation concentration (K_s) for the Monod equation averaged 164 mg free LCFA/l/d and 35 mg free LCFA/l, respectively. These parameters were used for all runs since particle size or pretreatment did not have a significant effect on neutral fat hydrolysis during anaerobic digestion, and lipase pretreatment or particle size should not affect the free LCFA oxidation rate. Simulated data fitted the observed results relatively well, except for the experiment at a D_{in} of 175 μm which showed greater variability in results (Fig. 6.3).

A slightly different fat degradation pattern was observed in reactors fed control and enzyme pretreated substrates (Fig. 6.3). Hydrolysis, which is first order with respect to neutral fat concentration, was more rapid at the onset of the treatment period in ASBRs fed FSHW+F than FSHW+FE. Consequently, free LCFA concentration increased slightly or remained relatively constant in the initial 30 h of digestion with FSHW+F (Fig. 6.3). On the other hand, initial free LCFA concentration was higher in FSHW+FE than

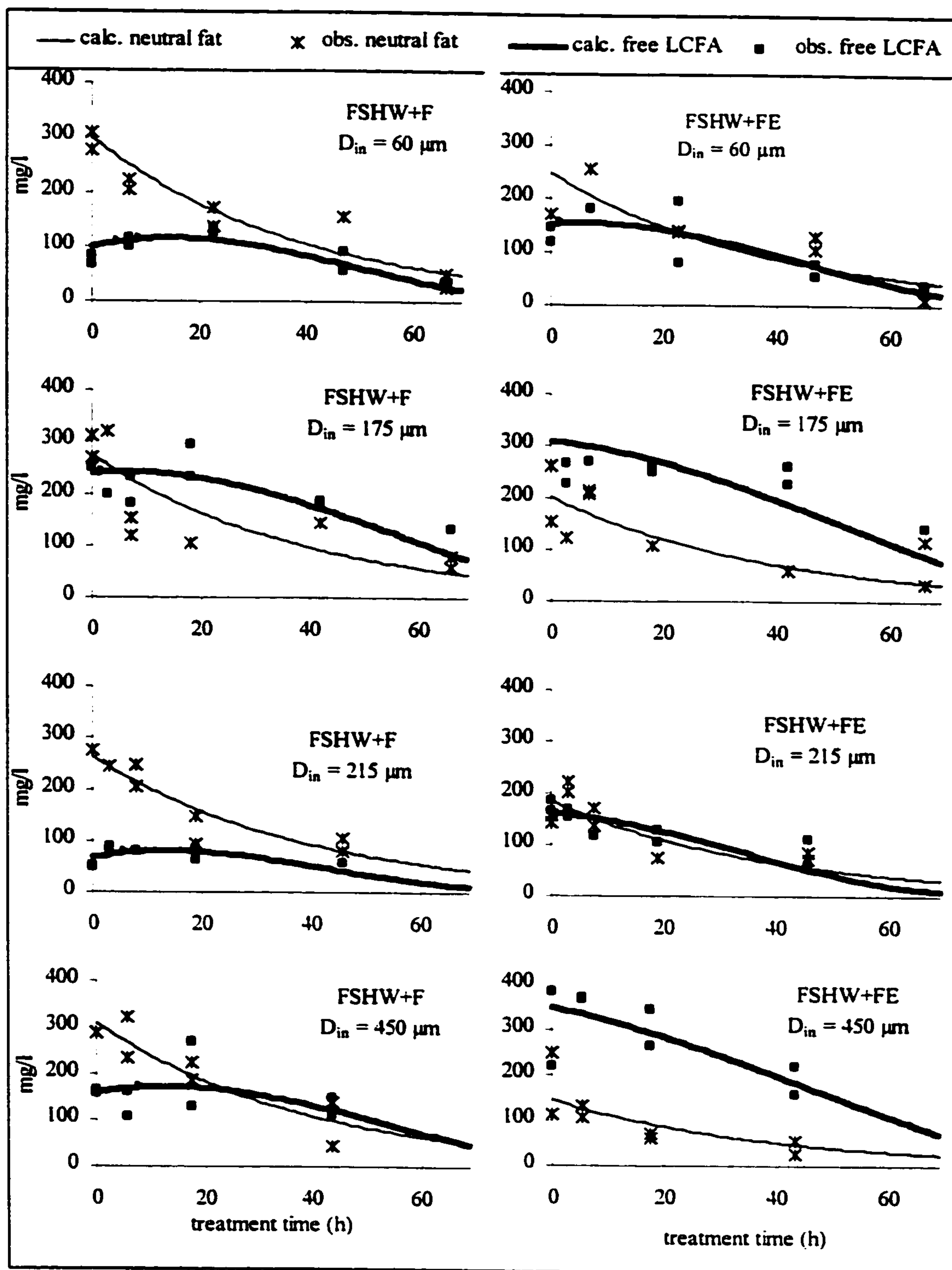

Figure 6. 3 Observed and calculated free LCFA and neutral fat concentrations during anaerobic digestion of FSHW+FE and FSHW+FE substrates

FSHW+F substrates. Thus, as predicted by Monod-type kinetics, LCFA removal rate was faster in reactors fed FSHW+FE in the early phase of digestion. However, the half-saturation concentration (K_s) estimated for the LCFA oxidation rate equation was low (35 mg/l), indicating a high affinity of the ASBR biomass for the LCFA substrate. The maximum oxidation rate (k_{max}) was thus reached at low free LCFA concentrations and increasing the free LCFA concentration had limited impact on the LCFA oxidation rate.

Based on modelled values, pork neutral fat and free LCFAs degradation after 69 h of anaerobic treatment ranged from 77% to 83% in ASBRs fed FSHW+F and from 78% to 88% in ASBRs fed FSHW+FE. On average, the initial neutral fat and free LCFA concentrations was reduced by 80% after 65 and 68 h of anaerobic treatment for pretreated and control substrates, respectively. Therefore, pretreating the substrate had only a small effect on fat particle digestion at 25°C, marked by a decrease of about 5% in digestion time to achieve the same level of treatment. However, it is possible that the effect of the enzymatic pretreatment be more significant for anaerobic digestion at higher temperatures, since LCFA oxidation rates should increase substantially with temperature. O'Rourke (1968) reported that the maximum LCFA removal rate (k_{max}) nearly doubled, while the half-saturation concentration (K_s) was reduced by over 50% when digester temperature was increased from 20°C to 35°C.

6.3.5 Biogas quality and methane yield

ASBR biogas contained 78.9% ± 1.4% of methane. There was no significant substrate or pretreatment effect on biogas methane content.

Figure 6.4 presents the fraction of input TCOD transformed into methane (g CH_4-COD per g of $TCOD_{in}$) during the third cycle of each experiment in FSHW+F and

FSHW+FE reactors. After 69 h of treatment, the fraction of input COD transformed into methane ranged from 0.77 to 0.79 with FSHW+F and from 0.80 to 0.83 with FSHW+FE. Although the difference was small, it was significant at $P < 0.001$, and was mostly due to higher methane production in the early phase of digestion from reactors fed FSHW+FE rather than FSHW+F.

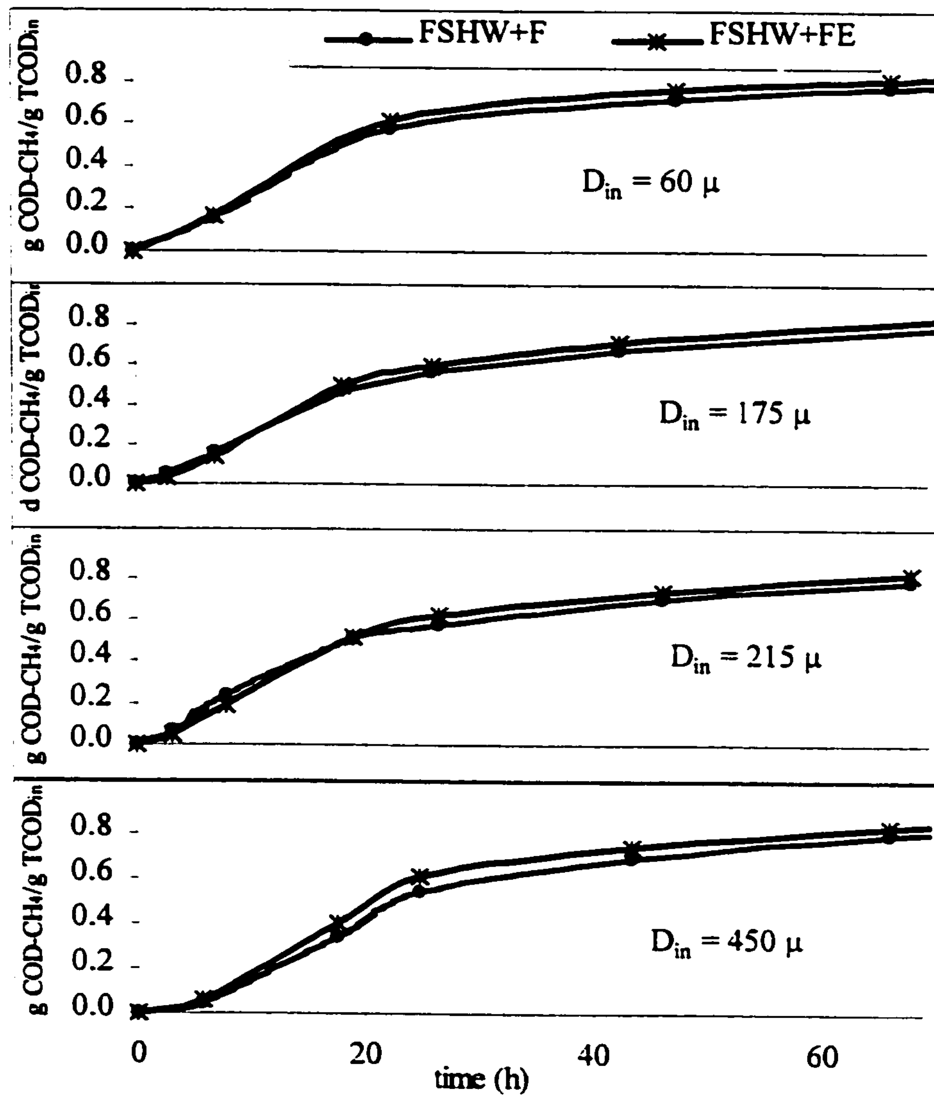

Figure 6. 4 Specific methane yield during anaerobic digestion of FSHW+F and FSHW+FE substrates

Figure 6.5 presents the difference in methane production rate between reactors fed FSHW (no fat or enzyme) and those fed FSHW+F and FSHW+FE in three periods of the ASBR treatment cycle. The data points represent the additional methane production that was obtained by supplementing the filtered slaughterhouse wastewater with untreated and enzyme pretreated fat particles. With FSHW+F, methane production in the initial 20 h of digestion decreased linearly with increasing D_{in}, from 1.56 ± 0.25 l/d at a D_{in} of 60 μm to -0.53 ± 0.30 l/d at 450 μm (Fig. 6.5a). The decrease in methane production with particle size was significant ($P < 0.05$) except between 175 and 215 μm. The negative value reported at a D_{in} of 450 μm indicated that methane production in the initial 20 h of digestion was lower in reactors fed FSHW+F than FSHW, although the former substrate contained almost 50% more COD than the latter. Thus, the larger fat particles seemed to inhibit methane production from fat particles as well as from filtered slaughterhouse wastewater. However, low methane production at a D_{in} of 450 μm could not be attributed to a decrease in fat hydrolysis rate, which would have limited the supply of free LCFAs to the oxidising bacteria, since initial free LCFA concentration in the ASBR mixed-liquor was higher at a D_{in} of 450 μm than 60 μm (Fig. 6.3). Although largely unexplained. retardation in methane production in the first few hours of digestion appeared to be the main significant effect of particle size on anaerobic digestion of slaughterhouse wastewater. Angelidaki et al. (1990) also reported lower methane production from bioreactors fed oil and cattle manure than from reactors fed manure only, indicating that the presence of lipids inhibited the methanization of substrate other than oil.

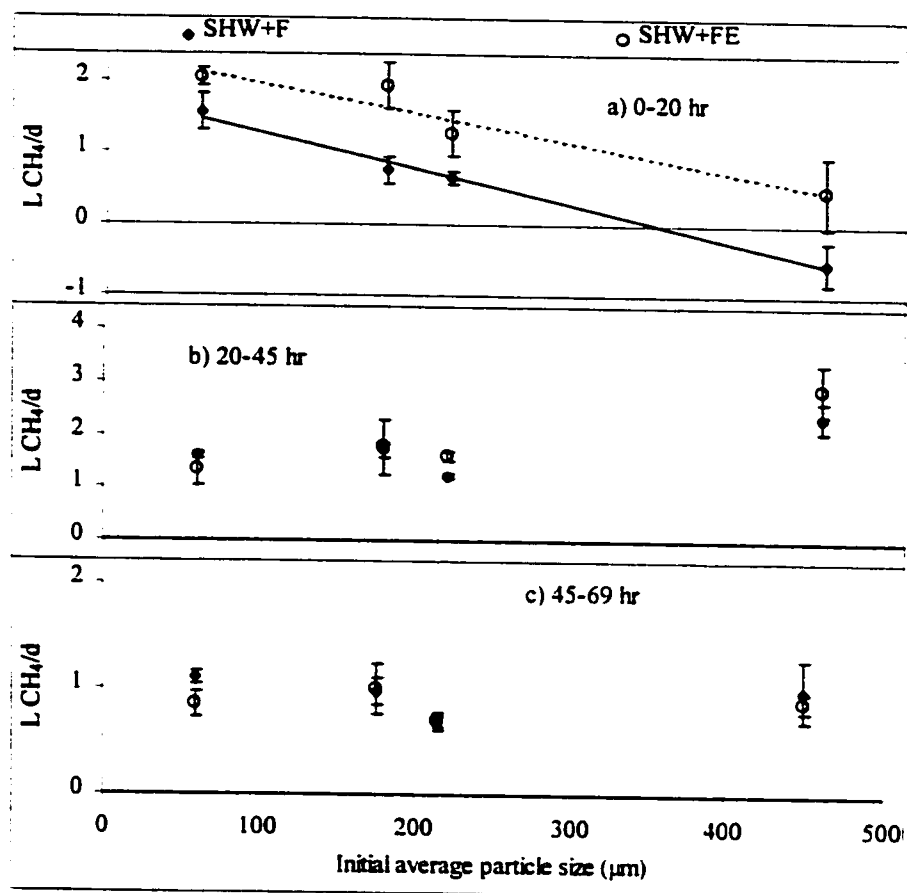

Figure 6. 5 Difference in methane production between reactors fed FSHW and those fed FSHW+F and FSHW+FE in three periods during the ASBR treatment cycle. The data points represent the additional methane production that was obtained by supplementing the slaughterhouse wastewater with untreated and enzyme pretreated fat particles

The difference in methane production rate between reactors fed FSHW and FSHW+FE in the initial 20 h of digestion decreased from 2.05 ± 0.11 l/d at 60 μm to 0.46 ± 0.49 l/d at 450 μm (Fig. 6.5a). The decrease in methane production with particle size was significant ($P < 0.05$) except between 60 and 175 μm. However, at every D_{in} except 215 μm, methane production rate was significantly higher ($P < 0.01$) in reactors receiving enzyme pretreated than control substrates. Since larger fat particles appeared to inhibit early methane production, pork fat particle size reduction during pretreatment with PL-

250 may have allowed an increase in methane production in the 0-20 h period. Based on results from previous experiments, 5.5 h of mixing with 250 mg/l of PL-250 would reduce D_{in} of 60, 175, 215 and 450 μm to 51, 117, 136 and 240 μm, respectively (Masse et al., 2001b). On Figure 6.6, differences in methane production rate in the initial 20 h of digestion between reactors fed FSHW and those FSHW+F and FSHW+FE were plotted with respect to the D_{in} following pretreatment. The regression line shown on Figure 6.6 was determined using results from FSHW+F runs only. The data from FSHW+FE runs also fitted the regression curve relatively well, suggesting that the higher methane production rate from FSHW+FE substrate can be partly attributed to a decrease in average particle size during pretreatment.

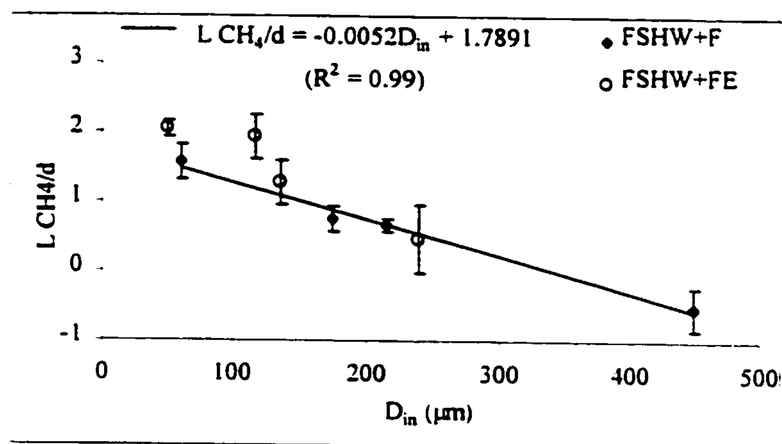

Figure 6.6 Difference in methane production between reactors fed FSHW and those fed FSHW+F and FSHW+FE in the initial 20h of anaerobic digestion. The data for FSHW+FE have been plotted according to the D_{in} obtained following 5.5 h of pretreatment with PL-250

In the 20-45 h digestion period, methane production from reactors fed FSHW+FE was similar to that from reactors fed FSHW+F at every D_{in} tested (Fig. 6.5b). However, with

both substrates, methane production rate tended to be higher at a D_{in} of 450 μm than at smaller D_{in}. It seemed that once the inhibition period was over, methane production was more vigorous in reactors that had experienced the longest inhibition period. In the 45-69 h digestion period, methane production rates were similar at all D_{in} and for both control and pretreated substrates (Fig. 6.5c).

6.3.6 SCOD and VFA concentration

Soluble COD averaged 2295 ± 219, 2294 ± 225 and 2507 ± 263 mg/l in FSHW, FSHW+F and FSHW+FE, respectively (Table 6.1). SCOD tended to be slightly higher in pretreated than in both control substrates, but the difference was not significant. Figure 6.7 presents SCOD concentrations during ASBR cycles at D_{in} of 60, 215 and 450 μm. All three substrates showed similar rates of SCOD reduction during anaerobic treatment. Mixed-liquor SCOD concentration decreased to 233 ± 60 mg/l within the first 20 h of the ASBR cycle, and ranged between 100 and 200 mg/l in the effluent. Substrate SCOD reduction during the 3-d ASBR cycles averaged 94% ± 1% in all reactors and at all D_{in}.

The three substrates also showed similar VFA concentrations in the mixed-liquor during treatment time (Fig. 6.8). Within the first 20 h of anaerobic treatment, average VFA concentrations had dropped below 25 mg/l in most bioreactors. They remained low for the remaining part of the cycle. VFA oxidation did not seem to be a limiting factor during treatment.

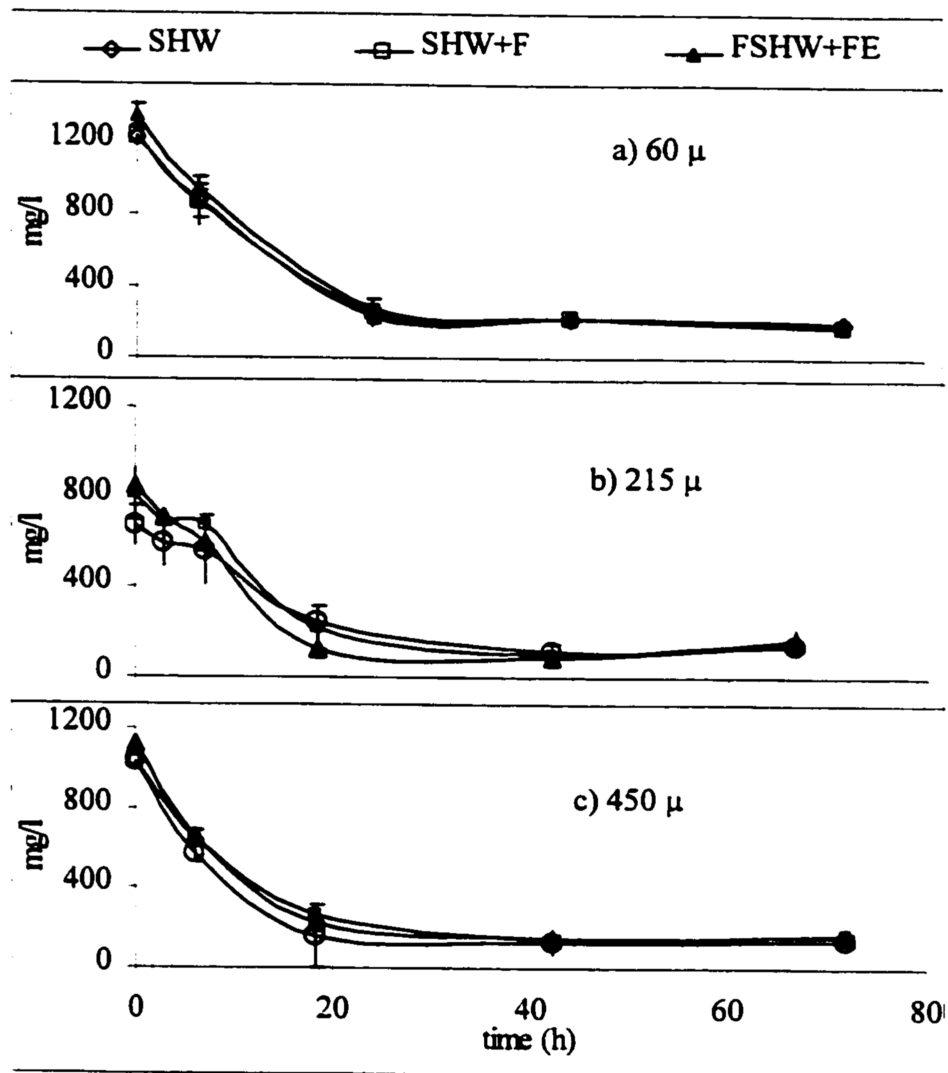

Figure 6.7 Soluble COD concentration in ASBRs fed FSHW, FSHW+F and FSHW+FE substrates at D_{in} of a) 60 μm, b) 215 μm, and c) 450 μm

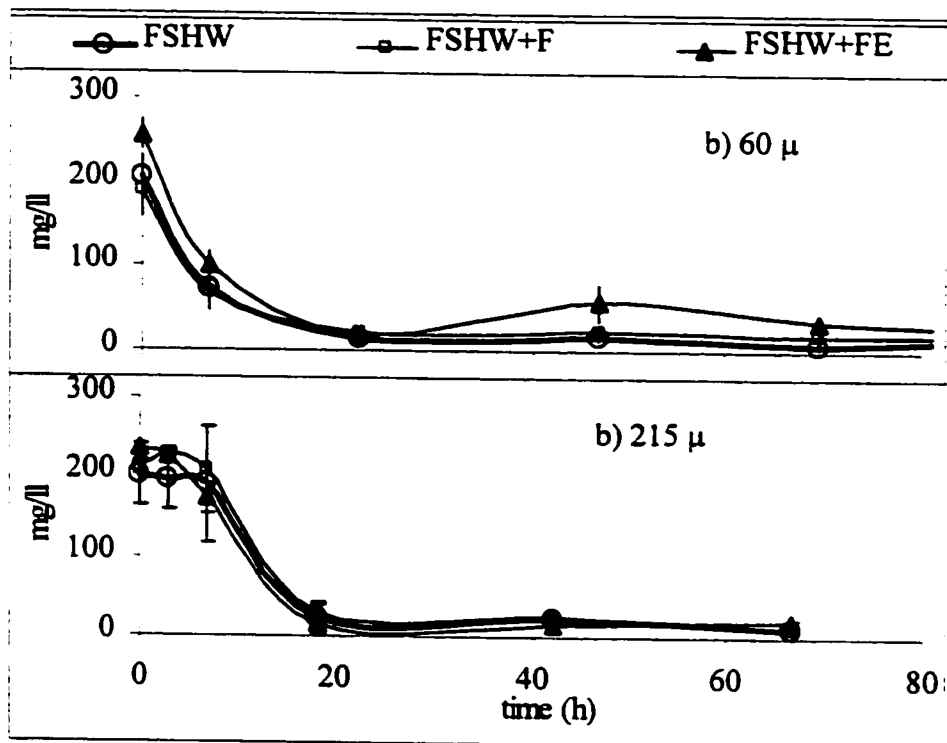

Figure 6.8 Volatile fatty acid concentration in ASBRs fed FSHW, FSHW+F and FSHW+FE substrates at D_{in} of a) 60 μm and b) 215 μm

6.4 Conclusion

Slaughterhouse wastewater containing fat particles was pretreated with PL-250 and fed to ASBRs operated at 25°C. About 35% of the neutral fat was hydrolysed during the 5.5-h pretreatment with 250 mg/l of PL-250. During anaerobic treatment, the first-order hydrolysis rates were estimated at 0.63 ± 0.07 and 0.50 ± 0.13 d^{-1} for control (FSHW+F) and pretreated (FSHW+FE) substrate, respectively. There was no significant particle size or pretreatment effect on the hydrolysis rate of pork fat particles in ASBRs. Transformation of influent total COD into methane was slightly higher in ASBRs fed FSHW+FE (82%) than FSHW+F (78%) substrates after 69 h of digestion. The difference

was mainly due to lower methane production in reactor fed FSHW+F than FSHW+FE substrates in the initial 20 h of anaerobic treatment. It was estimated that pretreating the substrate had only a small effect on fat particle digestion at 25°C, marked by a decrease of about 5% (3 h) in digestion time to achieve 80% reduction in substrate neutral fat and LCFA concentration. However, effects of pretreatment could be more important in reactors operated at higher temperatures, since LCFA removal rates would be increased.

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CHAPTER 7

SUMMARY AND CONCLUSION

7.1 Summary of Results

This research project was based on two main hypotheses. The first stated that fat particle hydrolysis was the rate-controlling step of slaughterhouse wastewater anaerobic degradation. Previous research reported conflicting conclusions as to which, of lipid hydrolysis or long-chain fatty acid (LCFA) oxidation, was the rate-limiting step of lipid degradation. However, particle liquefaction as the controlling process of slaughterhouse wastewater anaerobic degradation had been proposed by Sayed et al. (1988), based on an in-depth investigation of slaughterhouse wastewater digestion. The second hypothesis was that fat hydrolysis rate was inversely proportional to particle size. The relation between particle size and hydrolysis had been documented by many researchers, but there was no study specifically involving fat particles. Based on these two hypotheses, it was proposed that anaerobic digestion of slaughterhouse wastewater could be accelerated by the application of an hydrolysis pretreatment that could not only liquefy a fraction of the lipid but also reduce the size of fat particles.

Four hydrolysis pretreatments were investigated. Sodium hydroxide was selected because it had produced positive results with sludges. However, pretreatment with 5 to 400 meq NaOH/l did not increase soluble chemical oxygen demand (SCOD) in slaughterhouse wastewater containing fat particles. The average initial size (D_{in}) of fat particles was reduced by about 25% at NaOH concentrations ranging from 150 to 300

meq/l, but given the high doses of NaOH required and the resulting increase in pH to more than 13, alkaline pretreatment was not recommended as an hydrolysis pretreatment for fat particles. Three enzymatic products were also tested, Pancreatic Lipase 250 (PL-250), Lipase G-1000 (LG-1000), and EcoSystem Plus (ESP). No particle size reduction or SCOD increase was measured after 4 and 24 h of treatment with ESP. LG-1000 was found to decrease D_{in}, but high doses (> 1000 mg/l) or long treatment time were required. PL-250, on the other hand, could reduce D_{in} by a maximum of 40% after 4 h of mixing at room temperature. Although SCOD was not significantly increased during pretreatment, low concentrations of free LCFAs into solution of pretreated substrate indicated solubilization of some of the lipids with PL-250.

The effect of pretreatment with PL-250 on fat particles in slaughterhouse wastewater was further studied for various enzyme doses, treatment time, and D_{in}. The rate as well as the extent of pork fat particle size reduction during pretreatment with PL-250 increased with D_{in}. This conclusion apparently contradicted the assumption that hydrolysis was inversely proportional to particle size. However, it was observed that fat particles became more filamentous and plate-like as their size was increased, while smaller fat particles were denser and more spherical. The larger fat particles could probably be more easily broken at weak points, which explained the higher and faster particle size reduction in substrates with larger D_{in}. Particle size reduction also increased at higher PL-250 concentration, but the benefit of adding more enzyme diminished greatly as enzyme dose was increased: quadrupling PL-250 concentration from 250 to 1000 mg/l only increased maximum pork fat particle size reduction by 8%.

The maximum free LCFA concentration in filtered samples was detected after 4 to 6 h of pretreatment with PL-250. It ranged from 8.2 to 34.9 mg/l and represented approximately 1.7% of the initial fat particle concentration (2000 mg/l). The estimated linear rate of LCFA released in solution ranged from 39.4 to 169.9 mg/l/d, without any apparent particle size effect. However, it was later realised that most free LCFAs released during pretreatment remained adsorbed on solids surface and could not be recovered in filtered samples. When an analytical method which desorbed free LCFA from solids surface was used, results indicated that 35% of the initial neutral fat was hydrolysed into free LCFAs during a 5.5-h pretreatment at room temperature with 250 mg/l of PL-250 in a substrate containing approximately 2000 mg/l of fat particles. The adsorption of most of the free LCFAs on the fat particle surface partly explained why no increase in SCOD was observed during previous work with enzymatic pretreatment.

Pretreatment effect on subsequent anaerobic digestion of slaughterhouse wastewater was evaluated in anaerobic sequencing batch reactors (ASBRs) operated at 25°C. The low digestion temperature was selected for two main reasons: firstly slaughterhouses may want to operate the anaerobic reactor at room temperature to lower treatment costs and, secondly, fat degradation was reported to be more problematic at low temperatures (O'Rourke, 1968; Sayed et al., 1988). Three substrates were fed in duplicate to the ASBRs: filtered slaughterhouse wastewater (FSHW), filtered slaughterhouse wastewater containing pork fat particles (FSHW+F) and filtered slaughterhouse wastewater containing pork fat particles pretreated with PL-250 (FSHW+FE). Treatment time (5.5 h) and enzyme dose (250 mg/l for a substrate containing 2000 mg/l of fat particles) were based on optimum results from the study on pretreatment characterisation. The substrates

were fed for three 3-d cycles at each of four D_{in}: 60, 175, 215 and 450 μm. The FSHW and FSHW+F substrates were fed to the ASBRs for three additional cycles at a D_{in} of 110 μm.

Results from the FSHW+F runs were used to characterise and quantify neutral fat hydrolysis and LCFA oxidation rates during anaerobic digestion of slaughterhouse wastewater. Neutral fat hydrolysis was modelled using first-order kinetics. A first-order hydrolysis constant (k_h) was estimated for each D_{in} separately, in order to establish a relation between the hydrolysis rate and fat particle size. Estimated value of k_h ranged from 0.58 to 0.72 d^{-1}, but there was no significant particle size effect on neutral fat hydrolysis rate. It was suggested that the specific surface area (m^2/m^3) available for hydrolysis on pork fat particles was not inversely proportional to size. The fat particles were basically filamentous and bacteria could probably colonise the inside as well as the outside of the particles. Accordingly, contrary to our initial hypothesis, reducing fat particle size did not provide significantly more specific surface area for enzymatic reaction. Data from all D_{in} were thus pooled and k_h averaged 0.63 ± 0.07 d^{-1}. This value for lipid hydrolysis rate was similar to that estimated by Shimizu et al. (1993) for fat in lysed WAS digested in mesophilic continuously-fed anaerobic reactors (0.76 d^{-1}).

LCFA oxidation was modelled using Monod-type kinetics. The maximum substrate degradation rate (k_{max}) and the half-saturation concentration (K_s) averaged 164 ± 37 mg LCFA /l/d and 35 ± 31 mg LCFA/l, respectively. When reported on a VSS basis (0.077 mg COD/mg VSS/d), the value of k_{max} was much lower than values reported in the literature. It was suggested that k_{max} on a VSS basis was underestimated because biomass was in excess of substrate concentration in the reactors. The half-saturation concentration

(K_s) was also lower than values reported in the literature, except for that of Angelidaki et al. (1999). A low K_s value suggested a high affinity of the ASBR biomass for the substrate and indicated that the maximum degradation rate was reached at a much lower concentration than estimated in previous studies.

In the reactor runs with FSHW+FE, the first-order neutral fat hydrolysis rate ranged from 0.43 to 0.68 d^{-1}. As with FSHW+F, there was no particle size effect on the hydrolysis rate. There was also no pretreatment effect on fat hydrolysis rate in the reactor, which was consistent with the absence of correlation between particle size and hydrolysis rate. One of the main reported effects of PL-250 was to decrease fat particle size. However, since fat hydrolysis rate is not a function of particle size, enzymatic pretreatment of the substrate showed no beneficial effect on neutral fat hydrolysis rate once the substrate was fed to the ASBRs

Since particle size or pretreatment did not have a significant effect on neutral fat hydrolysis during anaerobic digestion, and lipase pretreatment or particle size should not affect the free LCFA oxidation rate, results from the FSHW+FE runs were used to validate the rate equations and the parameters estimated using the FSHW+F substrate. The neutral fat and free LCFA concentration measured during FSHW+FE fitted relatively well the simulated curves of fat degradation, except for the experiment at a D_{in} of 175 μm which showed greater variability in results. Based on modelled values, it was estimated that pretreating the substrate had only a small effect on fat particle digestion at 25°C, marked by a decrease of about 5% in digestion time to achieve 80% reduction in initial neutral fat and LCFA concentration. However, pretreatment effect could be more

significant on anaerobic digestion at higher temperatures, since LCFA oxidation rate should increase with temperature.

Observed results showed that transformation of influent COD into methane after 69 h of anaerobic treatment averaged 82% and 78% with FSHW+FE and FSHW+F substrates, respectively. Although the difference was small, it was significant at $p < 0.001$, and was mostly due to higher methane production from reactors fed FSHW+FE than FSHW+F in the early phase of digestion. In the FSHW+F runs, it was observed that methane production in the initial 20-h of digestion significantly decreased with increasing D_{in}. At the largest D_{in} tested (450 μm), methane production from reactors fed FSHW+F was lower than that from reactors fed FSHW, suggesting that the presence of large fat particles inhibited methane production from filtered slaughterhouse wastewater as well as from fat particles. Since particle size seemed to affect methane production rate in the 0-20 h period, particle size reduction during pretreatment could partially explain the higher methane production rate from reactors fed FSHW+FE than FSHW+F in the early phase of digestion. However, the decrease in methane production in the early phase of digestion with increasing D_{in} remains to be elucidated.

7.2 Main conclusions

The main conclusions from this research are:

- Anaerobic degradation of pork fat particles is mainly controlled by LCFA oxidation and, in ASBRs operated at 25°C, near maximum oxidation rate is reached at low free LCFA concentration (low K_s value). Consequently, increasing the initial free LCFA concentration by prehydrolysing the substrate will have limited effect on the fat

degradation rate. Research should concentrate on creating the most favourable conditions for LCFA oxidation.

- At D_{in} ranging from 60 to 450 µm, fat hydrolysis rate in anaerobic reactor is not a function of particle size.
- Neutral fat hydrolysis and free LCFA oxidation can be adequately modelled using first-order and Monod-type kinetics, respectively.
- Hydrolysis rate of fat will be underestimated if based on the increase in soluble compounds with respect to particulate organics. as done in most reported research on hydrolysis. An analytical method that removes bound LCFAs from solids surface (as was done in this work) must be used to measure lipid hydrolysis.

7.3 Further Research

In light of the findings of this thesis, the following avenues for further research are suggested:

- Quantify free LCFA production during enzymatic and alkaline pretreatment taking into account adsorbed free LCFAs and determine the effect of hydrolysis pretreatment on digestion at higher temperatures;
- Investigate the reason for the decrease in methane production rate with increasing fat particle size in the early phase of slaughterhouse wastewater digestion;
- Estimate hydrolysis and oxidation rate during anaerobic digestion of various fats (beef fat for example), and establish a correlation between the type of LCFAs and the oxidation rate;
- Quantify neutral fat hydrolysis and free LCFA oxidation rates with respect to biomass and/or enzyme concentration and with respect to digestion temperature;

- Further investigate the effect of fat particle size on hydrolysis rate by using a wider range of sizes.

7.4 Bibliography

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Appendix A

Appendix A presents graphs and tables that were not included in the original text of Chapter 3. Following is a description of the tables and figures.

- Table A.1 presents the data that were used in evaluating the accuracy of the serial filtration method used for particle size analysis, and in assessing cake formation when sample size was increased from 50 to 100 ml. These data were discussed in Section 3.2.3.
- Tables A.2 to A.5 and Figure A.1 present data collected during the experiments with NaOH. These results were discussed in Section 3.3.1. The SAS analysis used to estimate changes in particle size concentration during pretreatment with NaOH is given in Table A.6. Parameter estimates and confidence interval were used to built Figure 3.1.
- Tables A.7 to A.22 and Figures A.2 and A.3 present data collected during the experiments with PL-250. They include SCOD and particle size data for experiments with 2.5, 8.0 and 0.8 g/l of pork fat particles (Tables A.7 to A.12 and Figures A.2 and A.3) and with beef fat particles (Tables A.13 and A.14) as well as for experiments on the effect of NaCl on treatment efficiency (Tables A.15 and A.16). The non-linear least squares summary statistics for parameter estimates for Eqs. 3.2 and 3.5 on particle size reduction are presented in Tables A.17 and A.18. Calculations for statistical significance of Eq. 3.5, using Eq. 3.3, are shown in Table A.19. Free LCFA concentrations in samples following treatment are presented in Tables A.20 for pork

particles and A.22 for beef particles. Table A.21 shows each LCFAs of Table A.21 as a fraction of total free LCFAs.

- Table A.23 to A.25 and Figure A.4 present particle size distribution, particle size reduction and SCOD data collected during the experiment with Lipase G-1000.
- Table A.26 to A.27 present particle size distribution, particle size reduction and SCOD data collected during the experiment with EcoSystem Plus.

Table A.1 Filtration of 50 and 100-ml samples from various fat solutions

Avg. size (um)	Filtrate volume 50 ml (g/l)	100 ml	Avg. (g/l)	CV (%)	Avg. size (um)	Filtrate volume 50 ml (g/l)	100 ml	Avg. (g/l)	CV (%)
30	0.02	0.03	0.03	8.3	32	0.00	0.02	0.01	96.1
75	0.09	0.15	0.12	40.1	84	0.05	0.06	0.06	7.2
300	1.16	1.24	1.20	4.7	170	1.36	1.51	1.43	7.5
750	0.80	0.61	0.70	19.6	376	0.74	0.72	0.73	2.3
1168	1.11	1.11	1.11	0.0	726	0.25	0.24	0.25	2.7
					1168	0.16	0.17	0.17	2.7
Total	3.18	3.14	3.16	1.0		2.57	2.72	2.65	3.9
Avg. size	575	530	553	5.7		273	261	267.04	3.1

Avg. size (um)	Filtrate volume 50 ml g/l	100 ml	Avg. (g/l)	CV (%)	Filtrate volume 50 ml (g/l)	100 ml	Avg. (g/l)	CV (%)
6	0.42	0.40	0.41	4.1	0.35	0.29	0.32	13.9
32	0.01	0.01	0.01	4.9	0.01	0.01	0.01	34.2
84	0.08	0.11	0.10	24.1	0.04	0.06	0.05	27.8
170	0.70	0.91	0.80	18.6	0.68	0.89	0.78	19.0
376	1.50	1.96	1.73	19.1	1.58	1.50	1.54	3.3
726	0.94	1.14	1.04	13.8	1.04	1.07	1.06	2.1
1168	0.66	0.73	0.69	6.5	0.35	0.66	0.51	43.1
Total	4.31	5.26	4.78	14.1	4.04	4.47	4.26	7.1
Avg. size	291	309	299.98	4.5	292	331	311.20	8.9
6	0.38	0.33	0.36	9.5	0.37	0.37	0.37	1.4
32	0.01	0.01	0.01	58.1	0.01	0.01	0.01	45.3
84	0.14	0.05	0.10	64.8	0.06	0.07	0.06	4.5
170	0.61	0.66	0.63	5.3	0.60	0.82	0.71	22.6
376	1.77	1.61	1.69	6.7	1.50	1.60	1.55	4.8
726	0.96	1.34	1.15	23.3	0.83	0.95	0.89	9.2
1168	0.78	0.78	0.78	0.1	0.55	0.62	0.58	8.7
Total	4.65	4.77	4.71	1.9	3.91	4.43	4.17	8.9
Avg. size	315	357	335.97	8.7	293	298	295.41	1.3
32	0.28	0.27	0.27	2.1	0.44	0.50	0.47	8.6
84	0.55	0.69	0.62	16.4	0.44	0.53	0.48	13.7
170	0.36	0.42	0.39	11.1	0.32	0.29	0.30	8.8
376	0.38	0.35	0.36	4.9	0.44	0.41	0.42	4.8
726	0.16	0.14	0.15	12.5	0.14	0.11	0.12	19.8
1168	0.07	0.05	0.06	27.4	0.02	0.05	0.04	54.9
Total	1.79	1.92	1.85	4.7	1.81	1.88	1.85	3.0
Avg. size	153	139	145.86	6.5	132	121	126.32	6.1

Table A.2 Particle size distribution and average particle size after 4 h of mixing at room temperature with NaOH

Avg. size (μm)	NaOH concentration (meq/l)							
	0	350	300	50	0	150	100	250
	Particle concentration (g/l)							
5.5	0.27	0.39	0.37	0.31	0.27	0.55	0.42	0.41
31.5	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01
83.5	0.05	0.15	0.14	0.11	0.07	0.14	0.14	0.17
169.5	1.13	1.27	1.31	1.19	1.06	1.14	1.24	1.16
375.5	1.33	1.45	1.20	1.40	1.34	1.55	1.28	1.50
725.5	0.67	0.72	0.66	0.54	0.56	0.42	0.52	0.48
1167.5	0.29	0.14	0.16	0.20	0.26	0.19	0.24	0.16
Total (g/l)	3.74	4.13	3.86	3.76	3.58	4.00	3.85	3.89
Avg. dia. (μm)	259.74	216.54	210.12	229.98	249.44	178.58	201.82	200.38
$D_{treated}$:D_{in}		0.83	0.81	0.89		0.72	0.81	0.80

Avg. size (μm)	NaOH concentration (meq/l)						
	0	0	250	250	0	60	120
	Particle concentration (g/l)						
5.5	0.24	0.25	0.38	0.38	0.40	0.36	0.49
31.5	0.07	0.02	0.07	0.06	0.01	0.02	0.01
83.5	0.59	0.56	0.54	0.52	0.24	0.37	0.39
169.5	0.46	0.56	0.48	0.55	0.79	0.90	0.87
375.5	0.50	0.50	0.56	0.63	0.54	0.56	0.66
725.5	0.63	0.61	0.40	0.58	0.35	0.44	0.36
1167.5	0.36	0.42	0.17	0.30	0.25	0.18	0.23
Total (g/l)	2.85	2.93	2.60	3.02	2.59	2.83	3.01
Avg. dia. (μm)	212.25	223.74	145.00	173.00	161	164	144
$D_{treated}$:D_{in}			0.67	0.79		1.02	0.90

Avg. size (μm)	NaOH concentration (meq/l)			
	0	200	300	400
	Particle concentration (g/l)			
5.5	0.26	0.41	0.43	0.32
31.5	0.01	0.01	0.01	0.01
83.5	0.06	0.09	0.10	0.06
169.5	0.44	0.54	0.53	0.56
375.5	0.57	1.03	0.95	0.91
725.5	0.89	0.89	0.74	0.83
1167.5	0.51	0.36	0.24	0.27
Total (g/l)	2.74	3.32	2.98	2.96
Avg. dia. (μm)	325.85	250.68	217.08	262.70
$D_{treated}$:D_{in}		0.77	0.67	0.81

Table A.3 Particle size distribution and average particle size after various mixing times at room temperature with 250 meq/l NaOH

Avg. filter size (µm)	Treatment time (h) 0	0	24	24	0	0.25	0.67	1	2
	Particle concentration (g/L)								
5.5	0.32	0.29	0.29	0.28	0.32	0.50	0.48	0.47	0.47
31.5	0.05	0.06	0.26	0.28	0.02	0.02	0.03	0.01	0.01
83.5	0.62	0.63	0.61	0.65	0.05	0.10	0.10	0.12	0.11
169.5	0.49	0.54	0.45	0.45	0.49	0.56	0.52	0.53	0.51
375.5	0.41	0.47	0.69	0.78	0.81	0.97	0.86	0.87	0.82
725.5	0.37	0.70	0.80	0.76	1.08	0.87	1.12	0.98	0.89
1167.5	0.69	0.91	0.28	0.27	0.41	0.18	0.23	0.38	0.20
Total (g/l)	2.96	3.62	3.38	3.47	3.17	3.20	3.34	3.36	3.02
Avg. dia. (µm)	207	264	190	188	304	201	228	238	207
$D_{treated}:D_{in}$			0.81	0.80		0.66	0.75	0.78	0.68

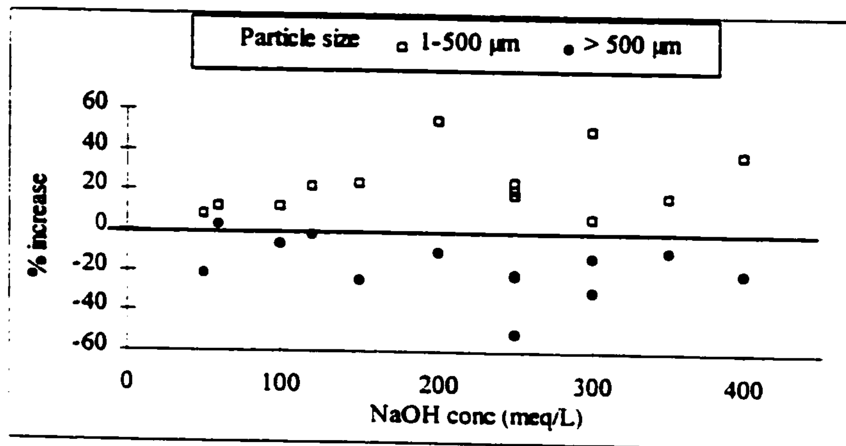


Figure A.1 Changes in the concentration of particles in two size ranges after 4 h of mixing with NaOH; % increase = (initial conc. – final conc.)/initial conc.*100%; a negative number indicates a decrease in the concentration of particles

Table A.4 SCOD and pH in samples mixed for 4 h with different NaOH concentrations

Avg. dia. mg/l	NaOH meq/l	pH	SCOD mg/l	$SCOD_{treated}$: $SCOD_{in}$
260	0	7.2	2053	
230	50	11.4	1687	0.82
210	300	13.3	2290	1.12
217	350	13.4	2201	1.07
249	0	7.3	2133	
202	100	12.9	1965	0.92
179	150	13.1	2108	0.99
200	250	13.3	2106	0.99
161	0	7.2	2200	
164	60	11.0	2309	1.05
144	120	12.6	2113	0.96
326	0	7.2	2507	
251	200	13.2	2415	0.96
217	300	13.3	2431	0.97
263	400	13.4	2433	0.97
218	0	7.3	1188	
159	250	13.2	1212	1.02

Table A.5 SCOD concentrations after different periods of mixing with 250 meq/l of NaOH

time h	SCOD mg/l	$SCOD_{treated}$: $SCOD_{in}$
0	1188	
24	1284	1.08
0	2280	
0.25	2551	1.12
0.67	2502	1.10
1	2535	1.11
2	2568	1.13

Table A.6 SAS analysis for the quadratic equation (Eq. 3.4) representing changes in NaOH concentration ($D_{treated}/D_{in}$ = NaOH concentration) with treatment time

Response Surface for Variable red

Response Mean	0.796923
Root MSE	0.069072
R-Square	0.6124
Coefficient of Variation	8.6674

Regression	DF	Type I Sum of Squares	R-Square	F Value	Pr > F
Linear	1	0.032901	0.2673	6.90	0.0253
Quadratic	1	0.042466	0.3450	8.90	0.0137
Crossproduct	0	0	0.0000	.	.
Total Model	2	0.075367	0.6124	7.90	0.0088

Residual	DF	Sum of Squares	Mean Square	F Value	Pr > F
Lack of Fit	7	0.033210	0.004744	0.98	0.5601
Pure Error	3	0.014500	0.004833		
Total Error	10	0.047710	0.004771		

Parameter	DF	Estimate	Standard Error	t Value	Pr > |t|	Parameter Estimate from Coded Data
Intercept	1	1.075759	0.073479	14.64	<.0001	0.733872
naoh	1	-0.002683	0.000760	-3.53	0.0055	-0.062384
naoh*naoh	1	0.000005169	0.000001733	2.98	0.0137	0.158299

Factor	DF	Sum of Squares	Mean Square	F Value	Pr > F
naoh	2	0.075367	0.037684	7.90	0.0088

Table A6 (continued)

naoh	PREDICT	RESIDUAL	U95M	L95M
60	0.93342	0.08658	1.02218	0.84465
120	0.82829	0.07171	0.88530	0.77128
250	0.72819	-0.05819	0.78697	0.66941
250	0.72819	0.06181	0.78697	0.66941
50	0.95456	-0.06456	1.05313	0.85599
100	0.85920	-0.04920	0.92154	0.79686
150	0.78968	-0.06968	0.84580	0.73357
250	0.72819	0.07181	0.78697	0.66941
350	0.77008	0.05992	0.84755	0.69261
200	0.74601	0.02399	0.80577	0.68626
250	0.72819	-0.04819	0.78697	0.66941
300	0.73621	-0.06621	0.79401	0.67842
400	0.82979	-0.01979	0.95803	0.70154

Table A.7 Particle size distribution and average particle size reduction in slaughterhouse wastewater containing approximately 2.5 g/l of pork fat particles and mixed for 4 h with the pancreatic lipase PL-250

Avg. size (µm)	PL-250 concentration (mg/L)							
	0	0	30	100	250	500	1000	3000
	Particle concentration (g/L)							
5.50	0.30	0.35	0.38	0.39	0.50	0.52	0.55	0.56
31.50	1.02	0.94	0.80	0.72	0.70	0.92	0.81	0.74
83.50	0.51	0.53	0.44	0.45	0.49	0.40	0.37	0.43
203.00	0.24	0.27	0.24	0.33	0.28	0.25	0.24	0.23
409.00	0.11	0.10	0.12	0.15	0.11	0.10	0.16	0.12
725.50	0.19	0.10	0.09	0.10	0.11	0.10	0.12	0.06
1167.50	0.26	0.18	0.12	0.17	0.11	0.06	0.10	0.04
Total (g/l)	2.63	2.48	2.20	2.31	2.29	2.36	2.35	2.19
Avg. dia (µm)	73.94	61.66	55.49	64.98	51.98	43.22	48.13	39.66
$D_{treated}:D_{in}$			0.82	0.96	0.77	0.64	0.71	0.59

Avg. size (µm)	PL-250 concentration (mg/L)					
	0	0	80	250	350	500
	Particle concentration (g/L)					
5.50	0.30	0.28	0.31	0.32	0.34	0.29
31.50	0.51	0.68	0.75	0.64	0.66	0.74
83.50	0.41	0.43	0.36	0.30	0.45	0.43
203.00	0.18	0.17	0.14	0.23	0.18	0.18
409.00	0.22	0.23	0.26	0.19	0.17	0.21
725.50	0.24	0.28	0.17	0.21	0.21	0.21
1167.50	0.29	0.27	0.31	0.22	0.12	0.13
Total (g/l)	2.16	2.34	2.29	2.10	2.12	2.19
Avg. dia (µm)	104.73	100.78	89.31	85.42	69.44	74.52
$D_{treated}:D_{in}$			0.87	0.83	0.68	0.73

Avg. size (µm)	PL-250 concentration (mg/L)						
	0	0	5	20	60	80	3000
	Particle concentration (g/L)						
5.50	0.26	0.28	0.27	0.27	0.31	0.30	0.42
31.50	0.36	0.30	0.37	0.49	0.34	0.36	0.66
83.50	0.51	0.52	0.53	0.54	0.45	0.50	0.54
203.00	0.53	0.48	0.46	0.46	0.43	0.47	0.43
409.00	0.36	0.40	0.55	0.45	0.46	0.47	0.34
725.50	0.19	0.23	0.21	0.14	0.17	0.14	0.07
1167.50	0.11	0.13	0.07	0.06	0.04	0.02	0.01
Total (g/l)	2.33	2.34	2.46	2.40	2.20	2.26	2.48
Avg. dia (µm)	111.49	120.03	117.95	97.77	101.33	97.17	63.49
$D_{treated}:D_{in}$			1.02	0.84	0.88	0.84	0.55

Table A7 (continued)

Avg. size (µm)	PL-250 concentration (mg/L)						
	0	0	10	25	50	200	500
	Particle concentration (g/L)						
5.50	0.28	0.30	0.35	0.38	0.36	0.42	0.46
31.50	0.16	0.17	0.24	0.30	0.11	0.26	0.35
83.50	0.58	0.54	0.60	0.57	0.58	0.58	0.48
203.00	0.62	0.61	0.61	0.57	0.68	0.61	0.45
409.00	0.34	0.38	0.67	0.75	0.66	0.65	0.61
725.50	0.34	0.41	0.24	0.23	0.28	0.20	0.10
1167.50	0.23	0.26	0.09	0.06	0.08	0.05	0.03
Total (g/l)	2.55	2.67	2.79	2.86	2.74	2.78	2.49
Avg. dia (µm)	151.41	159.62	126.86	118.73	139.12	108.69	85.60
$D_{treated}$:D_{in}			0.82	0.76	0.89	0.70	0.55

Avg. size (µm)	PL-250 concentration (mg/L)				
	0	0	250	400	1000
	Particle concentration (g/L)				
5.50	0.24	0.26	0.39	0.39	0.48
31.50	0.15	0.13	0.23	0.27	0.31
83.50	0.57	0.63	0.64	0.52	0.53
203.00	0.59	0.63	0.51	0.51	0.44
409.00	0.41	0.46	0.36	0.29	0.44
725.50	0.31	0.40	0.09	0.08	0.10
1167.50	0.18	0.18	0.07	0.03	0.03
Total (g/l)	2.46	2.68	2.28	2.08	2.33
Avg. dia (µm)	154.35	162.39	88.42	77.25	76.16
$D_{treated}$:D_{in}			0.56	0.49	0.48

Avg. size (µm)	PL-250 concentration (mg/L)				
	0	0	150	500	1000
	Particle concentration (g/L)				
5.50	0.27	0.26	0.41	0.55	0.43
31.50	0.07	0.07	0.04	0.05	0.05
83.50	0.15	0.15	0.13	0.16	0.22
203.00	0.14	0.16	0.26	0.32	0.20
409.00	0.42	0.41	0.58	0.68	0.53
725.50	0.50	0.49	0.36	0.63	0.35
1167.50	0.95	0.84	0.77	0.61	0.37
Total (g/l)	2.49	2.38	2.54	3.00	2.14
Avg. dia (µm)	352.41	334.03	250.05	212.95	170.23
$D_{treated}$:D_{in}			0.73	0.62	0.50

Avg. size (µm)	PL-250 concentration (mg/L)							
	0	0	25	80	150	500	1000	3000
	Particle concentration (g/L)							
5.50	0.29	0.29	0.36	0.33	0.35	0.44	0.48	0.46
31.50	0.07	0.05	0.08	0.05	0.04	0.04	0.04	0.05
83.50	0.18	0.20	0.16	0.13	0.12	0.18	0.18	0.25
203.00	0.18	0.19	0.23	0.20	0.22	0.24	0.26	0.24
409.00	0.41	0.49	0.57	0.56	0.51	0.60	0.61	0.50
725.50	0.90	0.66	0.57	0.48	0.39	0.37	0.43	0.41
1167.50	1.03	1.27	0.79	0.56	0.53	0.91	0.59	0.60
Total (g/l)	3.06	3.15	2.75	2.31	2.17	2.78	2.60	2.49
Avg. dia (µm)	380.88	395.21	285.40	263.67	236.22	257.25	205.92	197.89
$D_{treated}$:D_{in}			0.74	0.68	0.61	0.66	0.53	0.51

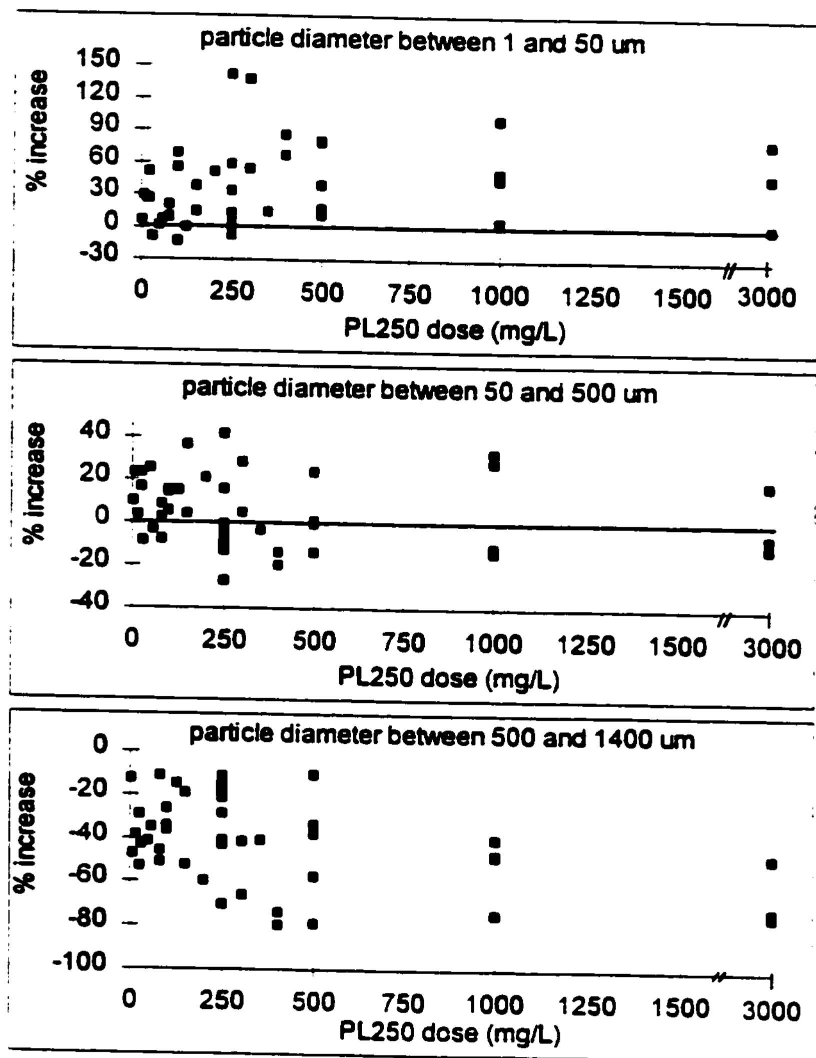


Figure A. 2 Changes in the concentration of particles in three size ranges after 4 h of mixing with PL-250; % increase = (initial conc. – final conc.)/initial conc.*100%; a negative number indicates a decrease in the concentration of particles

Table A.8 SCOD concentration in slaughterhouse wastewater containing approximately 2.5 g/l of pork fat particles and mixed for 4 h with the pancreatic lipase PL-250. The column 'less enzyme' corresponds to measured SCOD minus the COD added as enzyme (1.05 g COD per g enzyme)

PL250 mg/l	D_{in} µm	SCOD (mg/l) in sample	SCOD (mg/l) less enzyme	$SCOD_{treated}$: $SCOD_{in}$
0	68	1214	1214	
30	68	1484	1453	1.20
100	68	1568	1463	1.21
250	68	1838	1576	1.30
500	68	1986	1461	1.20
1000	68	2400	1350	1.11
0	103	1359	1359	
80	103	1621	1537	1.13
250	103	1731	1469	1.08
350	103	1884	1517	1.12
500	103	2215	1690	1.24
0	116	1414	1414	
5	116	1445	1439	1.02
20	116	1351	1330	0.94
60	116	1538	1475	1.04
80	116	1433	1349	0.95
0	156	1432	1432	
10	156	1536	1526	1.07
25	156	1360	1334	0.93
50	156	1427	1374	0.96
200	156	1467	1257	0.88
500	156	1760	1235	0.86
0	158	1430	1430	
250	158	1693	1431	1.00
400	158	1653	1233	0.86
1000	158	2267	1217	0.85
0	383	1449	1449	
25	383	1553	1527	1.05
80	383	1597	1513	1.04
150	383	1597	1440	0.99
500	383	2034	1509	1.04
1000	383	2450	1400	0.97
3000	383	4481	1331	0.92

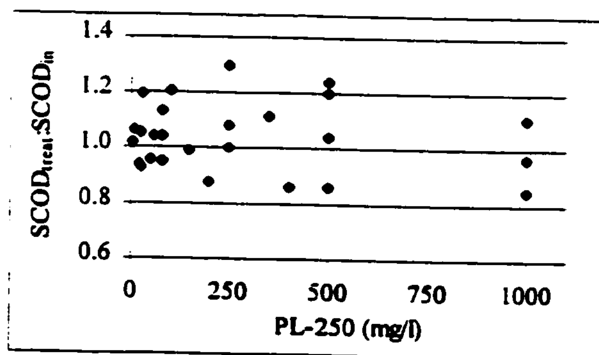


Figure A. 3 Effect of 4 h of mixing with the pancreatic lipase PL-250 on SCOD concentration in slaughterhouse wastewater containing approximately 2.5 g/l of pork fat particles

Table A. 9 Particle size distribution and average particle size reduction in slaughterhouse wastewater containing approximately 0.8 g/l of pork fat particles and mixed for 4 h with the pancreatic lipase PL-250

Avg. size (µm)	PL-250 concentration (mg/L) 0	0	100	150	200	300	450
	Particle concentration (g/L)						
5.50	0.25	0.24	0.28	0.30	0.34	0.36	0.34
31.50	0.07	0.10	0.11	0.11	0.13	0.09	0.10
83.50	0.21	0.20	0.17	0.20	0.15	0.13	0.13
203.00	0.21	0.17	0.17	0.14	0.12	0.14	0.12
409.00	0.14	0.09	0.15	0.11	0.14	0.11	0.09
725.50	0.09	0.06	0.04	0.05	0.05	0.03	0.03
1167.50	0.07	0.05	0.02	0.01	0.00	0.00	0.00
Total (g/l)	1.04	0.92	0.94	0.91	0.94	0.86	0.81
Avg. Dia. (µm)	84.06	65.62	56.21	46.87	43.73	35.53	35.42
$D_{treated}$:D_{in}			0.75	0.63	0.58	0.47	0.47

Table A.10 SCOD concentrations in slaughterhouse wastewater containing approximately 0.8 g/l of pork fat particles and mixed for 4 h with the pancreatic lipase PL-250. The column 'less enzyme' corresponds to measured SCOD minus the COD added as enzyme (1.05 g COD per g enzyme)

PL250 mg/l	SCOD (mg/l) in sample	less enzyme	$SCOD_{treated}$:$SCOD_{in}$
0	1374	1451	
50	1433	1380	0.95
150	1398	1241	0.85
275	1503	1214	0.84
400	1700	1280	0.88
500	1934	1409	0.97

Table A.11 Particle size distribution and average particle size reduction in slaughterhouse wastewater containing approximately 8 g/l of pork fat particles and mixed for 4 h with the pancreatic lipase PL-250

Avg. size (µm)	PL-250 concentration (mg/L) 0	0	100	150	200	300	450
	Particle concentration (g/L)						
5.50	0.40	0.34	0.40	0.22	0.38	0.56	0.47
31.50	0.77	1.20	1.53	1.32	1.51	1.68	1.68
83.50	2.46	2.51	2.18	1.99	1.94	2.26	1.88
203.00	1.74	1.79	1.83	1.51	1.73	1.24	1.27
409.00	0.94	1.08	1.27	1.17	1.09	0.93	1.05
725.50	0.54	0.68	0.38	0.29	0.27	0.22	0.25
1167.50	0.31	0.40	0.07	0.16	0.07	0.10	0.22
Total (g/l)	7.15	8.00	7.65	6.67	7.00	6.98	6.82
Avg. Dia. (µm)	124.42	127.75	104.75	114.21	99.35	82.29	93.61
$D_{treated}:D_{in}$			0.83	0.91	0.79	0.65	0.74

Avg. size (µm)	PL-250 concentration (mg/L) 0	0	50	275	400	500
	Particle concentration (g/L)					
5.50	0.22	0.37	0.51	0.38	0.51	0.62
31.50	0.40	0.91	0.89	1.76	1.89	1.73
83.50	3.80	2.48	2.81	1.86	1.82	1.98
203.00	1.48	2.16	1.67	1.31	1.42	1.48
409.00	1.04	0.86	1.07	1.10	0.93	1.10
725.50	0.54	0.73	0.27	0.36	0.29	0.26
1167.50	0.27	0.43	0.16	0.16	0.15	0.12
Total (g/l)	7.75	7.94	7.39	6.93	7.01	7.28
Avg. Dia. (µm)	132.66	133.59	104.35	98.64	86.67	87.06
$D_{treated}:D_{in}$			0.78	0.74	0.65	0.65

Table A.12 SCOD concentrations in slaughterhouse wastewater containing approximately 0.8 g/l of pork fat particles and mixed for 4 h with the pancreatic lipase PL-250. The column 'less enzyme' corresponds to measured SCOD minus the COD added as enzyme (1.05 g COD per g enzyme)

PL250 mg/l	SCOD (mg/l) in sample	less enzyme	$SCOD_{treated}:SCOD_{in}$
0	1451	1451	
50	1637	1584	1.09
150	1711	1554	1.07
275	1711	1422	0.98
400	1959	1539	1.06
500	1947	1422	0.98
0	1329	1329	
100	1654	1549	1.17
150	1584	1427	1.07
200	1700	1490	1.12
300	1584	1269	0.95
450	1887	1415	1.06

Table A.13 Particle size distribution and average particle size reduction in slaughterhouse wastewater containing approximately 2.5 g/l of beef fat particles and mixed for 4 h with the pancreatic lipase PL-250

Avg. size (µm)	PL-250 concentration (mg/L)						
	0	0	25	100	350	500	1500
	Particle concentration (g/L)						
5.50	0.24	0.23	0.28	0.48	0.51	0.55	0.70
31.50	0.04	0.05	0.09	0.29	0.24	0.35	0.32
83.50	0.44	0.38	0.60	0.56	0.33	0.37	0.54
203.00	0.93	0.97	0.72	0.53	0.38	0.42	0.44
409.00	0.61	0.62	0.50	0.44	0.58	0.50	0.50
725.50	0.47	0.49	0.31	0.33	0.26	0.26	0.28
1167.50	0.22	0.25	0.19	0.10	0.11	0.10	0.11
Total (g/l)	2.95	3.00	2.69	2.74	2.41	2.54	2.90
vg. Dia. (µm)	208.84	219.74	160.37	103.30	103.41	88.32	80.03
$D_{treated}:D_{in}$			0.75	0.48	0.48	0.41	0.37

Avg. size (µm)	PL-250 concentration (mg/L)				
	0	0	50	500	5000
	Particle concentration (g/L)				
5.50	0.30	0.27	0.42	0.65	0.75
31.50	0.03	0.03	0.02	0.05	0.04
83.50	0.29	0.34	0.25	0.28	0.35
203.00	0.40	0.36	0.61	0.27	0.42
409.00	0.88	0.88	0.69	0.63	0.63
725.50	0.47	0.74	0.43	0.27	0.29
1167.50	0.31	0.30	0.18	0.08	0.08
Total (g/l)	2.68	2.91	2.61	2.24	2.55
vg. Dia. (µm)	218.67	252.30	160.98	88.78	83.29
$D_{treated}:D_{in}$			0.68	0.38	0.35

Table A.14 SCOD concentrations in slaughterhouse wastewater containing approximately 2.5 g/l of beef fat particles and mixed for 4 h with the pancreatic lipase PL-250. The column 'less enzyme' corresponds to measured SCOD minus the COD added as enzyme (1.05 g COD per g enzyme)

PL250 mg/l	SCOD (mg/l) in sample	SCOD (mg/l) less enzyme	$SCOD_{treated}:SCOD_{in}$
0	1572	1572	
25	1594	1568	1.00
350	2096	1728	1.10
500	2113	1588	1.01
0	1792	1792	
50	1922	1870	1.04
500	1982	1457	0.81

Table A.15 Particle size distribution and average particle size reduction in slaughterhouse wastewater containing a) pork and b)beef fat particles and mixed for 4 h with the pancreatic lipase PL-250 and 0 or 1 g/l of NaCl

a. Pork particle: size reduction

Avg. size (µm)	with 1 g/l NaCl			without NaCl		
	PL-250 concentration (mg/L)					
	0	100	300	0	100	300
	Particle concentration (g/L)					
5.50	0.23	0.41	0.60	0.25	0.40	0.43
31.50	0.15	0.26	0.35	0.18	0.22	0.19
83.50	0.69	0.49	0.52	0.52	0.51	0.50
203.00	0.55	0.50	0.51	0.52	0.57	0.58
409.00	0.23	0.69	0.84	0.42	0.59	0.44
725.50	0.24	0.23	0.22	0.30	0.24	0.13
1167.50	0.17	0.07	0.06	0.24	0.08	0.03
Total (g/l)	2.26	2.65	3.11	2.43	2.60	2.31
Avg. Dia. (µm)	133.61	114.73	96.38	158.99	115.31	92.48
$D_{treated}:D_{in}$		0.86	0.72		0.73	0.58

b. beef fat particles

Avg. size (µm)	with 1 g/l NaCl		without NaCl	
	PL-250 concentration (mg/L)			
	0	500	0	500
	Particle concentration (g/L)			
5.50	0.29	0.65	0.23	0.55
31.50	0.03	0.05	0.05	0.35
83.50	0.31	0.28	0.41	0.37
203.00	0.38	0.27	0.95	0.42
409.00	0.88	0.63	0.62	0.50
725.50	0.60	0.27	0.48	0.26
1167.50	0.30	0.08	0.24	0.10
Total (g/l)	2.80	2.24	2.98	2.54
Avg. Dia. (µm)	235.49	88.78	214.29	88.32
$D_{treated}:D_{in}$		0.38		0.41

Table A.16 SCOD concentration in slaughterhouse wastewater containing pork and fat particles and mixed for 4 h with the pancreatic lipase PL-250 and 0 or 1 g/l of NaCl; The column 'less enzyme' corresponds to measured SCOD minus the COD added as enzyme (1.05 g COD per g enzyme)

NaCl g/l	PL250 mg/l	SCOD (mg/l) in sample	SCOD (mg/l) less enzyme	$SCOD_{treated}:SCOD_{in}$
	0	1349	1349	
1	100	1589	1484	1.22
0	100	1403	1298	1.07
1	300	1509	1194	0.98
0	300	1589	1274	1.05

Table A.17 Non-linear least squares summary statistics: Parameter estimates (Eq. 3.2) for particle size reduction in slaughterhouse wastewater containing fat particles and mixed for 4 h with PL-250

a. Pork fat (2.5 g/l); D_{in} = 68 μm

Source	DF	Sum of Squares	Mean Square
Regression	2	19196.073704	9598.036852
Residual	5	210.512496	42.102499
Uncorrected Total	7	19406.586200	
(Corrected Total)	6	495.222543	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Confidence Interval Lower	Upper
DMIN	41.77975747	5.3959613224	27.909198351	55.650316591
B1	0.00277121	0.0015068850	-0.001102305	0.006644725

Asymptotic Correlation Matrix

Corr	DMIN	B1
DMIN	1	0.7202884149
B1	0.7202884149	1

b. Pork fat (2.5 g/l); D_{in} = 109 μm

Source	DF	Sum of Squares	Mean Square
Regression	2	118252.93995	59126.46998
Residual	13	510.23065	39.24851
Uncorrected Total	15	118763.17060	
(Corrected Total)	14	2954.90393	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Confidence Interval Lower	Upper
DMIN	67.22491309	4.9129476655	56.611141297	77.838684883
B1	0.00367790	0.0009847360	0.001550506	0.005805289

Table A.17 (continued)

Asymptotic Correlation Matrix

Corr	DMIN	B1
DMIN	1	0.8281861749
B1	0.8281861749	1

c. Pork fat (2.5 g/1); D_{in} =147 μm

Source	DF	Sum of Squares	Mean Square
Regression	2	154896.51763	77448.25882
Residual	12	1680.00367	140.00031
Uncorrected Total	14	156576.52130	
(Corrected Total)	13	4688.32609	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Confidence Interval Lower	Upper
DMIN	79.75035712	8.4422227256	61.356306841	98.144407401
B1	0.00637744	0.0019568376	0.002113848	0.010641026

Asymptotic Correlation Matrix

Corr	DMIN	B1
DMIN	1	0.8401181697
B1	0.8401181697	1

d. Pork fat (2.5 g/1); D_{in} = 359 μm

Source	DF	Sum of Squares	Mean Square
Regression	2	652796.32155	326398.16077
Residual	10	8419.15275	841.91528
Uncorrected Total	12	661215.47430	
(Corrected Total)	11	12173.07229	

Table A.17 (continued)

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Confidence Interval Lower	Upper
DMIN	223.9270734	9.7100771526	202.29153718	245.56260965
B1	0.0269600	0.0119761026	0.00027545	0.05364463

Asymptotic Correlation Matrix

Corr	DMIN	B1
DMIN	1	0.4235116877
B1	0.4235116877	1

e. Pork fat (8.0 g/l)

Source	DF	Sum of Squares	Mean Square
Regression	2	109022.03492	54511.01746
Residual	9	487.45068	54.16119
Uncorrected Total	11	109509.48560	
(Corrected Total)	10	1975.50742	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Confidence Interval Lower	Upper
X	0.6744424997	0.03498124916	0.59530874304	0.75357625635
Y	0.0000558192	0.00001962573	0.00001142233	0.00010021605

Asymptotic Correlation Matrix

Corr	X	Y
X	1	0.7779132205
Y	0.7779132205	1

Table A.17 (continued)

e. Pork fat (0.8 g/l)

Source	DF	Sum of Squares	Mean Square
Regression	2	15344.072581	7672.036290
Residual	4	42.584219	10.646055
Uncorrected Total	6	15386.656800	
(Corrected Total)	5	1117.530133	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Confidence Interval Lower	Upper
X	0.4953074375	0.03168041065	0.40734970651	0.58326516842
Y	0.0001365194	0.00003340064	0.00004378563	0.00022925319

Asymptotic Correlation Matrix

Corr	X	Y
X	1	0.6897550794
Y	0.6897550794	1

e. Beef fat (2.5 g/l)

Source	DF	Sum of Squares	Mean Square
Regression	2	118672.70958	59336.35479
Residual	8	1520.23212	190.02902
Uncorrected Total	10	120192.94170	
(Corrected Total)	9	8960.06081	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Confidence Interval Lower	Upper
X	0.4061688263	0.02471282154	0.34918040788	0.46315724480
Y	0.0000788567	0.00001623704	0.00004141364	0.00011629972

Asymptotic Correlation Matrix

Corr	X	Y
X	1	0.4459237059
Y	0.4459237059	1

Table A.18 Non-linear least squares summary statistics: Parameter estimates (Eq. 3.5) for particle size reduction in slaughterhouse wastewater containing 2.5 g/l of pork fat particles and mixed for 4 h with PL-250 ($X = D_{min}$ and Y = first order coefficient)

Source	DF	Sum of Squares	Mean Square
Regression	2	1005297.9247	502648.9624
Residual	50	14109.2149	282.1843
Uncorrected Total	52	1019407.1396	
(Corrected Total)	51	228546.1131	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Confidence Interval Lower	Upper
X	0.5985861136	0.01592711355	0.56659562722	0.63057659995
Y	0.0000487198	0.00000867328	0.00003129901	0.00006614053

Asymptotic Correlation Matrix

Corr	X	Y
X	1	0.6062796578
Y	0.6062796578	1

Table A. 19 Testing for model (Eq. 3.5) significance by comparing pure error with model error (Eq. 3.3)

PL-250 mg/l	D_{in} um	Dia. final (um)	ariance*df	PL-250 mg/l	D_{in} um	Dia. final (um)	ariance*df
250	67	60		100	146	115	0.17
30	68	55		100	146	115	
100	68	65		300	146	96	7.6
250	68	52	35	300	146	92	
500	68	43		10	156	127	
1000	68	48		25	156	119	
3000	68	40		50	156	139	
80	103	89	31	200	156	109	
250	103	85	7.4	500	156	86	
350	103	69		250	158	88	
500	103	75	50	400	158	77	
250	104	88		1000	158	76	
500	105	81		1000	254	156	1.9
500	105	71		1000	254	158	
125	111	98		150	340	251	1192
250	114	86		150	340	208	
5	116	118		250	343	252	
20	116	98		150	343	250	
60	116	101		500	343	213	981
80	116	97		1000	343	170	637
3000	116	63		80	388	264	
250	128	82		150	388	236	
400	138	85		500	388	257	
150	146	101	133	1000	388	206	
150	146	117		3000	388	198	
				25	388	285	

Step	Value
1. sum pure variance (S_e)	3078.1
2. d.f. S_e	14
3. d.f. model	50
4. SS_{res} model	14109
5. $(SS_{res}-SS_{Se})/(\text{d.f.model.}-\text{d.f.}S_e)$	306
6. Step 5/(Step 1/d.f. S_e)	1.39
7. f value (50,8,0.05)	2.25

Table A.20 Free LCFA concentration in filtered samples of slaughterhouse wastewater containing 2.5 g/l of pork fat particles and mixed with PL-250 for 4 h

PL 250	Din	16:0	18:2	18:1	18:0	10:0	14:0	16:1	Total	avg	std
mg/l	µm					mg/l					
0	68	0.00	0.00	0.16	0.00	0.00	0.00	0.00	0.16	0.42	0.37
0	68	0.09	0.12	0.21	0.05	0.05	0.07	0.09	0.68		
0	343	0.04	0.08	0.12	0.03	0.04	0.03	0.04	0.38	0.51	0.18
0	343	0.07	0.15	0.20	0.05	0.06	0.05	0.06	0.64		
25	68	0.08	0.13	0.22	0.04	0.02	0.00	0.03	0.52	1.47	1.34
25	68	0.36	0.75	0.90	0.12	0.00	0.10	0.19	2.41		
25	388	0.32	0.50	0.83	0.09	0.02	0.05	0.15	1.96	2.07	0.16
25	388	0.34	0.57	0.95	0.14	0.00	0.03	0.16	2.18		
80	388	1.69	1.42	3.75	0.32	0.00	0.00	0.25	7.42	7.84	0.59
80	388	2.02	1.55	4.09	0.26	0.00	0.00	0.34	8.26		
100	68	0.69	1.03	1.86	0.18	0.12	0.13	0.23	4.25	4.25	
125	112	1.21	0.56	3.06	0.24	0.00	0.09	0.26	5.43	6.57	0.86
125	112	1.49	0.74	3.95	0.31	0.00	0.10	0.23	6.83		
125	112	0.88	1.85	2.53	0.24	0.24	0.29	0.49	6.51		
125	112	0.97	2.23	2.93	0.24	0.34	0.35	0.43	7.50		
150	340	1.04	0.51	2.99	0.29	0.00	0.07	0.25	5.14	6.06	0.93
150	340	1.11	0.54	3.66	0.40	0.00	0.00	0.27	5.97		
150	340	1.23	0.58	3.35	0.33	0.00	0.00	0.28	5.76		
150	340	1.53	0.67	3.96	0.55	0.00	0.07	0.57	7.35		
150	343	1.16	1.45	2.84	0.26	0.08	0.17	0.38	6.34	6.34	
150	388	2.04	2.09	4.52	0.35	0.04	0.02	0.53	9.59	9.65	0.09
150	388	1.93	2.21	4.78	0.31	0.00	0.09	0.41	9.72		
250	67	2.13	2.26	4.66	0.50	0.12	0.25	0.57	10.48	11.12	2.86
250	68	1.57	1.92	4.01	0.31	0.08	0.22	0.52	8.62		
250	68	2.55	3.09	6.57	0.57	0.25	0.37	0.85	14.24		
250	114	2.70	1.60	10.21	0.29	0.00	0.00	0.42	15.22	13.53	2.39
250	114	2.04	2.59	6.40	0.41	0.00	0.00	0.40	11.85		
250	128	2.03	1.78	5.83	0.08	0.00	0.00	0.31	10.03	10.03	
250	383	2.49	3.35	6.58	0.51	0.00	0.12	0.53	13.58	15.44	2.63
250	383	3.24	4.59	7.78	0.72	0.06	0.18	0.75	17.30		
500	68	0.91	0.88	2.21	0.19	0.00	0.10	0.20	4.49	6.15	2.34
500	68	1.66	1.40	3.78	0.30	0.08	0.29	0.30	7.80		
500	105	2.46	1.28	5.30	0.62	0.08	0.09	0.57	10.39	10.60	0.18
500	105	2.46	1.34	5.49	0.68	0.00	0.19	0.50	10.66		
500	105	2.50	1.35	5.73	0.75	0.00	0.00	0.40	10.73		
500	343	1.00	1.05	2.25	0.24	0.07	0.13	0.19	4.93	4.93	1.14
500	388	0.58	0.73	1.74	0.15	0.00	0.00	0.11	3.32	4.98	2.35
500	388	1.34	1.45	3.20	0.30	0.00	0.07	0.28	6.65		
1000	53	3.84	1.00	7.32	0.66	0.00	0.20	0.56	13.57	14.39	1.16
1000	53	4.21	1.09	7.98	0.84	0.09	0.29	0.72	15.21		
1000	68	1.52	1.11	3.38	0.22	0.00	0.14	0.21	6.57	7.50	1.31
1000	68	1.81	1.45	4.23	0.40	0.00	0.19	0.35	8.43		
1000	254	1.07	0.94	2.16	0.32	0.00	0.45	0.39	5.33	8.88	5.02
1000	254	3.15	1.85	4.90	2.12	0.00	0.00	0.42	12.43		
1000	343	1.04	1.56	2.56	0.28	0.07	0.17	0.33	6.01	6.01	
1000	388	1.15	1.72	4.41	0.36	0.00	0.00	0.26	7.89	9.56	2.36
1000	388	1.68	2.39	6.14	0.61	0.00	0.00	0.42	11.23		
3000	68	2.04	1.42	4.19	0.45	0.00	0.23	0.38	8.71	8.71	2.36
3000	388	1.26	0.93	2.17	0.48	0.00	0.00	0.52	5.37	5.41	0.07
3000	388	1.04	0.96	2.17	0.36	0.19	0.15	0.59	5.46		

Table A. 21 Proportion of each free LCFA in filtered samples of slaughterhouse wastewater containing 2.5 g/l of pork fat particles and mixed with PL-250 for 4 h

PL 250	Din	Long-chain fatty acid						
		16:0	18:2	18:1	18:0	10:0	14:0	16:1
mg/l	μm	mg/l						
0	68	0.00	0.00	1.00	0.00	0.00	0.00	0.00
0	68	0.13	0.18	0.30	0.07	0.08	0.10	0.14
0	343	0.11	0.21	0.31	0.08	0.10	0.08	0.12
0	343	0.11	0.23	0.32	0.08	0.09	0.08	0.09
25	68	0.16	0.25	0.42	0.08	0.03	0.00	0.06
25	68	0.15	0.31	0.37	0.05	0.00	0.04	0.08
25	388	0.16	0.26	0.42	0.05	0.01	0.02	0.08
25	388	0.15	0.26	0.44	0.06	0.00	0.01	0.07
80	388	0.23	0.19	0.51	0.04	0.00	0.00	0.03
80	388	0.24	0.19	0.49	0.03	0.00	0.00	0.04
100	68	0.16	0.24	0.44	0.04	0.03	0.03	0.05
125	112	0.22	0.10	0.56	0.04	0.00	0.02	0.05
125	112	0.22	0.11	0.58	0.05	0.00	0.01	0.03
125	112	0.13	0.28	0.39	0.04	0.04	0.04	0.08
125	112	0.13	0.30	0.39	0.03	0.04	0.05	0.06
150	340	0.20	0.10	0.58	0.06	0.00	0.01	0.05
150	340	0.19	0.09	0.61	0.07	0.00	0.00	0.04
150	340	0.21	0.10	0.58	0.06	0.00	0.00	0.05
150	340	0.21	0.09	0.54	0.08	0.00	0.01	0.08
150	343	0.18	0.23	0.45	0.04	0.01	0.03	0.06
150	388	0.21	0.22	0.47	0.04	0.00	0.00	0.06
150	388	0.20	0.23	0.49	0.03	0.00	0.01	0.04
250	67	0.20	0.22	0.44	0.05	0.01	0.02	0.05
250	68	0.18	0.22	0.46	0.04	0.01	0.02	0.06

Table A. 22 Free LCFA concentration in filtered samples of slaughterhouse wastewater containing 3.0 g/l of beef fat particles and mixed with PL-250 for 4 h

PL 250 mg/l	16:0	18:2	18:1	18:0	10:0	14:0	16:1	Total	avg	std
	long-chain fatty acid mg/l									
0	0.05	0.08	0.10	0.04	0.00	0.06	0.07	0.40	0.52	0.18
0	0.09	0.09	0.18	0.07	0.00	0.10	0.11	0.65		
25	3.59	1.28	7.32	0.64	0.51	0.59	0.91	14.84	14.84	
25										
100	0.75	0.38	2.00	0.28	0.00	0.07	0.22	3.71	8.55	6.85
100	2.91	1.17	6.24	0.74	0.57	0.75	1.02	13.40		
250	2.41	0.85	4.82	0.31	0.23	0.40	0.44	9.46	11.99	3.58
250	3.72	1.26	7.19	0.57	0.33	0.64	0.82	14.53		
500	0.89	0.31	1.78	0.16	0.12	0.16	0.23	3.65	6.75	4.38
500	2.51	0.78	4.48	0.42	0.52	0.55	0.59	9.84		
1500	1.36	0.54	3.27	0.25	0.35	0.30	0.53	6.60	12.22	7.95
1500	3.36	1.54	9.39	0.57	0.69	0.89	1.41	17.84		

Table A.23 Particle size distribution and average particle size reduction in slaughterhouse wastewater containing approximately 2.5 g/l of pork fat particles and mixed for 4 h with the Lipase G-1000

Avg. Size (µm)	Lipase G-1000 concentration (mg/L) 0	0	1250	2750	0	0	250	800
	Particle concentration (g/L)							
5.50	0.24	0.26	0.61	0.77	0.25	0.24	0.29	0.33
31.50	0.15	0.13	0.33	0.27	0.10	0.10	0.13	0.06
83.50	0.57	0.63	0.47	0.33	0.59	0.58	0.60	0.55
203.00	0.59	0.63	0.52	0.36	0.71	0.62	0.71	0.82
409.00	0.41	0.46	0.39	0.27	0.37	0.40	0.49	0.57
725.50	0.31	0.40	0.17	0.13	0.42	0.39	0.28	0.16
1167.50	0.18	0.18	0.07	0.02	0.12	0.14	0.09	0.07
Total (g/l)	2.46	2.68	2.55	2.15	2.57	2.48	2.59	2.58
Avg. dia. (µm)	154.35	162.39	73.32	45.58	158.47	160.57	138.13	131.50
$D_{treated}:D_{in}$			0.46	0.29			0.87	0.82

Avg. Size (µm)	Lipase G-1000 concentration (mg/L) 0	0	50	500	5000
	Particle concentration (g/L)				
5.50	0.16	0.15	0.17	0.16	0.54
31.50	0.18	0.13	0.04	0.09	0.31
83.50	0.63	0.61	0.55	0.64	0.20
203.00	0.45	0.52	0.59	0.59	0.11
409.00	0.61	0.59	0.63	0.76	0.33
725.50	0.31	0.41	0.30	0.27	0.19
1167.50	0.16	0.24	0.09	0.11	0.10
Total (g/l)	2.50	2.65	2.38	2.63	1.77
Avg. dia. (µm)	165.57	194.76	175	172	62
$D_{treated}:D_{in}$			0.97	0.95	0.35

Table A.24 Particle size distribution and average particle size reduction in slaughterhouse wastewater containing approximately 2.5 g/l of pork fat particles and mixed for 4 and 24 h with the Lipase G-1000

Avg. Size (µm)	4 h of treatment				24 h of treatment			
	Lipase G-1000 concentration (mg/L)							
	0	500	2000	3500	0	500	2000	3500
5.50	0.18	0.23	0.34	0.51	0.38	0.70	0.83	0.94
31.50	0.02	0.01	0.03	0.03	0.04	0.03	0.06	0.03
83.50	0.29	0.11	0.16	0.24	0.30	0.21	0.29	0.18
203.00	0.44	0.85	0.69	0.46	0.34	0.40	0.25	0.20
409.00	1.02	1.06	0.74	0.66	0.84	0.61	0.45	0.26
725.50	0.54	0.39	0.42	0.39	0.50	0.29	0.29	0.22
1167.50	0.30	0.19	0.13	0.12	0.45	0.10	0.06	0.09
Total (g/l)	2.78	2.85	2.53	2.42	2.84	2.33	2.22	1.93
Avg. dia. (µm)	271	231	179	130	215	89	61	42
$D_{treated}:D_{in}$		0.85	0.66	0.48		0.41	0.28	0.19

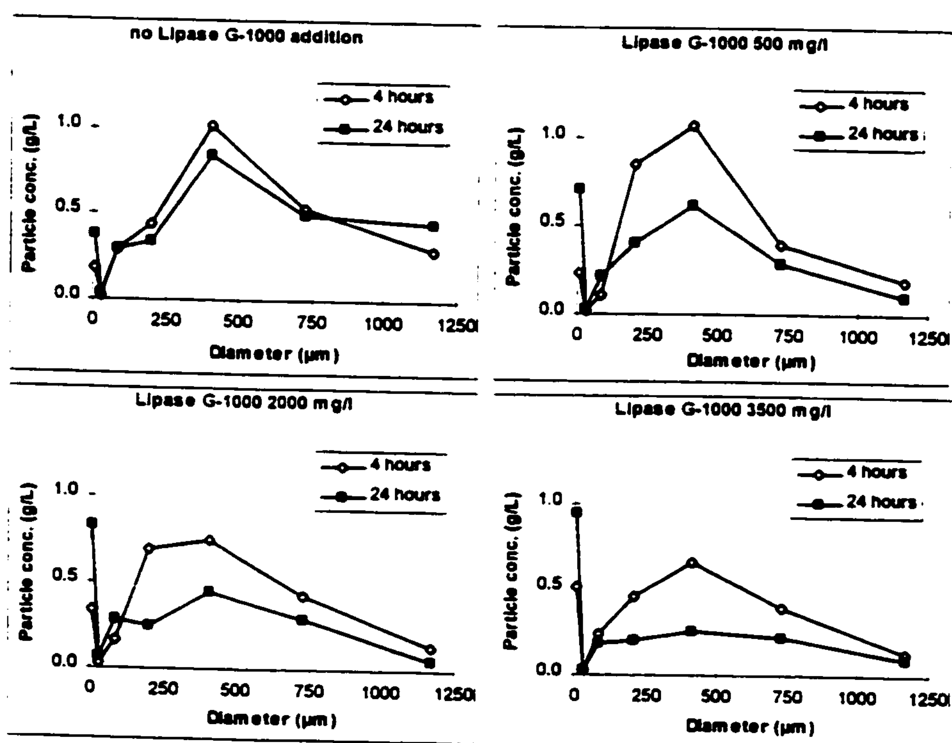


Figure A. 4 Particle size distribution in slaughterhouse wastewater containing 2.5 g/l of pork fat particles and mixed for 4 and 24 h with Lipase G-1000

Table A.25 SCOD in slaughterhouse wastewater containing approximately 2.5 g/l of pork fat particles and mixed for 4 and 24 h with the Lipase G-1000

LG-1000 mg/l	Treat. time (h)	SCOD (mg/l) in sample	SCOD (mg/l) less enzyme	$SCOD_{treated}$: $SCOD_{in}$
0	4	1208		
50	4	1356	1352	1.12
500	4	1332	1291	1.07
5000	4	1404	994	0.82
0	4	1430		
1250	4	1333	1231	0.86
2750	4	1307	1081	0.76
0	4	1434		
250	4	1403	1382	0.96
800	4	1429	1364	0.95
0	4	1305		
50	4	1464	1464	1.12
500	4	1668	1668	1.28
5000	4	1620	1620	1.24
0	24	1164		
500	24	1548	1507	1.29
2000	24	1668	1504	1.29
3500	24	2076	1789	1.54

Table A.26 Particle size distribution and particle size reduction in slaughterhouse wastewater containing approximately 3.0 g/l of pork fat particles and mixed with EcoSystem Plus for 4 and 24 h.

Avg. size (μm)	4 h of treatment Ecosystem Plus (μl/l)				24 h of treatment Ecosystem Plus (μl/l)			
	0	50	100	500	0	50	100	500
6	0.20	0.214	0.308	0.201	0.421	0.350	0.379	0.367
32	0.00	0.012	0.018	0.008	0.008	0.013	0.013	0.010
84	0.05	0.052	0.059	0.076	0.081	0.040	0.140	0.062
203	0.80	0.848	0.720	0.677	0.697	0.675	0.608	0.596
409	1.00	1.220	1.125	0.544	1.498	1.575	1.767	1.496
726	0.64	0.768	0.776	0.415	0.940	1.039	0.965	0.830
1168	0.32	0.347	0.315	0.340	0.457	0.352	0.776	0.546
Total (g/l)	3.01	3.46	3.32	2.26	4.10	4.04	4.65	3.91
Avg. dia. (μm)	289	299	304	256	271	292	315	293
$D_{treated}$:D_{in}		1.04	1.05	0.89		1.08	1.16	1.08

Avg. size (μm)	4 h of treatment Ecosystem Plus (μl/l)			
	0	0	5000	5000
6	0.24	0.25	0.28	0.20
32	0.07	0.02	0.03	0.02
84	0.59	0.56	0.56	0.55
203	0.46	0.56	0.49	0.54
409	0.50	0.50	0.52	0.52
726	0.63	0.61	0.51	0.45
1168	0.36	0.42	0.59	0.31
Total (g/l)	2.85	2.93	2.98	2.58
Avg. dia. (μm)	212	224	226	211
$D_{treated}$:D_{in}			1.04	0.97

Table A.27 SCOD in slaughterhouse wastewater containing approximately 3.0 g/l of pork fat particles and mixed for 4 and 24 h with EcoSystem Plus

Eco+	treat.	SCOD (mg/l)		$SCOD_{treated}$:
μl/l	time (h)	in sample	less enzyme	$SCOD_{in}$
0	4	1800		
50	4	2293	2283	1.27
100	4	1720	1700	0.94
500	4	1720	1620	0.90
0	4	1188		
5000	4	1932	932	0.78
0	24	1800		
50	24	1933	1923	1.07
100	24	2013	1993	1.11
500	24	1960	1860	1.03

Appendix B

Appendix B presents graphs and tables that were not included in the original text of Chapter 4. Following is a description of the tables and figures:

- Table B.1 presents data collected on control samples which showed that particle size or free LCFA concentration in solution was not modified by simply mixing at room temperature. These data were used to build Figure 4.1.
- Tables B.2 and B.3 present particle size distribution, particle size reduction and SCOD data from the experiment on PL-250 effect with time.
- Tables B.4 and B.6 present the non-linear least squares summary statistics used to estimate the parameters of Eqs. 4.1 to 4.3 on particle size reduction with various treatment times and concentrations of PL-250. Figures B.1 and B.2 were used to establish the relation between the parameter estimates (D_{min} and b_I) and the experimental conditions (D_{in} and PL-250 concentration), which were used to build equation 4.3.
- Tables B.7 presents the raw data on free LCFA concentrations in filtered samples during the experiments on PL-250 effect on pork fat particles. Data on individual LCFAs were used to describe the distribution on free LCFAs in solution, while the data on total LCFAs in solution were used to estimate parameters for Eq. 4.5. These parameters are presented in Table B.8.
- Table B.8 presents the raw data on free LCFA concentrations in filtered samples during the experiments on PL-250 effect on beef fat particles.

Table B.1 Particle size distribution, average particle size and free LCFA concentration in control samples (no PL-250 addition) mixed for various time periods at room temperature

Avg. size (μm)	Mixing time (hour) 0	7	0	3	27	2	24
	Particle concentration (g/L)						
6	0.24	0.26	0.27	0.26	0.23	0.25	0.3
32	0.15	0.13	0.07	0.07	0.07	0.05	0.06
84	0.57	0.63	0.15	0.15	0.16	0.57	0.63
203	0.59	0.63	0.14	0.16	0.17	0.51	0.52
409	0.41	0.46	0.42	0.41	0.49	0.5	0.44
726	0.31	0.4	0.5	0.49	0.51	0.62	0.53
1168	0.18	0.18	0.95	0.84	1.13	0.39	0.8
Total (g/l)	2.45	2.69	2.5	2.38	2.76	2.89	3.28
Avg. dia. (μm)	154	162	352	334	399	218	236
free LCFAs (mg/l)			0.45	0.51	0.09		

Table B.2 Particle size distribution and average particle size reduction in slaughterhouse wastewater containing 2.5 g/l of pork fat particles and mixed at room temperature for various time periods with the Pancreatic Lipase PL-250

Avg. size (μm)	time (min) (PL250 conc = 250 mg/l) 0	0	10	35	75	240	1560
	Particle concentration (g/L)						
6	0.23	0.26	0.30	0.32	0.33	0.39	0.55
32	0.95	0.82	0.67	0.81	0.77	0.71	1.00
84	0.58	0.55	0.52	0.43	0.48	0.38	0.34
203	0.28	0.23	0.18	0.20	0.24	0.30	0.18
409	0.13	0.10	0.10	0.12	0.10	0.15	0.08
726	0.10	0.13	0.10	0.11	0.10	0.08	0.08
1168	0.13	0.15	0.17	0.12	0.11	0.14	0.07
Total (g/l)	2.40	2.24	2.03	2.10	2.13	2.15	2.30
Avg. dia. (μm)	67	68	64	58	59	60	38
$D_{treated}:D_{in}$			0.96	0.87	0.87	0.90	0.56

Avg. size (μm)	time (min) (PL250 conc = 250 mg/l) 0	0	15	35	120	240	450	1470	2880
	Particle concentration (g/L)								
6	0.27	0.22	0.20	0.26	0.27	0.30	0.31	0.43	0.48
32	0.47	0.68	0.66	0.65	0.53	0.53	0.53	0.64	0.84
84	0.43	0.42	0.36	0.42	0.33	0.41	0.31	0.41	0.37
203	0.22	0.17	0.13	0.13	0.13	0.19	0.17	0.26	0.18
409	0.20	0.19	0.19	0.19	0.17	0.17	0.17	0.16	0.13
726	0.28	0.25	0.29	0.22	0.18	0.19	0.14	0.09	0.10
1168	0.23	0.25	0.20	0.17	0.15	0.22	0.15	0.20	0.20
Total (g/l)	2.10	2.17	2.03	2.04	1.75	2.02	1.77	2.19	2.30
Avg. dia. (μm)	109	99	101	83	79	88	72	63	54
$D_{treated}:D_{in}$			0.98	0.80	0.76	0.85	0.69	0.61	0.52

Table B.2 (continued)

Avg. size (µm)	time (min) (PL250 conc = 250 mg/l)						
	0	0	15	70	240	420	1500
	Particle concentration (g/L)						
6	0.30	0.29	0.35	0.34	0.36	0.43	0.42
32	0.39	0.41	0.39	0.38	0.42	0.43	0.40
84	0.50	0.48	0.45	0.43	0.32	0.29	0.25
203	0.39	0.41	0.32	0.30	0.27	0.27	0.26
409	0.33	0.30	0.32	0.33	0.28	0.25	0.24
726	0.21	0.28	0.21	0.18	0.16	0.16	0.10
1168	0.18	0.22	0.14	0.24	0.16	0.17	0.13
Total (g/l)	2.30	2.40	2.19	2.20	1.98	2.02	1.80
Avg. dia. (µm)	109	119	96	105	86	78	68
$D_{treated}:D_{in}$			0.84	0.92	0.75	0.68	0.60

Avg. size (µm)	time (min) (PL250 conc = 250 mg/l)								
	0	0	5	15	35	75	240	435	1500
	Particle concentration (g/L)								
6	0.25	0.29	0.25	0.28	0.30	0.24	0.43	0.47	0.60
32	0.40	0.38	0.41	0.35	0.36	0.42	0.29	0.27	0.51
84	0.49	0.49	0.49	0.52	0.40	0.44	0.31	0.28	0.36
203	0.42	0.40	0.29	0.33	0.29	0.31	0.26	0.21	0.29
409	0.33	0.29	0.36	0.35	0.33	0.32	0.30	0.23	0.32
726	0.27	0.21	0.34	0.31	0.24	0.21	0.12	0.14	0.13
1168	0.29	0.29	0.20	0.23	0.29	0.23	0.18	0.12	0.15
Total (g/l)	2.44	2.35	2.34	2.37	2.22	2.17	1.90	1.72	2.35
Avg. dia. (µm)	134	122	127	129	126	120	82	67	63
$D_{treated}:D_{in}$			0.99	1.01	0.99	0.93	0.64	0.52	0.49

Avg. size (µm)	time (min) (PL250 conc = 250 mg/l)						
	0	0	30	75	240	420	1500
	Particle concentration (g/L)						
6	0.27	0.26	0.27	0.28	0.39	0.50	0.63
32	0.07	0.07	0.07	0.05	0.05	0.06	0.09
84	0.15	0.15	0.19	0.15	0.17	0.17	0.16
203	0.14	0.16	0.16	0.23	0.28	0.25	0.23
409	0.42	0.41	0.57	0.52	0.55	0.62	0.43
726	0.50	0.49	0.57	0.41	0.47	0.37	0.41
1168	0.95	0.84	0.91	0.73	0.71	0.60	0.91
Total (g/l)	2.49	2.38	2.73	2.38	2.63	2.56	2.86
Avg. dia. (µm)	352	334	347	299	252	194	191
$D_{treated}:D_{in}$			1.01	0.87	0.73	0.57	0.56

Table B.2 (continued)

Avg. size (µm)	time (min) (PL250 conc = 250 mg/l)						
	0	0	25	35	75	420	1500
	Particle concentration (g/L)						
6	0.20	0.20	0.28	0.20	0.35	0.37	0.49
32	0.04	0.03	0.04	0.04	0.05	0.07	0.08
84	0.19	0.18	0.16	0.21	0.16	0.17	0.14
203	0.15	0.18	0.19	0.22	0.21	0.18	0.17
409	0.37	0.39	0.46	0.41	0.45	0.42	0.38
726	0.52	0.46	0.53	0.50	0.42	0.32	0.40
1168	0.96	0.81	0.68	0.59	0.77	0.49	0.60
Total (g/l)	2.42	2.26	2.33	2.17	2.41	2.01	2.26
Avg. dia. (µm)	402	363	308	315	272	198	183
$D_{treated}$:D_{in}			0.81	0.82	0.71	0.52	0.48

Avg. size (µm)	time (min) (PL250 conc = 1000 mg/l)										
	0	0	15	30	90	120	225	240	405	1320	1500
	Particle concentration (g/L)										
6	0.27	0.26	0.29	0.32	0.37	0.33	0.37	0.43	0.49	0.67	0.87
32	0.06	0.05	0.06	0.08	0.07	0.05	0.06	0.11	0.06	0.09	0.13
84	0.25	0.21	0.23	0.30	0.25	0.28	0.27	0.35	0.30	0.25	0.23
203	0.22	0.23	0.32	0.34	0.42	0.44	0.42	0.42	0.40	0.37	0.24
409	0.46	0.46	0.53	0.52	0.55	0.57	0.53	0.69	0.58	0.37	0.36
726	0.48	0.49	0.42	0.42	0.33	0.25	0.30	0.35	0.35	0.40	0.49
1168	0.54	0.40	0.45	0.34	0.35	0.39	0.20	0.28	0.29	0.30	0.35
Total (g/l)	2.29	2.10	2.29	2.33	2.34	2.32	2.15	2.63	2.47	2.44	2.68
Avg. dia. (µm)	259	248	233	195	183	191	156	158	148	109	93
$D_{treated}$:D_{in}			0.92	0.77	0.72	0.75	0.62	0.62	0.58	0.43	0.37

Avg. size (µm)	time (min) (PL250 conc = 500 mg/l)									
	0	0	30	60	120	240	240	390	1410	1530
	Particle concentration (g/L)									
6	0.21	0.19	0.43	0.29	0.31	0.27	0.27	0.41	0.45	0.47
32	0.54	0.58	0.37	0.40	0.53	0.63	0.76	0.64	0.81	0.81
84	0.42	0.42	0.33	0.30	0.29	0.45	0.43	0.31	0.24	0.22
203	0.16	0.22	0.15	0.21	0.23	0.30	0.22	0.17	0.12	0.11
409	0.15	0.18	0.17	0.11	0.16	0.13	0.13	0.11	0.09	0.10
726	0.29	0.26	0.30	0.30	0.30	0.18	0.13	0.18	0.10	0.10
1168	0.22	0.17	0.20	0.20	0.20	0.16	0.16	0.20	0.20	0.17
Total (g/l)	1.98	2.03	1.96	1.81	2.02	2.12	2.10	2.02	2.02	1.98
Avg. dia. (µm)	107	102	85	102	96	81	71	66	50	47
$D_{treated}$:D_{in}			0.81	0.98	0.92	0.77	0.67	0.63	0.47	0.45

Table B.2 (continued)

Avg. size (μm)	time (min) (PL250 conc = 150 mg/l)									
	0	0	30	60	120	240	240	400	1470	1590
	Particle concentration (g/L)									
6	0.15	0.15	0.16	0.17	0.20	0.21	0.20	0.27	0.43	0.48
32	0.03	0.02	0.02	0.03	0.02	0.02	0.02	0.05	0.10	0.09
84	0.23	0.18	0.27	0.24	0.24	0.23	0.34	0.31	0.30	0.28
203	0.34	0.35	0.44	0.47	0.51	0.60	0.60	0.54	0.38	0.33
409	0.53	0.38	0.59	0.70	0.63	0.69	0.64	0.53	0.54	0.60
726	0.45	0.58	0.52	0.40	0.42	0.33	0.23	0.27	0.31	0.28
1168	0.52	0.54	0.40	0.31	0.40	0.34	0.21	0.27	0.43	0.41
Total (g/l)	2.25	2.20	2.41	2.32	2.42	2.42	2.24	2.24	2.49	2.47
Avg. dia. (μm)	326	354	297	268	273	251	208	191	167	156
$D_{treated}:D_{in}$			0.87	0.79	0.80	0.74	0.61	0.56	0.49	0.46

Avg. size (μm)	time (min) (PL250 conc = 150 mg/l)									
	0	0	30	60	120	240	240	400	1470	1590
	Particle concentration (g/L)									
6	0.16	0.16	0.21	0.22	0.28	0.29	0.32	0.36	0.44	0.57
32	0.32	0.35	0.22	0.28	0.23	0.41	0.38	0.47	0.55	0.46
84	0.52	0.54	0.41	0.36	0.38	0.40	0.42	0.38	0.33	0.33
203	0.39	0.35	0.37	0.32	0.38	0.38	0.39	0.34	0.28	0.30
409	0.26	0.28	0.41	0.31	0.39	0.37	0.36	0.35	0.40	0.37
726	0.30	0.29	0.27	0.22	0.20	0.13	0.15	0.15	0.18	0.17
1168	0.25	0.20	0.18	0.19	0.16	0.15	0.31	0.14	0.18	0.12
Total (g/l)	2.21	2.17	2.06	1.91	2.02	2.12	2.33	2.20	2.35	2.32
Avg. dia. (μm)	152	140	151	133	126	101	117	88	83	68
$D_{treated}:D_{in}$			1.04	0.91	0.86	0.69	0.80	0.61	0.57	0.47

Avg. size (μm)	time (min) (PL250 conc = 125 mg/l)							
	0	0	15	35	75	240	420	1620
	Particle concentration (g/L)							
6	0.36	0.35	0.30	0.40	0.40	0.50	0.44	0.53
32	0.47	0.41	0.45	0.39	0.35	0.29	0.30	0.33
84	0.40	0.38	0.33	0.31	0.25	0.30	0.28	0.32
203	0.10	0.10	0.10	0.08	0.15	0.26	0.28	0.23
409	0.22	0.18	0.21	0.19	0.21	0.23	0.27	0.19
726	0.21	0.22	0.27	0.26	0.24	0.16	0.18	0.16
1168	0.44	0.43	0.33	0.36	0.39	0.39	0.30	0.35
Total (g/l)	2.21	2.07	2.00	1.98	2.01	2.13	2.04	2.13
Avg. dia. (μm)	110	114	115	103	112	98	99	85
$D_{treated}:D_{in}$			1.03	0.92	1.00	0.88	0.89	0.76

Table B.3 SCOD concentration and SCOD changes in slaughterhouse wastewater containing approximately 2.5 g/l of pork fat particles and mixed for various time periods with PL-250

PL250 mg/l	Time h	Din μm	SCOD (mg/l) in sample	SCOD (mg/l) less enzyme	$SCOD_{treated}$: $SCOD_{in}$
0	0	67	1425	1425	
250	10	67	1657	1395	0.98
250	35	67	1716	1454	1.02
250	75	67	1732	1469	1.03
250	250	67	1534	1271	0.89
250	420	67	1801	1539	1.08
250	1590	67	1539	1277	0.90
0	0	114	1424	1424	
250	0	114	1437	1175	0.82
250	5	114	1673	1411	0.99
250	15	114	1769	1507	1.06
250	35	114	1692	1430	1.00
250	70	114	1762	1499	1.05
250	240	114	1870	1607	1.13
250	420	114	1828	1566	1.10
250	1500	114	1751	1489	1.05
0	0	128	1614	1614	
250	10	128	1837	1575	0.98
250	15	128	1877	1615	1.00
250	35	128	1897	1634	1.01
250	75	128	1871	1609	1.00
250	240	128	2032	1770	1.10
250	435	128	2238	1975	1.22
250	1500	128	1943	1680	1.04
0	0	383	1461	1461	
0	0	383	1387	1387	
250	10	383	1666	1404	0.99
250	25	383	1569	1306	0.92
250	70	383	1724	1462	1.03
250	240	383	1773	1510	1.06
250	420	383	1763	1501	1.05
250	1440	383	1641	1379	0.97
0	0	112	1358	1358	
0	0	112	1489	1489	
125	10	112	1613	1482	1.04
125	25	112	1588	1457	1.02
125	70	112	1644	1513	1.06
125	240	112	1599	1468	1.03
125	420	112	1580	1448	1.02
125	1440	112	1580	1448	1.02

Table B.4 Non-linear least squares summary statistics: Parameter estimates (Eq. 4.1) for particle size reduction in slaughterhouse wastewater containing approximately 2.5 g/l of pork fat particles and mixed for various time periods with PL-250

a. D_{in} = 68 µm PL-250 = 250 mg/l

Source	DF	Sum of Squares	Mean Square
Regression	2	17397.232886	8698.616443
Residual	4	173.163014	43.290754
Uncorrected Total	6	17570.395900	
(Corrected Total)	5	689.885483	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Conf. Interval Lower	Upper
DMIN	36.80532283	6.8870434292	17.684083540	55.926562128
B1	0.00319407	0.0018031095	-0.001812092	0.008200242

Asymptotic Correlation Matrix

Corr	DMIN	B1
DMIN	1	0.7165782499
B1	0.7165782499	1

b. D_{in} = 104 µm PL-250 = 250 mg/l

Source	DF	Sum of Squares	Mean Square
Regression	2	95318.033857	47659.016929
Residual	11	836.154043	76.014004
Uncorrected Total	13	96154.187900	
(Corrected Total)	12	4027.595831	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Conf. Interval Lower	Upper
DMIN	62.13228901	5.1442345869	50.809861778	73.454716233
B1	0.00336392	0.0010511906	0.001050259	0.005677586

Table B.4 (continued)

Asymptotic Correlation Matrix

Corr	DMIN	B1
DMIN	1	0.6468530095
B1	0.6468530095	1

c. D_{in} = 128 µm PL-250 = 250 mg/l

Source	DF	Sum of Squares	Mean Square
Regression	2	77862.116953	38931.058476
Residual	5	200.752647	40.150529
Uncorrected Total	7	78062.869600	
(Corrected Total)	6	5414.278971	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Conf. Interval Lower	Upper
DMIN	59.08415660	6.2684527738	42.970819313	75.197493892
B1	0.00385820	0.0009343080	0.001456521	0.006259882

Asymptotic Correlation Matrix

Corr	DMIN	B1
DMIN	1	0.7169563893
B1	0.7169563893	1

d. D_{in} = 363 µm PL-250 = 250 mg/l

Source	DF	Sum of Squares	Mean Square
Regression	2	681785.08252	340892.54126
Residual	8	6005.82008	750.72751
Uncorrected Total	10	687790.90260	
(Corrected Total)	9	33372.64404	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Conf. Interval Lower	Upper
DMIN	195.9584185	14.585024130	162.32496808	229.59186895
B1	0.0084179	0.002555451	0.00252496	0.01431084

Table B.4 (continued)

Asymptotic Correlation Matrix

Corr	DMIN	B1
DMIN	1	0.5698999566
B1	0.5698999566	1

e. D_{in} = 254 μm PL-250 = 1000 mg/l

Source	DF	Sum of Squares	Mean Square
Regression	2	251782.31919	125891.15959
Residual	7	2584.93971	369.27710
Uncorrected Total	9	254367.25890	
(Corrected Total)	8	15203.87702	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Conf. Interval Lower	Upper
DMIN	108.9584232	12.985324059	78.252764499	139.66408188
B1	0.0051974	0.001282996	0.002163591	0.00823125

Asymptotic Correlation Matrix

Corr	DMIN	B1
DMIN	1	0.6856451291
B1	0.6856451291	1

f. D_{in} = 105 μm PL-250 = 500 mg/l

Source	DF	Sum of Squares	Mean Square
Regression	2	48881.899699	24440.949849
Residual	6	274.910901	45.818484
Uncorrected Total	8	49156.810600	
(Corrected Total)	7	3109.790150	

Table B.4 (continued)

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Conf. Interval Lower	Upper
DMIN	46.39641806	5.7706324803	32.276178866	60.516657259
B1	0.00250500	0.0006238114	0.000978589	0.004031414

Asymptotic Correlation Matrix

Corr	DMIN	B1
DMIN	1	0.7117749046
B1	0.7117749046	1

g. D_{in} = 340 μm PL-250 = 150 mg/l

Source	DF	Sum of Squares	Mean Square
Regression	2	428068.48671	214034.24336
Residual	6	2403.72119	400.62020
Uncorrected Total	8	430472.20790	
(Corrected Total)	7	19533.09259	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Conf. Interval Lower	Upper
DMIN	164.0887616	13.788569556	130.34932301	197.82820022
B1	0.0046613	0.001004276	0.00220397	0.00711873

Asymptotic Correlation Matrix

Corr	DMIN	B1
DMIN	1	0.675704505
B1	0.675704505	1

h. D_{in} = 146 μm PL-250 = 150 mg/l

Source	DF	Sum of Squares	Mean Square
Regression	2	99337.045373	49668.522686
Residual	6	427.098527	71.183088
Uncorrected Total	8	99764.143900	
(Corrected Total)	7	5549.250288	

Table B.4 (continued)

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Conf. Interval Lower	Upper
DMIN	73.91894396	6.3991956151	58.260665075	89.577222855
B1	0.00308841	0.0007036274	0.001366698	0.004810129

Asymptotic Correlation Matrix

Corr	DMIN	B1
DMIN	1	0.6691223796
B1	0.6691223796	1

i. D_{in} = 112 μm PL-250 = 125 mg/l

Source	DF	Sum of Squares	Mean Square
Regression	2	50524.761099	25262.380550
Residual	3	80.134801	26.711600
Uncorrected Total	5	50604.895900	
(Corrected Total)	4	478.816520	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Conf. Interval Lower	Upper
DMIN	84.37876719	6.5841598435	63.424678486	105.33285589
B1	0.00210790	0.0012227988	-0.001783662	0.00599945

Asymptotic Correlation Matrix

Corr	DMIN	B1
DMIN	1	0.7571666304
B1	0.7571666304	1

Table B.5 Non-linear least squares summary statistics used to establish relation between parameter estimates of Eq. 4.1 (D_{min} and b_l) and D_{in} for slaughterhouse wastewater containing 2.5 g/l of pork fat particles and mixed for various time periods with PL-250. Based on Eq. 4.1, $D_{min} = X*D_{in}$ and $b_l = Y*D_{in}$.

a. PL-250 = 250 mg/l, all D_{in}

Source	DF	Sum of Squares	Mean Square
Regression	2	870399.75698	435199.87849
Residual	34	9178.59902	269.95879
Uncorrected Total	36	879578.35600	
(Corrected Total)	35	270163.93350	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Conf. Interval Lower	Upper
X	0.5322030559	0.02346344876	0.48451988677	0.57988622500
Y	0.0000228846	0.00000375470	0.00001525421	0.00003051507

Asymptotic Correlation Matrix

Corr	X	Y
X	1	0.5958660351
Y	0.5958660351	1

b. PL-250 = 1000 mg/l, all D_{in}

Source	DF	Sum of Squares	Mean Square
Regression	2	251782.31919	125891.15959
Residual	7	2584.93971	369.27710
Uncorrected Total	9	254367.25890	
(Corrected Total)	8	15203.87702	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Conf. Interval Lower	Upper
X	0.4296642631	0.05120593752	0.30858048933	0.55074803682
Y	0.0000204954	0.00000505936	0.00000853187	0.00003245901

Table B.5 (continued)

Asymptotic Correlation Matrix

Corr	X	Y
X	1	0.685645246
Y	0.685645246	1

c. PL-250 = 150 mg/l, all D_{in}

Source	DF	Sum of Squares	Mean Square
Regression	2	527131.90509	263565.95254
Residual	14	3104.44671	221.74619
Uncorrected Total	16	530236.35180	
(Corrected Total)	15	80894.04290	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Conf. Interval Lower	Upper
X	0.4873155531	0.02841712406	0.42636702717	0.54826407898
Y	0.0000146543	0.00000224901	0.00000983071	0.00001947798

Asymptotic Correlation Matrix

Corr	X	Y
X	1	0.685736143
Y	0.685736143	1

d. PL-250 = 125 mg/l, all D_{in}

Source	DF	Sum of Squares	Mean Square
Regression	2	50524.761099	25262.380550
Residual	3	80.134801	26.711600
Uncorrected Total	5	50604.895900	
(Corrected Total)	4	478.816520	

Table B.5 (continued)

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Conf. Interval Lower	Upper
X	0.7537183819	0.05881336979	0.56654483257	0.94089193128
Y	0.0000188289	0.00001092273	-.00001593267	0.00005359049

Asymptotic Correlation Matrix

Corr	X	Y
X	1	0.7571664814
Y	0.7571664814	1

e. PL-250 = 500 mg/l, all D_{in}

Source	DF	Sum of Squares	Mean Square
Regression	2	48881.899699	24440.949849
Residual	6	274.910901	45.818484
Uncorrected Total	8	49156.810600	
(Corrected Total)	7	3109.790150	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Conf. Interval Lower	Upper
X	0.4433062578	0.05513691270	0.30839099533	0.57822152033
Y	0.0000239347	0.00000596036	0.00000935018	0.00003851918

Asymptotic Correlation Matrix

Corr	X	Y
X	1	0.7117746976
Y	0.7117746976	1

Table B.6 Non-linear least squares summary statistics used to estimate parameters for Eq. 4.3. The parameter X is the exponent of C shown in Eq. 4.2 and Y is as explained in Table B.5. Parameters X and Y were estimated using data al all D_{in} and all PL-250 concentrations

Source	DF	Sum of Squares	Mean Square
Regression	2	1746770.8719	873385.4360
Residual	72	17172.8013	238.5111
Uncorrected Total	74	1763943.6732	
(Corrected Total)	73	428781.3812	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic 95 % Confidence Interval	
			Lower	Upper
X	0.1238257726	0.00253140079	0.11877950499	0.12887204012
Y	0.0000192828	0.00000188063	0.00001553388	0.00002303181

Asymptotic Correlation Matrix

Corr	X	Y
X	1	-0.658588076
Y	-0.658588076	1

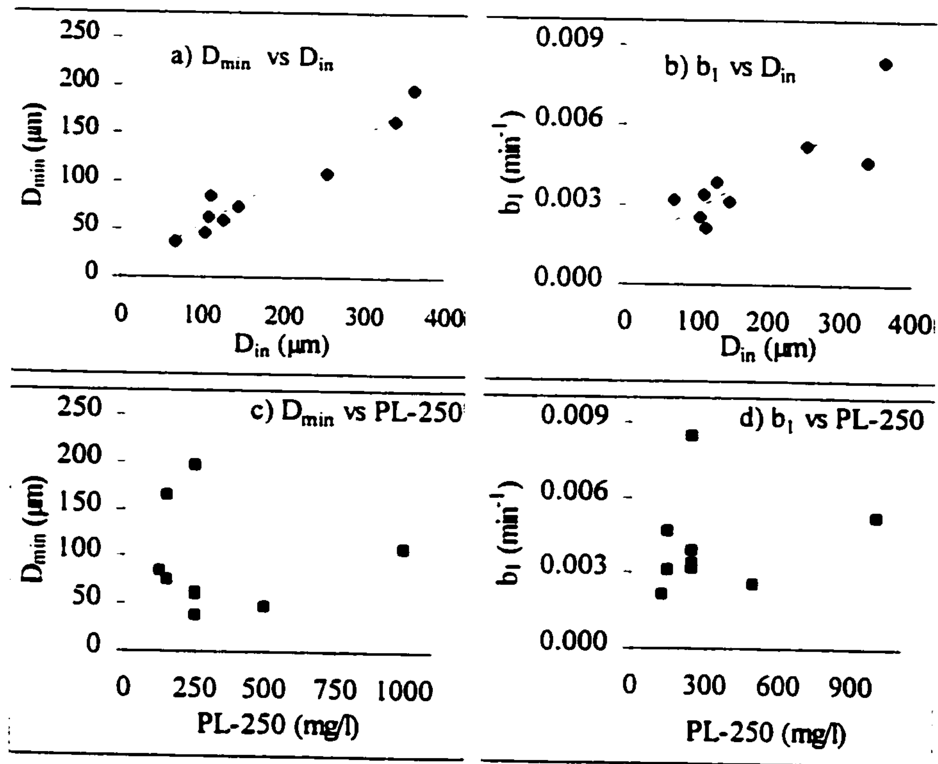


Figure B.1 Relation between estimates of parameters D_{min} and b_l and experimental conditions (D_{in} and PL-250 concentration); points are from the estimates of Tables B.4a to B.4i

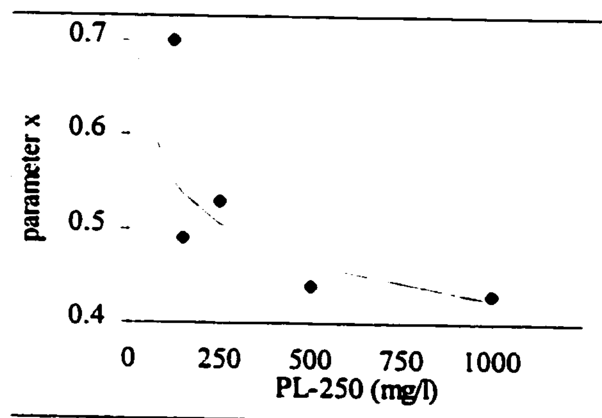


Figure B.2 Relation between parameter X and PL-250 concentration; points are from the estimates shown on Table B.5

Table B.7 Free LCFA concentrations in filtered samples of slaughterhouse wastewater containing approximately 2.5 g/l of pork fat particles and mixed for various time periods with PL-250

Time	D_{in}	PL-250	Long-chain fatty acid									
min	μm	mg/l	16:0	18:2	18:1	18:0	10:0	14:0	16:1	Total	avg	std
			mg/l									
0	112	125	0.08	0.07	0.19	0.07	0.02	0.02	0.03	0.48	0.51	0.04
0	112	125	0.12	0.05	0.14	0.06	0.04	0.04	0.09	0.54		
10	112	125	0.05	0.12	0.25	0.10	0.00	0.00	0.06	0.58	0.64	0.08
10	112	125	0.16	0.12	0.24	0.10	0.00	0.02	0.06	0.70		
25	112	125	0.87	1.10	1.73	0.30	0.27	0.44	0.41	5.12	4.33	1.10
25	112	125	0.53	0.81	1.26	0.34	0.03	0.27	0.31	3.55		
70	112	125	0.41	0.84	0.94	0.12	0.51	0.16	0.31	3.29	4.07	1.11
70	112	125	0.60	1.21	1.36	0.44	0.36	0.50	0.38	4.85		
240	112	125	0.97	2.23	2.93	0.24	0.34	0.35	0.43	7.50	7.01	0.70
240	112	125	0.88	1.85	2.53	0.24	0.24	0.29	0.49	6.51		
405	112	125	1.13	2.52	3.91	0.26	0.39	0.48	0.75	9.45	8.18	1.80
405	112	125	0.85	1.75	3.26	0.17	0.00	0.33	0.54	6.91		
1440	112	125	1.25	1.31	3.55	0.46	0.00	0.07	0.71	7.35	6.71	0.90
1440	112	125	0.83	1.23	2.52	0.30	0.25	0.35	0.59	6.07		
30	150	146	0.38	0.41	0.87	0.12	0.00	0.00	0.16	1.94	1.84	0.14
30	150	146	0.30	0.36	0.82	0.10	0.00	0.00	0.16	1.74		
60	150	146	0.67	0.65	1.52	0.25	0.00	0.08	0.30	3.47	3.23	0.33
60	150	146	0.59	0.56	1.34	0.17	0.02	0.07	0.25	3.00		
120	150	146	0.88	0.63	1.50	0.24	0.00	0.05	0.24	3.53	4.40	1.22
120	150	146	1.00	0.76	2.86	0.30	0.00	0.05	0.29	5.26		
240	150	146	1.21	0.56	3.06	0.24	0.00	0.09	0.26	5.43	6.13	0.99
240	150	146	1.49	0.74	3.95	0.31	0.00	0.10	0.23	6.83		
265	150	146	3.10	1.33	7.96	0.70	0.08	0.17	0.64	13.99	10.46	4.98
265	150	146	1.88	0.63	3.77	0.28	0.00	0.00	0.39	6.94		
450	150	146	6.92	2.59	17.64	1.55	0.00	0.00	1.13	29.85	26.80	4.30
450	150	146	5.29	2.14	14.57	0.94	0.00	0.12	0.70	23.76		
1500	150	146	0.62	0.74	3.15	0.40	0.06	0.12	0.40	5.50	5.66	0.23
1500	150	146	0.65	0.78	3.22	0.35	0.08	0.16	0.59	5.82		
1590	150	146	0.66	0.75	2.58	0.37	0.08	0.17	0.38	4.98	5.06	0.11
1590	150	146	0.73	0.70	2.62	0.36	0.08	0.16	0.49	5.14		
30	150	340	0.08	0.03	0.12	0.06	0.00	0.00	0.03	0.31	0.62	0.43
30	150	340	0.23	0.10	0.26	0.14	0.03	0.07	0.09	0.92		
60	150	340	0.13	0.07	0.28	0.11	0.00	0.00	0.06	0.65	0.73	0.11
60	150	340	0.18	0.09	0.28	0.11	0.00	0.05	0.10	0.81		
120	150	340	0.39	0.26	0.99	0.18	0.00	0.00	0.18	2.01	2.11	0.15
120	150	340	0.43	0.29	1.11	0.22	0.00	0.00	0.17	2.22		
240	150	340	1.11	0.54	3.66	0.40	0.00	0.00	0.27	5.97	5.87	0.15
240	150	340	1.23	0.58	3.35	0.33	0.00	0.00	0.28	5.76		
240	150	340	1.53	0.67	3.96	0.55	0.00	0.07	0.57	7.35	6.25	1.57
240	150	340	1.04	0.51	2.99	0.29	0.00	0.07	0.25	5.14		
390	150	340	3.98	1.29	11.28	0.99	0.00	0.21	0.83	18.57	16.35	3.14
390	150	340	2.77	1.04	9.13	0.68	0.00	0.00	0.51	14.12		
1500	150	340	0.26	0.39	1.74	0.10	0.00	0.05	0.19	2.72	2.78	0.08
1500	150	340	0.31	0.40	1.70	0.10	0.00	0.08	0.24	2.84		
1620	150	340	0.51	0.62	3.04	0.22	0.06	0.15	0.37	4.98	4.98	

Table B.7 (continued)

Time	D_{in}	PL-250	Long-chain fatty acid									
min	μm	mg/l	16:0	18:2	18:1	18:0	10:0	14:0	16:1	Total	avg	std
			mg/l									
0	250	67	0.12	0.14	0.24	0.07	0.00	0.05	0.06	0.68	0.44	0.34
0	250	67	0.04	0.06	0.08	0.02	0.00	0.00	0.00	0.20		
10	250	67	0.27	0.34	0.55	0.00	0.00	0.00	0.07	1.24	1.01	0.32
10	250	67	0.14	0.22	0.33	0.02	0.00	0.00	0.06	0.78		
35	250	67	0.26	0.33	0.58	0.08	0.00	0.03	0.06	1.33	2.29	1.35
35	250	67	0.64	0.81	1.42	0.15	0.00	0.06	0.17	3.24		
75	250	67	0.60	0.87	1.41	0.12	0.03	0.09	0.21	3.34	2.82	0.73
75	250	67	0.42	0.58	0.94	0.10	0.03	0.07	0.17	2.31		
240	250	67	2.13	2.26	4.66	0.50	0.12	0.25	0.57	10.48	10.48	
420	250	67	2.97	3.20	7.91	0.40	0.06	0.30	0.79	15.63	16.17	0.76
420	250	67	3.42	3.34	8.25	0.56	0.10	0.35	0.70	16.71		
1590	250	67	0.27	0.47	0.97	0.15	0.09	0.13	0.18	2.27	2.27	
0	250	104	0.09	0.00	0.25	0.06	0.05	0.00	0.04	0.49	0.43	0.268
0	250	104	0.09	0.09	0.18	0.06	0.00	0.05	0.06	0.53		
0	250	104	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.04		
0	250	104	0.15	0.11	0.18	0.10	0.00	0.06	0.06	0.65		
5	250	104	0.16	0.25	0.36	0.10	0.05	0.07	0.13	1.12	1.51	0.55
5	250	104	0.28	0.41	0.59	0.16	0.12	0.14	0.20	1.90		
15	250	104	0.23	0.36	0.44	0.09	0.04	0.06	0.13	1.35	1.76	0.58
15	250	104	0.35	0.59	0.72	0.14	0.06	0.10	0.22	2.17		
35	250	104	0.47	0.80	1.09	0.11	0.06	0.10	0.22	2.85	3.19	0.49
35	250	104	0.52	1.02	1.47	0.17	0.00	0.12	0.25	3.54		
80	250	104	0.49	0.80	1.36	0.14	0.00	0.09	0.18	3.06	4.75	2.39
80	250	104	1.05	1.70	2.67	0.28	0.13	0.19	0.42	6.44		
240	250	104	2.70	1.60	10.21	0.29	0.00	0.00	0.42	15.22	13.53	2.39
240	250	104	2.04	2.59	6.40	0.41	0.00	0.00	0.40	11.85		
420	250	104	3.35	2.81	11.53	0.56	0.00	0.00	0.80	19.06	17.43	2.31
420	250	104	3.01	2.22	9.77	0.32	0.00	0.00	0.49	15.80		
1470	250	104	0.84	0.59	2.52	0.36	0.00	0.00	0.69	5.01	5.37	0.51
1470	250	104	0.65	0.70	3.00	0.36	0.00	0.33	0.68	5.74		
0	250	128	0.07	0.11	0.33	0.03	0.05	0.04	0.07	0.70	0.49	0.19
0	250	128	0.07	0.05	0.18	0.04	0.07	0.00	0.00	0.41		
0	250	128	0.07	0.06	0.17	0.00	0.00	0.06	0.00	0.36		
5	250	128	0.11	0.09	0.19	0.05	0.04	0.04	0.04	0.55	0.62	0.09
5	250	128	0.13	0.11	0.22	0.06	0.06	0.06	0.05	0.68		
10	250	128	0.26	0.29	0.62	0.11	0.00	0.00	0.00	1.28	1.31	0.04
10	250	128	0.27	0.25	0.53	0.14	0.00	0.03	0.11	1.34		
15	250	128	0.18	0.26	0.56	0.08	0.05	0.08	0.10	1.32	1.18	0.19
15	250	128	0.14	0.21	0.46	0.06	0.03	0.06	0.09	1.05		
35	250	128	0.11	0.26	0.29	0.04	0.00	0.04	0.08	0.82	1.41	0.84
35	250	128	0.24	0.57	0.63	0.11	0.12	0.10	0.23	2.00		
75	250	128	0.83	1.01	2.13	0.17	0.00	0.00	0.21	4.36	4.40	0.06
75	250	128	0.85	0.96	2.03	0.15	0.08	0.11	0.26	4.44		
240	250	128	2.03	1.78	5.83	0.08	0.00	0.00	0.31	10.03	25.19	21.44
240	250	128	8.89	6.38	20.95	1.36	0.22	0.84	1.71	40.35		
1500	250	128	0.67	0.95	2.07	0.24	0.18	0.33	0.59	5.01	3.85	1.65
1500	250	128	0.37	0.51	1.10	0.08	0.14	0.20	0.27	2.68		

Table B.7 (continued)

Time min	D_{in} µm	PL-250 mg/l	16:0	18:2	18:1	18:0	10:0	14:0	16:1	Total	avg	std
			Long-chain fatty acid mg/l									
10	250	383	0.42	0.73	0.81	0.10	0.01	0.06	0.24	2.39	2.09	0.43
10	250	383	0.25	0.59	0.68	0.10	0.00	0.02	0.14	1.79		
25	250	383	0.38	0.71	1.02	0.09	0.00	0.12	0.13	2.45	3.74	1.83
25	250	383	0.98	1.45	1.87	0.20	0.03	0.09	0.42	5.03		
70	250	383	1.89	2.48	4.18	0.39	0.00	0.14	0.50	9.57	9.19	0.55
70	250	383	1.66	2.29	3.99	0.36	0.00	0.11	0.39	8.80		
240	250	383	3.24	4.59	7.78	0.72	0.06	0.18	0.75	17.30	15.44	2.63
240	250	383	2.49	3.35	6.58	0.51	0.00	0.12	0.53	13.58		
420	250	383	3.23	3.64	9.17	1.04	0.17	0.88	0.92	19.04	15.75	4.64
420	250	383	2.42	3.00	5.99	0.45	0.00	0.12	0.49	12.47		
1440	250	383	0.55	1.35	1.88	0.19	0.06	0.14	0.40	4.57	5.10	0.75
1440	250	383	0.77	1.50	2.32	0.23	0.08	0.19	0.55	5.63		
0	0	343	0.10	0.06	0.14	0.06	0.00	0.04	0.04	0.45	0.45	
240	0	343	0.07	0.15	0.20	0.05	0.06	0.05	0.06	0.64	0.51	0.18
240	0	343	0.04	0.08	0.12	0.03	0.04	0.03	0.04	0.38		
1440	0	343	0.03	0.00	0.02	0.00	0.00	0.00	0.00	0.05	0.09	0.06
1440	0	343	0.05	0.01	0.04	0.03	0.00	0.00	0.00	0.13		
25	250	343	0.20	0.32	0.45	0.11	0.07	0.07	0.08	1.28	1.35	0.09
25	250	343	0.22	0.36	0.49	0.11	0.07	0.07	0.10	1.42		
55	250	343	0.64	0.74	1.26	0.22	0.07	0.10	0.18	3.22	3.22	
420	250	343	2.22	3.15	5.90	0.41	0.19	0.31	0.68	12.87	12.87	
1440	250	343	0.71	1.56	2.21	0.39	0.38	0.39	0.58	6.22	6.22	
30	500	105	0.68	0.62	1.38	0.34	0.06	0.33	0.31	3.71	3.77	0.09
30	500	105	0.69	0.59	1.35	0.25	0.00	0.07	0.88	3.84		
60	500	105	0.37	0.31	0.89	0.13	0.00	0.00	0.11	1.82	2.03	0.30
60	500	105	0.45	0.38	1.08	0.17	0.00	0.00	0.17	2.24		
120	500	105	1.75	1.03	3.87	0.61	0.00	0.15	0.44	7.85	5.67	3.08
120	500	105	0.73	0.46	1.96	0.21	0.00	0.00	0.14	3.49		
240	500	105	2.46	1.34	5.49	0.68	0.00	0.19	0.50	10.66	10.53	0.19
240	500	105	2.46	1.28	5.30	0.62	0.08	0.09	0.57	10.39		
240	500	105	2.50	1.35	5.73	0.75	0.00	0.00	0.40	10.73	10.73	
390	500	105	7.98	3.64	19.21	1.57	0.00	0.29	1.59	34.28	36.19	2.71
390	500	105	8.77	4.18	22.05	1.63	0.00	0.00	1.47	38.11		
1470	500	105	0.36	0.31	1.97	0.21	0.00	0.00	0.17	3.02	4.19	1.65
1470	500	105	0.57	2.59	0.76	0.76	0.18	0.17	0.33	5.36		
1560	500	105	0.55	0.47	2.08	0.34	0.13	0.19	0.34	4.10	4.03	0.10
1560	500	105	0.52	0.45	1.97	0.31	0.17	0.20	0.33	3.96		

Table B.7 (continued)

Time	D_{in}	PL-250	Long-chain fatty acid									
min	μm	mg/l	16:0	18:2	18:1	18:0	10:0	14:0	16:1	Total	avg	std
			mg/l									
0	1000	254	0.04	0.03	0.08	0.04	0.00	0.00	0.00	0.19	0.19	
15	1000	254	0.27	0.18	0.48	0.16	0.00	0.00	0.10	1.18	1.11	0.10
15	1000	254	0.24	0.18	0.50	0.13	0.00	0.00	0.00	1.04		
30	1000	254	0.43	0.42	0.97	0.30	0.00	0.00	0.13	2.25	2.19	0.08
30	1000	254	0.47	0.38	0.87	0.27	0.00	0.03	0.12	2.13		
80	1000	254	0.46	0.36	0.85	0.21	0.00	0.00	0.13	2.02	1.86	0.22
80	1000	254	0.38	0.30	0.75	0.18	0.00	0.00	0.09	1.71		
120	1000	254	0.80	0.42	1.38	0.27	0.09	0.24	0.20	3.40	3.01	0.54
120	1000	254	0.70	0.23	1.03	0.24	0.00	0.21	0.22	2.63		
225	1000	254	1.07	0.94	2.16	0.32	0.00	0.45	0.39	5.33	7.12	4.68
225	1000	254	0.78	0.61	1.52	0.25	0.00	0.12	0.33	3.61		
240	1000	254	3.15	1.85	4.90	2.12	0.00	0.00	0.42	12.43		
420	1000	254	0.69	0.72	1.81	0.24	0.29	0.18	0.35	4.26	4.17	0.14
420	1000	254	0.64	0.87	1.56	0.25	0.11	0.27	0.37	4.07		
1380	1000	254	0.70	0.43	1.84	0.49	0.04	0.12	0.15	3.78	3.46	0.44
1380	1000	254	0.78	0.29	1.25	0.35	0.10	0.19	0.19	3.15		
1500	1000	254	0.81	0.42	1.76	0.29	0.14	0.28	0.18	3.86	5.32	2.06
1500	1000	254	1.23	0.86	3.66	0.71	0.00	0.10	0.22	6.77		
20	1000	53	0.37	0.31	0.64	0.14	0.00	0.00	0.30	1.74	2.38	0.90
20	1000	53	0.61	0.59	1.08	0.25	0.00	0.09	0.39	3.01		
60	1000	53	0.31	0.25	0.58	0.18	0.06	0.06	0.14	1.59	1.51	0.12
60	1000	53	0.23	0.29	0.49	0.09	0.04	0.06	0.23	1.42		
120	1000	53	0.38	0.49	1.09	0.20	0.00	0.08	0.31	2.55	2.55	
240	1000	53	0.27	0.26	0.85	0.14	0.00	0.05	0.14	1.71	2.10	0.55
240	1000	53	0.43	0.36	1.07	0.21	0.06	0.06	0.30	2.49		
240	1000	53	4.21	1.09	7.98	0.84	0.09	0.29	0.72	15.21	14.39	1.16
240	1000	53	3.84	1.00	7.32	0.66	0.00	0.20	0.56	13.57		
390	1000	53	0.69	0.32	1.75	0.17	0.00	0.00	0.17	3.10	4.37	1.80
390	1000	53	1.20	0.49	2.71	0.45	0.21	0.21	0.36	5.64		
1410	1000	53	0.59	0.25	1.65	0.20	0.08	0.11	0.15	3.03	2.60	0.61
1410	1000	53	0.37	0.17	1.21	0.13	0.05	0.08	0.15	2.17		
1500	1000	53	0.74	0.25	1.61	0.28	0.15	0.18	0.27	3.48	3.06	0.59
1500	1000	53	0.45	0.19	1.21	0.44	0.08	0.12	0.16	2.64		

Table B.8 Estimates of parameters for Eq. 4.5 on the release of free LCFAs in solution of slaughterhouse wastewater containing approximately 2.5 g/l of pork fat particles

a. Parameter a

D_{in}	PL-250	estimate	
μm	mg/l	min^{-1}	d^{-1}
53	1000	0.0011	1.6
67	250	0.0023	3.3
105	500	0.0015	2.2
114	250	0.0017	2.5
128	250	0.0020	2.8
146	150	0.0013	1.9
340	150	0.0029	4.2
343	250	0.0025	3.6
383	250	0.0017	2.4
	average	0.0019	2.72
	STD	0.0006	0.83
	CV (%)	30.71	30.71

b. Parameters k_h' and k_x (with a =2.72 day^{-1})

D_{in}	PL-250	k_h estimate		k_x estimate	
μm	mg/l	min^{-1}	d^{-1}	min^{-1}	d^{-1}
53	1000	0.0408	59	0.00247	3.56
67	250	0.0796	115	0.00247	3.56
105	500	0.1120	161	0.00225	3.24
112	125	0.0407	59	0.00124	1.79
121	250	0.1150	166	0.00286	4.12
146	150	0.0865	125	0.00186	2.68
254	1000	0.0274	39	0.00130	1.87
340	150	0.0495	71	0.00169	2.43
363	250	0.1010	145	0.00298	4.29

Table B.9 Free LCFAs in filtered samples of slaughterhouse wastewater containing approximately 2.5 g/l of beef fat particles and mixed for various time periods with PL-250

Time	Long-chain fatty acid									
min	16:0	18:2	18:1	18:0	10:0	14:0	16:1	Total	avg	std
	mg/l									
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.61	1.15
0	0.04	0.00	0.08	0.00	0.00	0.00	0.00	0.12		
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
0	0.56	0.22	1.00	0.00	0.17	0.19	0.21	2.34		
10	0.07	0.05	0.10	0.08	0.03	0.05	0.05	0.44	0.57	0.18
10	0.11	0.05	0.19	0.08	0.11	0.08	0.07	0.69		
15	0.40	0.46	1.02	0.12	0.14	0.19	0.47	2.80	2.51	0.41
15	0.35	0.34	0.78	0.09	0.14	0.15	0.35	2.21		
30	0.18	0.13	0.46	0.12	0.00	0.01	0.21	1.12	1.29	0.25
30	0.19	0.16	0.48	0.14	0.10	0.16	0.23	1.47		
40	0.75	0.64	1.89	0.30	0.57	0.79	0.63	5.57	4.62	1.35
40	0.57	0.49	1.46	0.17	0.20	0.24	0.54	3.67		
80	0.22	0.25	0.43	0.07	0.11	0.17	0.25	1.50	2.33	1.18
80	0.46	0.40	1.07	0.16	0.46	0.27	0.36	3.17		
90	0.60	0.61	1.91	0.15	0.00	0.25	0.42	3.95	3.70	0.35
90	0.50	0.51	1.66	0.11	0.13	0.17	0.37	3.46		
240	0.85	0.63	2.90	0.25	0.55	0.47	0.59	6.23	6.99	1.08
240	1.03	0.73	3.49	0.27	0.80	0.58	0.85	7.75		
240	2.58	1.18	6.04	0.41	0.55	0.62	0.99	12.38	11.59	1.12
240	2.31	1.35	5.77	0.34	0.00	0.32	0.71	10.80		
430	5.02	1.96	11.68	0.70	0.50	0.91	1.34	22.12	23.35	1.74
430	5.74	2.39	14.17	0.75	0.00	0.25	1.29	24.58		
450	2.65	1.02	7.26	0.48	0.38	0.62	1.07	13.48	14.33	1.20
450	3.02	1.14	8.14	0.70	0.31	0.72	1.15	15.17		
1470	0.49	0.54	1.69	0.20	1.02	0.69	0.59	5.22	4.76	0.65
1470	0.43	0.41	1.22	0.12	0.91	0.54	0.65	4.30		
1500	0.45	0.76	1.99	0.17	1.39	1.26	1.10	7.12	6.77	0.49
1500	0.33	0.65	1.69	0.13	1.47	1.09	1.06	6.42		

Appendix C

Appendix C presents graphs and tables that were not included in the original text of Chapters 5 and 6. Following is a description of the tables and figures:

- Tables C.1 to C.6 present background work that served to validate the method used to extract total fat, neutral fat and free LCFA.
- Table C.7 gives the particle size distribution in the fat solution used in Chapters 5 and 6. These data were used to built Figure 5.1. Table C.8 describes the substrates fed to the anaerobic sequencing batch reactors.
- All fat data are presented in Tables C.9 to C.13. Table C.9 gives fat distribution in sludge samples collected before each experiment. These data were used to build Figure 5.3. Average fraction of neutral fat in VSS for each experiment was used for parameter f_{ns} (Eq. 5.1). Table C.10 present total solids, total volatile solids and fat concentration in sludge samples collected throughout the experiments. Table C.11 presents fat distribution in the substrates. These results were used for Tables 5.1 and 6.3. Table C.12 gives concentration of neutral fat and LCFA in ASBR mixed-liquor samples for each experiment described in Chapters 5 and 6. Data from Table C.12 were used to build Figures 5.4, 5.6, 5.7 and 6.3. They were also used to calculate neutral fat hydrolysis and LCFA oxidation. Table C.13 presents free LCFA distribution in mixed-liquor samples.
- Tables C.14 to C.19 presents data and analyses used to model neutral fat hydrolysis and LCFA oxidation rates. The non-linear least squares summary statistics for parameter estimates for first-order neutral fat hydrolysis equation (Eq. 5.2) and

LCFA oxidation (Eq. 5.4) are presented in Tables C.14 and C.17, respectively. Data from Table C.16 were used to estimate parameters for Equation 5.4. Calculations for statistical significance of Eqs. 5.2 and 5.4 are shown in Tables C.15 and C.18, respectively. Statistical significance was evaluated using Eq. 3.3. Table C.19 gives the values used to built the simulation curves shown on Figure 6.3. It is preceded by an explanation of how each value and parameter was obtained.

- Tables C.20 and C.21 present SCOD and VFA concentrations measured in the second cycle of some of the experiments.
- Table C.22 presents methane content in the biogas from the ASBRs. Table C.23 and Figures C.1 to C.5 gives the production of methane from each bioreactors in the second and third cycle of each experiment, and Table C.24 presents the total transformation of input TCOD in methane during the 3-d ASBR cycle.
- Table C.25 and C.26 present methane production from ASBRs fed PL-250 dissolved in tap water and FSHW, respectively.

Table C.1 Fat content in slaughterhouse wastewater prior to the initiation of the experiment

Date	Total fat (mg/l)
21/1/00	512
27/1/00	488
17/2/00	310
17/2/00	330
Avg.	410
STD	104.865
CV (%)	26

Table C.2 Fat extracted vs number of ether extractions

Sample id.	Number of extraction 2	3	4
	Fat concentration (mg/l)		
1	1601	1841	
2	2166	2481	
3	1145	2208	2142
4	1776	2053	1989

Table C.3 Total fat recovery with ether extraction method

Medium	Fat added mg/l	Fat extracted mg/l	Recovery %
Water	846	813	96.1
Water	846	842	99.5
Water	387	451	116.6
Water	387	387	99.9
Water	123	96	77.7
Water	133	163	122.4
FSHW	183	163	89.2
FSHW	433	440	101.6
		average	100.4
		STD	14.2

FSHW is filtered slaughterhouse wastewater

Table C.4 COD content of fat particles added to the filtered slaughterhouse wastewater

Fat particle (mg/l)	SCOD (mg/l)	TCOD (mg/l)	mg COD/ mg gras
419	13.8	1061	2.53
419	0.0	1046	2.50
670		1643	2.45
716		1734	2.42
828	17.6	2259	2.73
828	27.5	1913	2.31
		Avg.	2.49
		STD	0.14

Table C.5 Efficiency of the column in retaining total fat.

Sample volume ml	First elution mg	Neutral fat in sample mg	First elution to neutral fat %
4.1	-0.2	5.4	-3.70
5.4	0.5	6.8	7.33
3.8	0.0	6.7	0.00
5.6	0.3	7.7	3.88
5.6	-0.2	11.0	-1.82
1.0	0.1	14.8	0.68
4.6	0.2	8.2	2.44
5.1	0.3	7.6	3.96
4.4	0.1	8.0	1.25
5.1	0.2	8.0	2.49
2.3	-0.1	6.5	-1.53
2.5	0.3	5.8	5.15
2.7	0.1	6.6	1.51
3.1	0.1	8.5	1.18
1.2	0.0	11.5	0.00
average	0.11		1.52
STD	0.20		2.85

First elution is the eluate that was collected from the first passage of the extracted fat resuspended in hexane in the Bond Elut column

Table C.6 Recovery of free LCFAs in samples spiked with LCFAs, passed through the Bond Elut columns, methylated and analysed on the GC

LCFA Type	g/l	Area/μg LCFA[1] standard	Area/μg LCFA[1] bond elut	Recovery %
14:0	1.60	247394.4	205563	83.09
16:0	0.98	268319.8	224291	83.59
18:0	0.79	309542.2	259087	83.70
overall	3.36	275085.5	229647	83.48
14:0	1.60	247394.4	234761	94.89
16:0	0.98	268319.8	262772	97.93
18:0	0.79	309542.2	306116	98.89
overall	3.36	275085.5	267883	97.38
14:0	0.85	280557.4	272665	97.19
16:0	1.01	318412	309463	97.19
18:0	1.09	336505.2	325884	96.84
overall	2.95	311825	302671	97.06

1 Area on GC chromatogram of standards and samples spiked with LCFAs, passed through Bond Elut columns and methylated

Table C.7 Pork fat particle distribution in the solutions used in the 5 experiments described in Chapters 5 and 6

Avg. size	Initial average particle size (μm)									
	60	112	175	213	452	60	112	175	213	452
μm	Particle conc. (mg/l)					% of total weight				
1	0.01	0.01	0.01	0.00	0.01	0.4	1.8	0.5	0.0	0.7
10	0.68	0.19	0.25	0.24	0.06	49.2	30.4	16.2	16.0	4.2
53	0.52	0.22	0.47	0.34	0.26	37.9	34.8	30.5	22.8	17.3
114	0.12	0.07	0.34	0.27	0.12	8.4	11.5	22.2	18.2	7.9
292	0.03	0.04	0.16	0.25	0.22	2.2	6.7	10.5	16.6	14.1
526	0.02	0.04	0.13	0.19	0.39	1.2	6.3	8.3	12.6	25.6
925	0.01	0.05	0.18	0.20	0.46	0.9	8.4	11.9	13.8	30.1
Total	1.38	0.63	1.55	1.48	1.52	100	100	100	100	100

Table C.8 Characteristics of the substrates fed to the anaerobic sequencing batch reactors

D_n	Fat (mg/L)			TCOD (mg/L)			SCOD (mg/L)			SS (mg/L)			VSS (mg/L)		
μm	FSHW	FSHW +F	FSHW +FE	FSHW	FSHW +F	FSHW +FE	FSHW	FSHW +F	FSHW +FE	FSHW	FSHW +F	FSHW +FE	FSHW	FSHW +F	FSHW +FE
110	213	751		3530	5229		2638	2546		489	1004		460	996	
	186	719		3308	5132		2299	2569		450	1031		437	1024	
	106	746					2277	2186							
Avg.	169	739		3419	5181		2405	2434		470	1018		448	1010	
STD	56	17		157	68		203	215		28	19		16	20	
175	141	730	666	3547	4778	4876	2165	2100	2249	408	1032	966	404	1002	955
	175	732	704	3625	5020	4845	2401	2220	2453	450	1091	1054	432	1042	983
	142	836		3220											
Avg.	153	766	685	3464	4899	4861	2283	2160	2351	429	1062	1010	418	1022	969
STD	19	61	27	215	171	22	167	85	144	30	42	62	20	28	20
215	169	804	774	3248	4216	4041	2151	2277	2457	332	698	688	312	672	642
	124	688	690	2913	4186	5022	2311	2138	2328	291	743	786	259	710	731
Avg.	146	746	732	3081	4201	4531	2231	2208	2393	312	721	737	286	691	687
STD	32	82	59	237	22	693	113	98	91	29	32	69	37	27	63
450	137	604	775	2822	4434	3955	2164	2509	2263	271	701	826	271	675	748
	254	725	604	2776	3540	4145	2009	2066	2496	296	884	890	281	822	
Avg.	195	664	689	2799	3987	4018	2086	2288	2379	284	793	858	276	749	748
STD	83	86	121	33	632	134	109	313	165	18	129	45	7	104	
60	193	690	737	3532	4682	4945	2715	2727	2895	486	817	730	468	833	711
	122	660	690	3196	4386	4531	2526	2312	2919	406	794	664	378	789	621
	115			3038			2210			451			405		
Avg.	143	675	714	3255	4534	4738	2484	2520	2907	448	806	697	417	811	666
STD	43	21	33	252	209	293	255	293	17	40	16	47	46	31	64
Overall averages and STD for the 5 experiments described in (1) Chapter 5 and the 4 experiments described in (2) Chapter 6															
(1) Avg.	160	724		3230	4560		2322	2332		394	880		373	857	
(1) STD	43	61		295	516		212	221		82	149		79	148	
(2) Avg.	157	719	705	3192	4405	4477	2295	2294	2507	377	845	825	357	818	770
(2) STD	42	71	57	306	451	426	219	225	263	80	148	137	77	141	144

Table C.9 Fat distribution in the sludge bed at the beginning of each experiment

Exp.	D_m	Lipids (mg/l)				VSS	as a fraction of VSS (g/g)				as a fraction of total fat (g/g)		
no.	µm	Total	Neutral	LCFA	Other	mg/l	Total	Neutral	LCFA	Other	Neutral	LCFA	Other
1	110	2167	1500	10	657	21850	0.099	0.069	0.000	0.030	0.692	0.005	0.303
1	110	1047	647	26	374	9019	0.116	0.072	0.003	0.041	0.618	0.025	0.357
1	110	1193	605	26	562	9295	0.128	0.065	0.003	0.060	0.507	0.022	0.471
2	175	939	554		385	7429	0.126	0.075		0.052	0.590	0.000	0.410
2	175	921	498		423	7772	0.119	0.064		0.054	0.541	0.000	0.459
3	215	800	302	40	458	6200	0.129	0.049	0.006	0.074	0.378	0.050	0.573
3	215	769	328	38	403	6350	0.121	0.052	0.006	0.063	0.427	0.049	0.523
4	450	920	351	74	495	5565	0.165	0.063	0.013	0.089	0.382	0.081	0.538
4	450	947	400	51	495	5625	0.168	0.071	0.009	0.088	0.423	0.054	0.523
4	450	972	429		543	5460	0.178	0.079		0.100	0.441	0.000	0.559
4	450	913	376	68	469	6127	0.149	0.061	0.011	0.077	0.412	0.075	0.513
4	450	775	407	97	271	5716	0.136	0.071	0.017	0.047	0.526	0.125	0.350
4	450	910	328	40	541	5967	0.152	0.055	0.007	0.091	0.361	0.044	0.595
4	450	824	365	22	437	5583	0.148	0.065	0.004	0.078	0.443	0.026	0.531
4	450	750	415	20	316	5765	0.130	0.072	0.003	0.055	0.553	0.026	0.421
5	60	2167	1292	27	848	15153	0.143	0.085	0.002	0.056	0.596	0.013	0.391
5	60	2033	1117	12	904	15227	0.134	0.073	0.001	0.059	0.549	0.006	0.445
5	60	1974	1179	84	712	15238	0.130	0.077	0.006	0.047	0.597	0.043	0.360
		Averages											
all		1168	616	42	516	8852	0.137	0.068	0.006	0.065	0.502	0.036	0.462
1	110	1469	917	21	531	13388	0.115	0.068	0.002	0.044	0.606	0.017	0.377
2	175	930	526		404	7601	0.122	0.069		0.053	0.565		0.435
3	215	785	315	39	430	6275	0.125	0.050	0.006	0.069	0.402	0.050	0.548
4	450	876	384	53	446	5726	0.153	0.067	0.009	0.078	0.442	0.054	0.504
5	60	2058	1196	41	821	15206	0.135	0.079	0.003	0.058	0.581	0.020	0.399
		STD											
all		517	379	27	171	4761	0.020	0.009	0.005	0.019	0.097	0.033	0.087
1	110	609	505	9	144	7330	0.015	0.003	0.001	0.015	0.093	0.011	0.086
2	175	12	39		27	242	0.006	0.007		0.002	0.035		0.035
3	215	22	19	1	39	106	0.006	0.002	0.000	0.007	0.035	0.000	0.035
4	450	82	35	28	101	223	0.016	0.007	0.005	0.018	0.066	0.039	0.080
5	60	99	89	38	99	46	0.007	0.006	0.003	0.007	0.027	0.020	0.043

Table C.10 Sludge bed characteristics at different periods during the experiment

D_{in} µm	before cycle	ASBR	total fat (mg/l) FSHW	FSHW +F	FSHW +FE	total solids mg/L FSHW	FSHW +F	FSHW +FE	Volatile solids mg/l FSHW	FSHW +F	FSHW +FE	Total fat/STV (%) FSHW	FSHW +F	FSHW +FE
60	1	1	2201	2281	2194	20352	20759	20823	15153	15073	15216	14.53	15.14	14.42
60	1	2	2174	2092	2160	20180	19206	17894	15227	14489		14.28	14.43	
60	2	1	2167	1974	2221	18718	20181	19924	14785	15238	15153	14.66	12.96	14.66
60	2	2	2033	1892	1980	18019	18163	18753	13872	14035	14350	14.66	13.48	13.80
60	3	1				18226	19010	19096	14120	14735	14815			
60	3	2				18567	17504	18991	14189	13718	14668			
60	4	1	2065	2507	2293	17548	18381	18396	13549	14228	13925	15.24	17.62	16.47
60	4	2	1860	2258	2331	15780	17173	17889	12172	13397	13925	15.28	16.86	16.74
110	1	1	2400	2350		26760	29364		20485	20768		11.72	11.32	
110	1	2	2400	2167		27082	27915		21051			11.40		
110	2	1				25041	20638		19463	16206				
110	2	2				22982	21867		18417	17500				
110	3	1		1664		23122	19394		17692	14920			11.15	
110	3	2		1845		21675	19139		16982	15278			12.08	
110	4	1	1523	1509		17379	15871		13206	12117		11.53	12.45	
110	4	2	1526	1689		16737	18307		12861	14135		11.86	11.95	
175	1	1	2045	1930	2086	22700	20634	22143	16670	15750	17226	12.27	12.25	12.11
175	1	2	2248		2483	23695	23664	24353	17995	17509	18280	12.49		13.59
175	2	1	1726	1752	2067	20718	16866	19526	15548	15548	15358	11.10	11.27	13.46
175	2	2	2000	2038	2218	22218	21766	23381	17173	16345	17680	11.65	12.47	12.55
175	3	1				16320	20261	17965	12124	15137	13624			
175	3	2				20449	19651	22966	15367	14453	17018			
215	1	1	1815	1728	1859	19875	19566	20466	14564	14593	15104	12.47	11.84	12.31
215	1	2	1992	2067	1817	19615	20296	19545	15005	15424	14689	13.28	13.40	12.37
215	2	1				19961	18172	18336	14836	13876	14005			
215	2	2				19881	19106	18435	15099	14617	14202			
215	3	1	1881	1881	1631	18415	18246	17515	13887	13981	13570	13.55	13.46	12.02
215	3	2	1742	1742	1672	19108	18343	16859	14510	14066	13064	12.00	12.38	12.80
450	1	1	1714	1819	1860	15368	15634	16306	11576	11571	12269	14.81	15.72	15.16
450	1	2	1973	1714	1950	16337	14894	16160	12504	11331	12420	15.78	15.13	15.70
450	2	1				17353	16516	16204	13043	12588	12540			
450	2	2				16203	16267	15042	12477	12620	11617			
450	3	1	1917	2123		16424	16264	16367	12532	12478	12661	15.29	17.01	
450	3	2	1950	2053	2000	15858	16040	15312	12194	12490	11772	15.99	16.44	16.99
			Average											
60			2083	2168	2196	18424	18797	18971	14133	14364	14579	14.77	15.08	15.22
110			1962	1871		22597	21562		17520	15846		11.63	11.79	
175			2005	1907	2213	21016	20474	21722	15813	15790	16531	11.88	12.00	12.92
215			1858	1854	1745	19476	18955	18526	14650	14426	14106	12.82	12.77	12.37
450			1889	1927	1937	16257	15936	15899	12388	12180	12213	15.47	16.08	15.95
overall			1971	1960	2048	19667	19266	18794	15010	14552	14366	13.45	13.67	14.07
60			128	226	123	1458	1246	1004	994	644	533	0.40	1.85	1.31
110			506	324		3896	4717		3081	2742		0.20	0.54	
175			284	160	192	2876	2223	2463	2187	1146	1728	0.54	0.52	0.71
215			106	158	110	606	859	1315	440	581	739	0.71	0.79	0.32
450			118	193	71	662	590	570	483	572	425	0.53	0.82	0.94
overall			241	250	235	3119	3101	2450	2457	1870	1790	1.63	2.04	1.72

Table C.11 Fat distribution in the substrates used for Experiments 1 (D_{in} = 110 μm) and 5 (D_{in} = 60 μm)

D_{in} μm	Substrate	Type of fat Total	Neutral	LCFA	Other	% of total fat Neutral	LCFA	Other
60	FSHW	217	110	84	23	0.51	0.39	0.11
60	FSHW	169	68	61	40	0.40	0.36	0.24
60	FSHW			91				
60	FSHW+F	714	457	55	202	0.64	0.08	0.28
60	FSHW+F	762	509	108	144	0.67	0.14	0.19
60	FSHW+FE	737	342	244	151	0.46	0.33	0.21
60	FSHW+FE	690	280	246	164	0.41	0.36	0.24
110	FSHW	213		99				
110	FSHW+F	751		83				
Overall								
Avg.	FSHW	200	89	81	29.28	0.45	0.37	0.15
	FSHW+F	742	483	82	176.99	0.65	0.11	0.24
	FSHW+FE	714	311	245	157.87	0.43	0.34	0.22
STD	FSHW	27	30	19	12	0.08	0.02	0.09
	FSHW+F	25	37	27	41	0.02	0.05	0.07
	FSHW+FE	33	44	2	9			
For Experiment 5 (D_{in} = 60 μm)								
Avg.	FSHW	193	89	79	32	0.45	0.37	0.17
	FSHW+F	738	483	82	173	0.65	0.11	0.24
	FSHW+FE	714	311	245	158	0.43	0.34	0.22
STD	FSHW	34	30	16	12	0.08	0.02	0.09
	FSHW+F	34	37	38	41	0.02	0.05	0.07
	FSHW+FE	33	44	2	9	0.04	0.02	0.02

Table C.12 Neutral fat and LCFA concentrations in the mixed-liquor samples of ASBRs fed FSHW, FSHW+F and FSHW+FE substrates

a) D_{in} = 110 µm — f_{ns} = 0.069

Substrate			Type of fat (mg/l)			
	ASBR	Time (h)	Neutral all[1]	Neutral substrate[2]	LCFA	VSS (g/l)
FSHW+F	14	3	961	298	67	9652
FSHW+F	14	22	789	145	97	9374
FSHW+F	14	46	659	41		8998
FSHW+F	14	0	737	275	71	6728
FSHW+F	14	7	654	191	87	6737
FSHW+F	14	25	629	136	97	7175
FSHW+F	14	49	554	57	40	7233
FSHW+F	16	0	915	243	57	9786
FSHW+F	16	3			72	9558
FSHW+F	16	7	861	206		9537
FSHW+F	16	22	856	201	90	9537
FSHW+F	16	0	849	288		8165
FSHW+F	16	7	800	268	65	7745
FSHW+F	16	25			132	7780
FSHW+F	16	49	581	47	43	7780

b) D_{in} = 175 µm — f_{ns} = 0.064

treat	ASBR	Time (h)	Type of fat (mg/l) Neutral all[1]	Neutral substrate[2]	LCFA	VSS (g/l)
FSHW+F	13	0	768	311		7137
FSHW+F	21	0	734	270	251	7245
FSHW+F	21	3	783	320	198	7245
FSHW+F	13	7	576	120	232	7133
FSHW+F	21	7	671	154	182	8070
FSHW+F	13	18			233	6932
FSHW+F	21	18	597	105	296	7682
FSHW+F	13	42			188	6635
FSHW+F	21	42	638	144	175	7715
FSHW+F	13	72	490	59		6725
FSHW+F	21	72	554	78	133	7429
FSHW+FE	15	0	734	262		7367
FSHW+FE	20	0	678	152		8216
FSHW+FE	15	3	786	326	226	7177
FSHW+FE	20	3	662	122	266	8430
FSHW+FE	15	7	659	206		7077
FSHW+FE	20	7	742	213	270	8263
FSHW+FE	15	18	576	108	264	7315
FSHW+FE	20	18	552	35	251	8082
FSHW+FE	15	42	630	180	227	7029
FSHW+FE	20	42			262	7918
FSHW+FE	15	72	546	117	142	6698

Table C.12 (continued)

c) D_{in} = 215 μm f_{ns} = 0.050

treat	ASBR	Time (h)	Type of fat (mg/l) Neutral all[1]	Neutral substrate[2]	LCFA	VSS (g/l)
FSHW+F	13	0	583	276	49	6135
FSHW+F	13	3			88	6233
FSHW+F	13	8	517	203		6271
FSHW+F	13	19	406	94	84	6251
FSHW+F	13	46	409	106	56	6060
FSHW+F	20	0	706	360	50	6914
FSHW+F	20	3	582	243	83	6777
FSHW+F	20	8	595	248	80	6944
FSHW+F	20	19	478	148	63	6603
FSHW+F	20	46	407	79	55	6563
FSHW+FE	15	0	481	142	166	6793
FSHW+FE	15	3	538	220	152	6351
FSHW+FE	15	8	461	137		6496
FSHW+FE	15	19	352	26	129	6524
FSHW+FE	15	46	397	85	62	6252
FSHW+FE	16	0	467	157	184	6212
FSHW+FE	16	3	524	201	167	6462
FSHW+FE	16	8	493	171	116	6432
FSHW+FE	16	19	397	74	105	6452
FSHW+FE	16	46	395	71	112	6473

d) D_{in} = 450 μm f_{ns} = 0.067

treat	ASBR	Time (h)	Type of fat (mg/l) Neutral all[1]	Neutral substrate[2]	LCFA	VSS (g/l)
FSHW	14	0	351		74	5565
FSHW	22	0	400	22	51	5625
FSHW	14	6	429	62	68	5460
FSHW	14	18	407	23	97	5716
FSHW	22	18			40	5967
FSHW	14	44	365		22	5583
FSHW	22	44	415	27	20	5765
FSHW+F	13	0			164	5418
FSHW+F	16	0	656	287	159	5498
FSHW+F	13	6	617	232	159	5727
FSHW+F	16	6	700	320	107	5642
FSHW+F	13	18	560	186	268	5559
FSHW+F	16	18	610	223	130	5746
FSHW+F	16	44	520	141	149	5645
FSHW+F	16	44	423	44	109	5645
FSHW+FE	15	0	485	113	385	5537
FSHW+FE	23	0			220	5664
FSHW+FE	15	6	532	133	368	5944
FSHW+FE	15	6	506	106	372	5944
FSHW+FE	15	18	449	62	344	5761
FSHW+FE	23	18	451	71	264	5655
FSHW+FE	15	44	412	27	158	5720
FSHW+FE	23	44	451	53	218	5914

Table C.12 (continued)

e) D_{in} = 60 μm			f_{ns} =	0.079		
			Type of fat (mg/l)			
treat	ASBR	Time (h)	Neutral all[1]	Neutral substrate[2]	LCFA	VSS (g/l)
FSHW	15	0	524	49	31	6032
FSHW	15	6	580	102	14	6078
FSHW	15	24	552	78	14	6025
FSHW	15	44	533	55	6	6078
FSHW	21	6			21	
FSHW	21	24			25	
FSHW	21	44			6	
FSHW+F	14	0	759	278	67	6117
FSHW+F	14	0	791	310	84	6117
FSHW+F	14	6	725	223	115	6376
FSHW+F	20	6	725	205	99	6605
FSHW+F	14	24	674	171	135	6385
FSHW+F	20	24	635	136	123	6343
FSHW+F	14	44	692	192	58	6353
FSHW+F	20	44	654	155	92	6343
FSHW+F	20	66	48	27	36	260
FSHW+F	14	66	70	50	38	249
FSHW+FE	13	0	661	169	117	6244
FSHW+FE	16	0	582	95	147	6188
FSHW+FE	13	6	780	254		6679
FSHW+FE	16	6	612	97	180	6549
FSHW+FE	13	24	658	137	81	6613
FSHW+FE	16	24	658	138	196	6599
FSHW+FE	13	44	658	129	76	6712
FSHW+FE	16	44	600	105	55	6290
FSHW+FE	13	66	32	11	37	261
FSHW+FE	16	66	52	32	27	261

1 All neutral fat extracted in mixed-liquor samples

2 Neutral fat from the pork fat particles, calculated using Equation 5.1 with the f_{ns} reported above each section of the Table

Table C.13 LCFA distribution in mixed-liquor samples from ASBRs fed control (FSHW+F) and enzyme pretreated (FSHW+FE) substrates

a) Control substrate (FSHW+F)

D_{in}	ASBR	Time	Type of LCFAs						Type of LCFA				
µm		h	14:0	16:1	16:0	18:1	18:0	Total	14:0	16:1	16:0	18:1	18:0
			mg/l						% of total concentration				
110	14	3	0.00	0.99	20.55	29.18	15.97	66.69	0.00	1.48	30.81	43.75	23.95
110	14	7	0.00	1.37	26.38	37.99	21.51	87.25	0.00	1.57	30.23	43.54	24.66
110	14	22	0.00	1.53	30.13	48.62	16.36	96.64	0.00	1.58	31.17	50.31	16.93
110	14	0	0.00	0.68	18.02	37.19	15.60	71.49	0.00	0.95	25.21	52.02	21.82
110	14	22	0.00	1.25	25.46	55.80	14.56	97.07	0.00	1.29	26.23	57.48	15.00
110	14	46	0.00	0.37	10.94	23.27	5.86	40.45	0.00	0.92	27.04	57.54	14.50
110	16	0	0.00	1.03	16.32	39.16	0.00	56.51	0.00	1.82	28.88	69.30	0.00
110	16	3	0.00	0.68	20.79	31.13	19.83	72.43	0.00	0.94	28.70	42.98	27.38
110	16	25	0.00	0.59	26.64	46.02	16.64	89.88	0.00	0.66	29.64	51.20	18.51
110	16	49	0.00	0.93	27.55	43.51	16.43	88.42	0.00	1.05	31.16	49.20	18.58
110	16	0	0.00	2.21	34.15	77.31	19.31	132.98	0.00	1.66	25.68	58.13	14.52
110	16	7	0.00	0.80	17.73	35.96	10.83	65.33	0.00	1.23	27.14	55.05	16.58
110	16	25	0.00	2.12	33.54	77.56	19.20	132.41	0.00	1.60	25.33	58.57	14.50
110	16	49	0.00	0.59	12.97	23.39	6.50	43.45	0.00	1.36	29.84	53.84	14.96
175	13	7	0.00	3.83	62.36	121.12	44.24	231.54	0.00	1.65	26.93	52.31	19.11
175	13	18	0.00	4.47	64.34	129.32	35.01	233.14	0.00	1.92	27.60	55.47	15.02
175	13	42	0.00	2.74	60.10	97.26	27.41	187.50	0.00	1.46	32.05	51.87	14.62
175	21	0	0.00	4.75	70.51	129.61	45.65	250.53	0.00	1.90	28.15	51.74	18.22
175	21	3	0.00	3.21	52.80	101.89	40.18	198.08	0.00	1.62	26.66	51.44	20.28
175	21	7	0.00	2.62	47.30	102.16	30.26	182.34	0.00	1.44	25.94	56.03	16.60
175	21	18	0.00	6.10	78.06	166.12	46.18	296.45	0.00	2.06	26.33	56.04	15.58
175	21	42	0.00	2.23	56.20	88.26	28.11	174.80	0.00	1.28	32.15	50.49	16.08
175	21	72	0.00	2.21	43.42	67.58	20.21	133.43	0.00	1.66	32.54	50.65	15.15
215	13	0	0.00	0.00	14.01	20.48	14.43	48.92	0.00	0.00	28.64	41.87	29.49
215	13	3	0.00	0.00	25.51	41.08	21.45	88.05	0.00	0.00	28.97	46.66	24.37
215	13	19	0.00	0.00	23.80	39.39	20.80	84.00	0.00	0.00	28.34	46.89	24.77
215	13	46	0.00	0.00	12.99	30.08	12.84	55.91	0.00	0.00	23.24	53.79	22.97
215	20	0	0.00	0.00	11.46	21.50	17.47	50.43	0.00	0.00	22.73	42.63	34.64
215	20	3	0.47	0.00	23.10	37.38	21.80	82.75	0.57	0.00	27.92	45.17	26.34
215	20	8	0.00	0.00	22.74	37.52	20.22	80.48	0.00	0.00	28.25	46.62	25.13
215	20	19	0.00	0.00	14.66	35.04	13.50	63.20	0.00	0.00	23.20	55.44	21.36
215	20	46	0.00	0.00	16.35	28.15	10.89	55.38	0.00	0.00	29.51	50.83	19.66
450	13	0	0.88	2.11	40.64	88.74	31.19	163.56	0.54	1.29	24.84	54.25	19.07
450	13	6	1.37	0.33	68.93	117.73	43.64	232.01	0.59	0.14	29.71	50.74	18.81
450	13	44	0.88	0.45	49.61	71.15	27.26	149.35	0.59	0.30	33.22	47.64	18.25
450	16	0	0.00	1.55	43.81	75.57	38.50	159.42	0.00	0.97	27.48	47.40	24.15
450	16	18	0.00	0.94	38.87	52.32	37.53	129.66	0.00	0.73	29.98	40.35	28.95
450	16	44	0.00	0.00	33.81	51.50	24.30	109.60	0.00	0.00	30.85	46.98	22.17
60	14a	0	0.00	0.75	17.02	32.66	16.94	67.37	0.00	1.11	25.26	48.49	25.14
60	14	6	0.00	1.18	31.55	52.06	29.76	114.55	0.00	1.03	27.55	45.45	25.98
60	20	6	0.00	0.97	25.36	48.85	23.85	99.03	0.00	0.98	25.60	49.33	24.09
60	14	24	0.00	0.85	38.83	60.83	34.09	134.60	0.00	0.63	28.85	45.19	25.33
60	20	24	2.14	0.76	33.57	57.50	28.89	122.86	1.74	0.62	27.32	46.81	23.52
60	14	44	0.00	0.00	17.63	23.41	16.63	57.67	0.00	0.00	30.56	40.60	28.84
60	20	44	0.00	0.00	27.58	40.61	24.13	92.32	0.00	0.00	29.87	43.99	26.14
60	14	66	0.00	0.00	12.27	13.76	10.07	36.09	0.00	0.00	33.99	38.12	27.89
60	20	66	0.00	0.22	12.38	14.69	10.91	38.19	0.00	0.57	32.40	38.45	28.57

Table C.13 (continued)

D_{in} µm	ASBR	Time h	14:0 (mg/l)	16:1 (mg/l)	16:0 (mg/l)	18:1 (mg/l)	18:0 (mg/l)	Total (mg/l)	14:0 (% of total concentration)	16:1 (% of total concentration)	16:0 (% of total concentration)	18:1 (% of total concentration)	18:0 (% of total concentration)
175	15	3	0.00	4.82	61.21	121.59	38.87	226.49	0.00	2.13	27.03	53.68	17.16
175	15	18	0.00	4.10	74.68	142.45	42.83	264.06	0.00	1.55	28.28	53.94	16.22
175	15	42	0.00	3.26	69.27	121.39	33.58	227.50	0.00	1.43	30.45	53.36	14.76
175	20	7	0.00	4.83	69.33	151.44	43.99	269.59	0.00	1.79	25.72	56.17	16.32
175	20	18	0.00	4.02	68.51	138.33	40.04	250.91	0.00	1.60	27.31	55.13	15.96
175	20	42	0.00	4.04	77.13	137.29	43.29	261.75	0.00	1.54	29.47	52.45	16.54
175	20	72	0.00	1.90	53.69	79.70	6.51	141.81	0.00	1.34	37.86	56.20	4.59
215	15	0	0.00	0.00	36.08	96.83	32.96	165.87	0.00	0.00	21.75	58.38	19.87
215	15	3	0.00	0.00	31.18	89.68	31.62	152.48	0.00	0.00	20.45	58.81	20.74
215	15	19	0.00	0.00	24.15	70.85	33.79	128.79	0.00	0.00	18.75	55.01	26.24
215	15	46	0.00	0.00	18.28	27.87	16.19	62.34	0.00	0.00	29.32	44.70	25.97
215	16	0	0.00	1.66	34.97	113.31	34.24	184.19	0.00	0.90	18.99	61.52	18.59
215	16	3	0.14	0.00	31.71	105.30	30.33	167.48	0.08	0.00	18.94	62.87	18.11
215	16	8	0.00	0.00	26.85	66.71	22.32	115.88	0.00	0.00	23.17	57.57	19.26
215	16	19	0.00	0.00	23.49	54.86	26.24	104.58	0.00	0.00	22.46	52.45	25.09
215	16	46	0.14	0.00	34.07	54.22	23.13	111.57	0.13	0.00	30.54	48.60	20.74
450	15	0	2.96	5.04	104.38	209.12	63.04	384.53	0.77	1.31	27.14	54.38	16.39
450	15	6	2.50	5.24	92.48	212.81	55.30	368.32	0.68	1.42	25.11	57.78	15.01
450	15	6	2.40	5.44	89.75	217.08	57.10	371.78	0.65	1.46	24.14	58.39	15.36
450	15	18	2.50	3.98	92.50	187.78	57.48	344.24	0.73	1.16	26.87	54.55	16.70
450	15	44	1.13	0.00	55.01	72.48	29.66	158.28	0.72	0.00	34.75	45.79	18.74
450	23	0	1.21	2.89	53.24	125.49	36.78	219.61	0.55	1.32	24.24	57.14	16.75
450	23	18	1.28	2.29	72.11	134.61	53.45	263.73	0.49	0.87	27.34	51.04	20.27
450	23	44	1.17	0.04	65.23	108.89	42.77	218.10	0.54	0.02	29.91	49.93	19.61
60	13	0	0.79	0.77	32.34	51.13	31.97	117.00	0.68	0.66	27.64	43.70	27.32
60	13	24	0.00	0.00	39.40	41.24	0.00	80.64	0.00	0.00	48.86	51.14	0.00
60	13	44	0.00	0.00	24.90	26.46	24.77	76.14	0.00	0.00	32.70	34.76	32.54
60	13	44	0.00	0.33	29.25	29.57	27.19	86.35	0.00	0.39	33.88	34.25	31.49
60	16	0	0.00	1.26	39.60	69.04	36.63	146.52	0.00	0.86	27.03	47.12	25.00
60	16	44	0.00	29.38	0.00	25.86	0.00	55.25	0.00	53.19	0.00	46.81	0.00
60	16	24	0.00	0.99	58.70	80.97	54.98	195.64	0.00	0.51	30.00	41.39	28.10
60	13	6	0.00	0.53	16.36	24.82	18.01	59.71	0.00	0.88	27.39	41.57	30.15
60	16	6	0.00	1.49	52.23	75.45	51.06	180.22	0.00	0.82	28.98	41.86	28.33
60	13	66	0	1.051	13.86	14.47	11.88	41.26	0.00	2.55	33.59	35.06	28.80
60	16	66	0.453	0	7.867	10.4	9.495	28.21	1.61	0.00	27.88	36.85	33.66

Table C.14 Non-linear least squares summary statistics: Parameter estimates (Eq. 5.2) for first-order neutral fat hydrolysis during digestion in ASBRs operated at 25°C of control (FSHW+F) and enzyme pretreated (FSHW+FE) substrates

a) D_{in} = 60 μm, FSHW+F substrate

Source	DF	Sum of Squares	Mean Square	F Value	Approx Pr > F
Regression	2	312881	156440	291.20	<.0001
Residual	6	3223.3	537.2		
Uncorrected Total	8	316104			
Corrected Total	7	71104.0			

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
F0	282.5	13.6396	249.1	315.9
k	0.0291	0.00409	0.0191	0.0391

Approximate Correlation Matrix

	F0	k
F0	1.0000000	0.5132748
k	0.5132748	1.0000000

b) D_{in} = 110 μm, FSHW+F substrate

Source	DF	Sum of Squares	Mean Square	F Value	Approx Pr > F
Regression	2	532805	266402	255.22	<.0001
Residual	11	11482.1	1043.8		
Uncorrected Total	13	544287			
Corrected Total	12	103054			

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
F0	281.2	14.7718	248.7	313.7
k	0.0296	0.00449	0.0197	0.0395

Approximate Correlation Matrix

	F0	k
F0	1.0000000	0.5387111
k	0.5387111	1.0000000

Table C.14 (continued)

c) D_{in} = 175 μm, FSHW+F substrate

Source	DF	Sum of Squares	Mean Square	F Value	Approx Pr > F
Regression	2	320711	160356	36.50	0.0002
Residual	7	30751.8	4393.1		
Uncorrected Total	9	351463			
Corrected Total	8	80716.2			

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
F0	255.4	34.2106	174.6	336.3
k	0.0241	0.0108	-0.00149	0.0496

Approximate Correlation Matrix

	F0	k
F0	1.0000000	0.4860777
k	0.4860777	1.0000000

d) D_{in} = 215 μm, FSHW+F substrate

Source	DF	Sum of Squares	Mean Square	F Value	Approx Pr > F
Regression	2	279582	139791	131.83	<.0001
Residual	6	6362.3	1060.4		
Uncorrected Total	8	285944			
Corrected Total	7	42342.0			

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
F0	268.7	21.5448	216.0	321.4
k	0.0298	0.00652	0.0139	0.0458

Approximate Correlation Matrix

	F0	k
F0	1.0000000	0.6404542
k	0.6404542	1.0000000

Table C.14 (continued)

e) D_{in} = 450 µm, FSHW+F substrate

Source	DF	Sum of Squares	Mean Square	F Value	Approx Pr > F
Regression	2	334183	167091	82.03	0.0002
Residual	5	10184.4	2036.9		
Uncorrected Total	7	344367			
Corrected Total	6	51829.7			

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
F0	307.5	31.3140	227.0	388.0
k	0.0255	0.00719	0.00706	0.0440

Approximate Correlation Matrix

	F0	k
F0	1.0000000	0.6420846
k	0.6420846	1.0000000

f) D_{in} = 60 µm, FSHW+FE substrate

Source	DF	Sum of Squares	Mean Square	F Value	Approx Pr > F
Regression	2	148885	74442.6	41.30	0.0003
Residual	6	10815.8	1802.6		
Uncorrected Total	8	159701			
Corrected Total	7	40872.9			

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
F0	222.8	32.3435	143.6	301.9
k	0.0202	0.00604	0.00540	0.0350

Approximate Correlation Matrix

	F0	k
F0	1.0000000	0.6524158
k	0.6524158	1.0000000

Table C.14 (continued)

g) D_{in} = 175 μm, FSHW+FE substrate

Source	DF	Sum of Squares	Mean Square	F Value	Approx Pr > F
Regression	2	293221	146610	28.34	0.0004
Residual	7	36209.3	5172.8		
Uncorrected Total	9	329430			
Corrected Total	8	57641.6			

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
F0	218.4	33.4133	139.4	297.4
k	0.0184	0.0120	-0.0101	0.0469

Approximate Correlation Matrix

	F0	k
F0	1.0000000	0.4965413
k	0.4965413	1.0000000

h) D_{in} = 215 μm, FSHW+FE substrate

Source	DF	Sum of Squares	Mean Square	F Value	Approx Pr > F
Regression	2	193408	96703.8	74.32	<.0001
Residual	7	9108.4	1301.2		
Uncorrected Total	9	202516			
Corrected Total	8	21040.0			

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
F0	176.7	17.2114	136.0	217.4
k	0.0180	0.00733	0.000680	0.0354

Approximate Correlation Matrix

	F0	k
F0	1.0000000	0.5392801
k	0.5392801	1.0000000

Table C.14 (continued)

i) D_{in} = 450 μm, FSHW+FE substrate

Source	DF	Sum of Squares	Mean Square	F Value	Approx Pr > F
Regression	2	52700.9	26350.5	93.04	0.0001
Residual	5	1416.1	283.2		
Uncorrected Total	7	54117.0			
Corrected Total	6	8513.4			

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
F0	125.0	11.8862	94.4173	155.5
k	0.0282	0.00712	0.00986	0.0465

Approximate Correlation Matrix

	F0	k
F0	1.0000000	0.6371271
k	0.6371271	1.0000000

j) All D_{in}, FSHW+F substrate

Source	DF	Sum of Squares	Mean Square	F Value	Approx Pr > F
Regression	2	1790938	895469	523.59	<.0001
Residual	44	75251.6	1710.3		
Uncorrected Total	46	1866190			
Corrected Total	45	354837			

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
F0	275.9	10.3065	255.1	296.7
k	0.0264	0.00287	0.0206	0.0322

Approximate Correlation Matrix

	F0	k
F0	1.0000000	0.5618310
k	0.5618310	1.0000000

Table C.14 (continued)

k) All D_{in}, FSHW+FE substrate

Source	DF	Sum of Squares	Mean Square	F Value	Approx Pr > F
Regression	2	656521	328260	114.03	<.0001
Residual	31	89243.5	2878.8		
Uncorrected Total	33	745764			
Corrected Total	32	163888			

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
F0	186.9	14.9213	156.5	217.4
k	0.0207	0.00526	0.00997	0.0314

Approximate Correlation Matrix

	F0	k
F0	1.0000000	0.5584402
k	0.5584402	1.0000000

Table C.15 Testing for significance of Equation 5.2 for control (FSWH+F) and enzyme pretreated (FSHW+FE) substrates by comparing pure error with model error (Eq. 3.3)

a) FSHW+F

	D_{in} = 60 µm			D_{in} = 110 µm			D_{in} = 175 µm			D_{in} = 215 µm			D_{in} = 450 µm		
	time	Neutral	var*d.f	time	Neutral	var*d.f	time	Neutral	var*d.f	time	Neutral	var*d.f	time	Neutral	var*d.f
	h	fat		h	fat		h	fat		h	fat		h	fat	
		mg/l			mg/l			mg/l			mg/l			mg/l	
	0	278	512	0	243	1110	0	311	847.91	0	276				
	0	310		0	275		0	270		3	243		0.0	287	
	7	223	162	0	288		3	320		8	203	1013	5.6	232	3929
	7	205		3	298		7	120	593.94	8	248		5.6	320	
	23	171	612.5	7	191	3329	7	154		19	94	1458	17.6	186	684
	23	136		7	268		18	105		19	148		17.6	223	
	47	192	684.5	7	206		42	144		46	79	354	43.5	140	4705
	47	155		23	145	2484	66	59	182.35	46	106		43.5	43	
	66	27	264.5	23	201		66	78							
	66	50		25	136										
				46	41	130.98									
				49	57										
				49	47										
1. Sum pure variance (S_e)/d.f. S_e			447.1			881.8			541.4			941			3106
2. d.f. S_e			5			8			3			3			3
3. d.f. model			10			13			9			8			7
4. SS_{res} model			3223			11482			30751			6362			10184
5. (SS_{res}-SS_{Se})/(d.f.model.-d.f.S_e)			329.167			1475.9			7281.7			1179.3			433.41
6. Step 5/(Step 1/d.f. S_e)			0.73623			1.6737			13.45			1.2527			0.1396
7. f value (n-p-v,v,.05)			6.59			3.86			9.12			9.28			9.28
Significant?			yes			yes			no			yes			yes

Table C.15 (continued)

b) FSHW+FE

	D_{in} = 60 μm			D_{in} = 175 μm			D_{in} = 215 μm			D_{in} = 450 μm		
	time	Neutral	var*d.f	time	Neutral	var*d.f	time	Neutral	var*d.f	time	Neutral	var*d.f
	h	fat		h	fat		h	fat		h	fat	
		mg/l			mg/l			mg/l			mg/l	
	0	169		0	262	6050	0	142	113	0.0	113	
	7	95		0	152		0	157		5.6	133	365
	23	137	0.5	3	326	20808	3	220	181	5.6	106	
	23	138		3	122		3	201		17.6	62	41
	47	129	288	7	206	24.5	8	136	613	17.6	71	
	47	105		7	213		8	171		43.5	27	338
	66	11	220.5	18	108		19	74		43.5	53	
	66	32		42	180		46	85	98			
				66	117		46	71				
1. Sum pure variance (S_e)/d.f. S_e			170			8961			334.5			248
2. d.f. S_e			3			3			3			3
3. d.f. model			8			9			9			7
4. SS_{res} model			10815			36209			9108			1416
5. (SS_{res}-SS_{Se})/(d.f.model.-d.f.S_e)			3435			2332			2026			336.5
6. Step 5/(Step 1/d.f. S_e)			20.25			0.26			6.06			1.36
7. f value (n-p-v,v,.05)			6.59			9.12			9.28			9.28
Significant?			no			yes			yes			yes

Table C.15 (continued)

c) Pooled data

time h	FSHW+FE Neutral fat mg/l	Var	df	Var*df
0	169	2577	5	12887
0	262			
0	152			
0	142.0			
0	157			
0	113			
3	326	7057	3	21171
3	122			
3	201			
3	220			
5.6	133	365	1	365
5.6	106			
7	206	1995	4	7978
7	213			
7	254			
8	136			
8	171			
17.6	62	406	3	1219
17.6	71			
18	108			
19	74			
23	137	1	1	1
23	138			
42	58	1200	6	7200
43.5	27			
43.5	53			
46	85			
46	92			
47	129			
47	105			
66	11	3150	2	6301
66	32			
66	117			

time h	FSHW+FE Neutral fat mg/l	Var	df	Var*df
0	278	435	8	3484
0	310			
0	243			
0	275			
0	288			
0	311			
0	270			
0	276.1			
0.0	287			
3	320	1567	2	3133
3	298			
3	243			
5.6	232	3929	1	3929
5.6	320			
7	191	2006	8	16049
7	268			
7	120			
7	154			
7	223			
7	205			
7	206			
8	203			
8	248			
17.6	186	2957	4	11827
17.6	223			
18	105			
19	94			
19	148			
23	171	790	4	3160
23	136			
23	145			
23	201			
25	136			
42	144	2200	8	17601
43.5	140			
43.5	43			
46	79			
46	106			
46	41			
47	155			
49	57			
49	47			
66	27	454	3	1363
66	50			
66	59			
66	78			

	FSHW+FE (left)	FSHW+FE (right)
1. Sum pure variance (S_e)/d.f. S_e	2285	1593
2. d.f. S_e	25	38
3. d.f. model	33	46
4. SS_{res} model	89243	75251
5. $(SS_{res}-SS_{Se})$/(d.f.model.-d.f.S_e)	5354	2451
6. Step 5/(Step 1/d.f. S_e)	2.34	1.54
7. f value (n-p-v.v..05)	3.63	3.32
Significant?	yes	yes

Table C.16 Data used to evaluate LCFA oxidation (Equations 5.2 and 5.3)

D_{in}	ASBR	Time		LCFA				
				Sample[1]	L_{avg}[2]	$L_{p(t=t)}$[3]	$L_{ox(t=t)}$[3]	r_{ox}[2]
µm		h	d	mg/l	mg/l	mg/l	mg/l	mg/l/t
60	1	0	0.0	67	.	0.00	0	.
60	1	6	0.3	115	91	38.26	-10	.
60	1	24	1.0	135	125	120.48	52	3.48
60	1	44	1.8	58	97	179.56	189	6.77
60	1	66	2.8	36	47	218.98	250	2.79
60	2	0	0.0	84	.	0.00	0	.
60	1	6	0.3	99	92	38.26	23	3.69
60	2	24	1.0	123	111	120.48	81	3.25
60	2	44	1.8	92	108	179.56	172	4.48
60	2	66	2.8	38	65	218.98	265	4.25
110	1	0	0.0	71	.	0.00	0	.
110	1	3	0.1	67	69	26.38	30	9.12
110	1	7	0.3	87	77	52.59	37	1.69
110	1	23	0.9	97	92	137.49	111	4.77
110	1	25	1.1	97	92	148.51	123	4.08
110	1	49	2.0	40	69	214.13	245	5.31
110	2	0	0.0	57	.	0.00	0	.
110	2	7	0.3	65	61	51.43	43	6.36
110	2	23	0.9	90	78	137.49	104	3.85
110	2	25	1.1	132	99	148.51	74	1.62
110	2	47	1.9	88	100	210.47	179	4.97
110	2	49	2.0	44	78	214.13	227	6.65
175	1	0	0.0	250	.	0.00	0	.
175	1	7	0.3	232	241	39.17	57	8.26
175	1	18	0.8	233	233	90.15	107	4.47
175	1	42	1.8	188	211	162.55	225	4.89
175	2	0	0.0	251	.	0.00	0	.
175	2	3	0.1	198	225	16.35	69	25.22
175	2	7	0.3	182	190	39.17	108	9.31
175	2	18	0.8	296	239	90.15	45	.
175	2	42	1.8	175	236	162.55	239	8.06
175	2	74	3.1	133	154	212.45	330	2.85
215	1	0	0.0	49	.	0.00	0	.
215	1	3	0.1	88	69	23.59	-15	.
215	1	19	0.8	84	86	115.38	80	4.28
215	1	46	1.9	56	70	200.29	193	4.18
215	2	0	0.0	50	.	0.00	0	.
215	2	3	0.1	83	67	23.59	-9	.
215	2	8	0.3	80	82	55.47	25	7.47
215	2	19	0.8	63	72	115.38	102	6.96
215	2	46	1.9	55	59	200.29	195	3.44
450	1	0	0.0	164	.	0.00	0	.
450	1	6	0.2	159	161	40.85	45	8.12
450	1	18	0.7	268	213	111.37	7	.
450	1	44	1.8	149	208	206.42	221	4.62
450	2	0	0.0	159.00	.	0.00	0	.
450	2	6	0.2	107.00	133	40.85	93	16.64
450	2	18	0.7	130.00	119	111.37	140	3.95
450	2	44	1.8	109.00	120	206.42	256	4.48

1 As measured in samples (from Table C.12)
2 Term from Equation 5.4
3 Term From Equation 5.3

Table C.17 Non-linear least squares summary statistics: Parameter estimates (Eq. 5.4) for LCFA oxidation during digestion in ASBRs operated at 25°C of slaughterhouse wastewater containing fat particles

Source	DF	Sum of Squares	Mean Square	F Value	Approx Pr > F
Regression	2	832.8	416.4	102.10	<.0001
Residual	30	122.4	4.0784		
Uncorrected Total	32	955.2			
Corrected Total	31	130.3			

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
kmax	6.8386	1.5520	3.6689	10.0082
ks	34.5985	30.9126	-28.5331	97.7301

Approximate Correlation Matrix

	kmax	ks
kmax	1.0000000	0.9512764
ks	0.9512764	1.0000000

Table C.18 Testing for significance of Equation 5.4 by comparing pure error with model error (Eq. 3.3)

D_{in} µm	ASBR	time h	LCFA Sample[1] mg/l	L_{avg}[2] mg/l	r_{ox}[2] mg/l/d	Var[3]	df	Var*df
215	2	46	55	59	3.4	2.3	2	4.5
110	2	7	65	61	6.4			
60	2	66	38	65	4.2			
110	1	49	40	69	5.3	4.6	3	13.8
110	1	3	67	69	9.1			
215	1	46	56	70	4.2			
215	2	19	63	72	7.0			
110	1	7	87	77	1.7	5.3	4	21.4
110	2	23	90	78	3.8			
110	2	49	44	78	6.7			
215	2	8	80	82	7.5			
215	1	19	84	86	4.3			
60	1	6	99	92	3.7	2.9	5	14.4
110	1	23	97	92	4.8			
110	1	25	97	92	4.1			
60	1	44	58	97	6.8			
110	2	25	132	99	1.6			
110	2	47	88	100	5.0			
60	2	44	92	108	4.5	0.8	1	0.8
60	2	24	123	111	3.3			
450	2	18	130.00	119	4.0	0.3	2	0.5
450	2	44	109.00	120	4.5			
60	1	24	135	125	3.5			
175	2	74	133	154	2.9	13.9	1	13.9
450	1	6	159	161	8.1			
175	2	7	182	190	9.3	6.9	2	13.8
450	1	44	149	208	4.6			
175	1	42	188	211	4.9			
175	1	18	233	233	4.5	4.5	2	9.1
175	2	42	175	236	8.1			
175	1	7	232	241	8.3			
			a. var /d.f.			4.2		
			b. d.f. Se			22		
			3. number samples			32		
			4. SSresidual			122		
			5. (SSres-1.*2.)/(2.-2-3.)			3.8		
			6. compared			0.9		
			7. f value (n-p-v,v,.05)			3.5		

1 As measured in samples (from Table C.12)
2 Term from Equation 5.4
3 Since the model related r_{ox} to L_{avg}, the replicates were considered as samples having similar values of L_{agv}, that is these samples should have had similar oxidation rate (r_{ox})

Table C. 19 Values used to simulate fat degradation at each D_{in} for FSHW+F and FSHW+FE substrates

The different values of Table C.19 were obtained as follows :

1. N is neutral fat concentration and was calculated using equation 5.2 with the parameters evaluated with the FSHW+F runs.
2. $L_{p\,(t=1)}$ is the free LCFA production in one hour and was equal to the hydrolysed neutral fat in that hour. Using first-order equation :

$$\frac{dN}{dt} = L_{p(t=1)} = k_h N = k_h N_0 e^{-k_h t} \quad \text{(C.1)}$$

   where dN is the amount of hydrolysed neutral fat or LCFA produced (mg/l); dt is equal to the 1 h time period; N_0 is equal to the neutral fat concentration shown in the previous column (mg/l); and t is equal to 1 h.
3. $L_{r\,(t=-1)}$ is equal to $L_{r\,(t=t)}$ for the previous hour.
4. $L_{r\,(t=t)}$ is the free LCFA concentration remaining in the reactor at time t and was calculated using Equation 5.3 as follows :

$$L_{r(t=t)} = L_{(t=-1)} - L_{ox(t=1)} + L_{p(t=1)} \quad \text{(C.2)}$$

   Where $L_{ox\,(t=1)}$ is the amount of LCFA oxidised in 1 hour, calculated using Equation 5.4 and the parameters estimated using the FSHW+F runs.

Initial neutral fat and free LCFA concentrations for the simulation are found at 0 h in the column for N and $L_{t=-1}$, respectively. They corresponded to the values that provided the least amount of residual error between observed and modelled data.

Time	D_{in} = 110 µm, FSHW+F				D_{in} = 175 µm, FSHW+F				D_{in} = 215 µm, FSHW+F			
h	N mg/l	$L_{p(t=1)}$ mg/l	$L_{(t=-1)}$ mg/l	$L_{(t=t)}$ mg/l	N mg/l	$L_{p(t=1)}$ mg/l	$L_{(t=-1)}$ mg/l	$L_{(t=t)}$ mg/l	N mg/l	$L_{p(t=1)}$ mg/l	$L_{(t=-1)}$ mg/l	$L_{(t=t)}$ mg/l
0	269	6.88	68.0	70.4	274	7.01	241.0	242.0	263	6.72	68.0	70.2
1	262	6.70	70.4	72.5	267	6.82	242.0	242.9	256	6.55	70.2	72.2
2	255	6.53	72.5	74.4	260	6.65	242.9	243.6	250	6.38	72.2	74.0
3	249	6.36	74.4	76.1	253	6.48	243.6	244.0	243	6.22	74.0	75.5
4	242	6.19	76.1	77.6	247	6.31	244.0	244.4	237	6.05	75.5	76.9
5	236	6.03	77.6	78.9	240	6.14	244.4	244.5	231	5.90	76.9	78.1
6	230	5.88	78.9	80.1	234	5.99	244.5	244.5	225	5.74	78.1	79.1
7	224	5.72	80.1	81.0	228	5.83	244.5	244.4	219	5.60	79.1	80.0
8	218	5.58	81.0	81.8	222	5.68	244.4	244.1	213	5.45	80.0	80.7
9	212	5.43	81.8	82.5	216	5.53	244.1	243.6	208	5.31	80.7	81.2
10	207	5.29	82.5	83.0	211	5.39	243.6	243.0	202	5.17	81.2	81.6
11	202	5.15	83.0	83.3	205	5.25	243.0	242.3	197	5.04	81.6	81.9
12	196	5.02	83.3	83.5	200	5.11	242.3	241.4	192	4.91	81.9	82.0
13	191	4.89	83.5	83.6	195	4.98	241.4	240.4	187	4.78	82.0	82.0
14	186	4.76	83.6	83.5	190	4.85	240.4	239.3	182	4.66	82.0	81.8
15	181	4.64	83.5	83.3	185	4.73	239.3	238.1	177	4.54	81.8	81.6
16	177	4.52	83.3	83.0	180	4.60	238.1	236.7	173	4.42	81.6	81.2
17	172	4.40	83.0	82.6	175	4.48	236.7	235.3	168	4.30	81.2	80.7
18	168	4.29	82.6	82.1	171	4.37	235.3	233.7	164	4.19	80.7	80.2
19	163	4.18	82.1	81.5	166	4.25	233.7	232.0	160	4.08	80.2	79.5
20	159	4.07	81.5	80.8	162	4.14	232.0	230.2	156	3.98	79.5	78.7

Table C.19 (continued)

Time	D_{in} = 110 µm, FSHW+F				D_{in} = 175 µm, FSHW+F				D_{in} = 215 µm, FSHW+F			
h	N	$L_{p(t=1)}$	$L_{(t=-1)}$	$L_{(t=t)}$	N	$L_{p(t=1)}$	$L_{(t=-1)}$	$L_{(t=t)}$	N	$L_{p(t=1)}$	$L_{(t=-1)}$	$L_{(t=t)}$
	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l
21	155	3.96	80.8	80.0	158	4.04	230.2	228.3	152	3.88	78.7	77.9
22	151	3.86	80.0	79.1	154	3.93	228.3	226.3	148	3.77	77.9	76.9
23	147	3.76	79.1	78.1	150	3.83	226.3	224.2	144	3.68	76.9	75.9
24	143	3.66	78.1	77.0	146	3.73	224.2	222.0	140	3.58	75.9	74.8
25	140	3.57	77.0	75.9	142	3.63	222.0	219.7	136	3.49	74.8	73.6
26	136	3.48	75.9	74.7	138	3.54	219.7	217.4	133	3.40	73.6	72.4
27	132	3.39	74.7	73.4	135	3.45	217.4	214.9	129	3.31	72.4	71.1
28	129	3.30	73.4	72.1	131	3.36	214.9	212.4	126	3.22	71.1	69.7
29	126	3.21	72.1	70.7	128	3.27	212.4	209.8	123	3.14	69.7	68.3
30	122	3.13	70.7	69.3	125	3.19	209.8	207.1	120	3.06	68.3	66.8
31	119	3.05	69.3	67.8	121	3.11	207.1	204.4	117	2.98	66.8	65.3
32	116	2.97	67.8	66.2	118	3.02	204.4	201.6	114	2.90	65.3	63.8
33	113	2.89	66.2	64.6	115	2.95	201.6	198.7	111	2.83	63.8	62.2
34	110	2.82	64.6	63.0	112	2.87	198.7	195.7	108	2.75	62.2	60.6
35	107	2.74	63.0	61.4	109	2.80	195.7	192.7	105	2.68	60.6	58.9
36	105	2.67	61.4	59.7	106	2.72	192.7	189.7	102	2.61	58.9	57.2
37	102	2.60	59.7	58.0	104	2.65	189.7	186.5	100	2.55	57.2	55.5
38	99	2.54	58.0	56.2	101	2.58	186.5	183.4	97	2.48	55.5	53.8
39	97	2.47	56.2	54.5	98	2.52	183.4	180.1	94	2.42	53.8	52.1
40	94	2.41	54.5	52.7	96	2.45	180.1	176.9	92	2.35	52.1	50.4
41	92	2.34	52.7	51.0	93	2.39	176.9	173.5	90	2.29	50.4	48.6
42	89	2.28	51.0	49.2	91	2.33	173.5	170.2	87	2.23	48.6	46.9
43	87	2.22	49.2	47.4	89	2.27	170.2	166.8	85	2.18	46.9	45.1
44	85	2.17	47.4	45.7	86	2.21	166.8	163.3	83	2.12	45.1	43.4
45	83	2.11	45.7	43.9	84	2.15	163.3	159.8	81	2.06	43.4	41.7
46	80	2.06	43.9	42.2	82	2.09	159.8	156.3	79	2.01	41.7	40.0
47	78	2.00	42.2	40.4	80	2.04	156.3	152.8	77	1.96	40.0	38.3
48	76	1.95	40.4	38.7	78	1.99	152.8	149.2	75	1.91	38.3	36.6
49	74	1.90	38.7	37.0	76	1.94	149.2	145.6	73	1.86	36.6	35.0
50	72	1.85	37.0	35.3	74	1.89	145.6	142.0	71	1.81	35.0	33.4
51	71	1.80	35.3	33.7	72	1.84	142.0	138.3	69	1.76	33.4	31.8
52	69	1.76	33.7	32.1	70	1.79	138.3	134.6	67	1.72	31.8	30.3
53	67	1.71	32.1	30.6	68	1.74	134.6	131.0	65	1.67	30.3	28.8
54	65	1.67	30.6	29.0	66	1.70	131.0	127.3	64	1.63	28.8	27.3
55	63	1.62	29.0	27.6	65	1.65	127.3	123.5	62	1.59	27.3	25.9
56	62	1.58	27.6	26.1	63	1.61	123.5	119.8	60	1.55	25.9	24.5
57	60	1.54	26.1	24.7	61	1.57	119.8	116.1	59	1.51	24.5	23.2
58	59	1.50	24.7	23.4	60	1.53	116.1	112.4	57	1.47	23.2	22.0
59	57	1.46	23.4	22.1	58	1.49	112.4	108.6	56	1.43	22.0	20.7
60	56	1.42	22.1	20.9	57	1.45	108.6	104.9	54	1.39	20.7	19.6
61	54	1.39	20.9	19.7	55	1.41	104.9	101.2	53	1.36	19.6	18.5
62	53	1.35	19.7	18.6	54	1.38	101.2	97.5	52	1.32	18.5	17.5
63	51	1.32	18.6	17.6	52	1.34	97.5	93.8	50	1.29	17.5	16.5
64	50	1.28	17.6	16.6	51	1.31	93.8	90.1	49	1.25	16.5	15.5
65	49	1.25	16.6	15.6	50	1.27	90.1	86.5	48	1.22	15.5	14.6
66	48	1.22	15.6	14.7	48	1.24	86.5	82.9	47	1.19	14.6	13.8
67	46	1.18	14.7	13.9	47	1.21	82.9	79.2	45	1.16	13.8	13.0
68	45	1.15	13.9	13.1	46	1.18	79.2	75.7	44	1.13	13.0	12.3
69	44	1.12	13.1	12.3	45	1.15	75.7	72.1	43	1.10	12.3	11.6

Table C.19 (continued)

Time	D_{in} =450 μm, FSHW+F				D_{in} = 60 μm, FSHW+F				D_{in} = 175 μm, FSHW+FE			
h	N	$L_{p(t=1)}$	$L_{(t=-1)}$	$L_{(t=t)}$	N	$L_{p(t=1)}$	$L_{(t=-1)}$	$L_{(t=t)}$	N	$L_{p(t=1)}$	$L_{(t=-1)}$	$L_{(t=t)}$
	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l
0	308	7.88	160.0	162.3	300	7.67	99.0	101.6	201	5.14	310.0	309.0
1	300	7.67	162.3	164.3	292	7.47	101.6	104.0	196	5.01	309.0	307.9
2	292	7.47	164.3	166.1	285	7.28	104.0	106.2	191	4.88	307.9	306.6
3	285	7.28	166.1	167.8	277	7.09	106.2	108.1	186	4.75	306.6	305.2
4	277	7.09	167.8	169.2	270	6.91	108.1	109.8	181	4.63	305.2	303.7
5	270	6.91	169.2	170.4	263	6.73	109.8	111.4	176	4.51	303.7	302.1
6	263	6.73	170.4	171.5	256	6.55	111.4	112.7	172	4.39	302.1	300.3
7	256	6.55	171.5	172.4	250	6.38	112.7	113.9	167	4.28	300.3	298.5
8	250	6.38	172.4	173.1	243	6.22	113.9	114.9	163	4.17	298.5	296.5
9	243	6.22	173.1	173.6	237	6.06	114.9	115.7	159	4.06	296.5	294.5
10	237	6.06	173.6	174.0	231	5.90	115.7	116.3	155	3.95	294.5	292.3
11	231	5.90	174.0	174.2	225	5.75	116.3	116.8	151	3.85	292.3	290.0
12	225	5.75	174.2	174.2	219	5.60	116.8	117.2	147	3.75	290.0	287.7
13	219	5.60	174.2	174.1	213	5.45	117.2	117.4	143	3.65	287.7	285.2
14	213	5.45	174.1	173.9	208	5.31	117.4	117.4	139	3.56	285.2	282.7
15	208	5.31	173.9	173.5	202	5.17	117.4	117.3	136	3.47	282.7	280.1
16	202	5.17	173.5	173.0	197	5.04	117.3	117.1	132	3.38	280.1	277.4
17	197	5.04	173.0	172.3	192	4.91	117.1	116.7	129	3.29	277.4	274.6
18	192	4.91	172.3	171.6	187	4.78	116.7	116.2	125	3.20	274.6	271.7
19	187	4.78	171.6	170.7	182	4.66	116.2	115.6	122	3.12	271.7	268.8
20	182	4.66	170.7	169.6	177	4.54	115.6	114.9	119	3.04	268.8	265.8
21	177	4.54	169.6	168.5	173	4.42	114.9	114.1	116	2.96	265.8	262.7
22	173	4.42	168.5	167.3	168	4.31	114.1	113.2	113	2.88	262.7	259.6
23	168	4.31	167.3	165.9	164	4.19	113.2	112.1	110	2.81	259.6	256.3
24	164	4.19	165.9	164.5	160	4.09	112.1	111.0	107	2.74	256.3	253.1
25	160	4.09	164.5	162.9	156	3.98	111.0	109.8	104	2.67	253.1	249.7
26	156	3.98	162.9	161.3	152	3.88	109.8	108.5	102	2.60	249.7	246.3
27	152	3.88	161.3	159.5	148	3.78	108.5	107.1	99	2.53	246.3	242.9
28	148	3.78	159.5	157.7	144	3.68	107.1	105.6	96	2.46	242.9	239.3
29	144	3.68	157.7	155.8	140	3.58	105.6	104.1	94	2.40	239.3	235.8
30	140	3.58	155.8	153.8	136	3.49	104.1	102.4	91	2.34	235.8	232.2
31	137	3.49	153.8	151.7	133	3.40	102.4	100.7	89	2.28	232.2	228.5
32	133	3.40	151.7	149.5	130	3.31	100.7	99.0	87	2.22	228.5	224.8
33	130	3.31	149.5	147.3	126	3.23	99.0	97.1	85	2.16	224.8	221.0
34	126	3.23	147.3	145.0	123	3.14	97.1	95.3	82	2.11	221.0	217.2
35	123	3.14	145.0	142.6	120	3.06	95.3	93.3	80	2.05	217.2	213.4
36	120	3.06	142.6	140.2	117	2.98	93.3	91.3	78	2.00	213.4	209.5
37	117	2.98	140.2	137.7	114	2.90	91.3	89.3	76	1.95	209.5	205.6
38	114	2.90	137.7	135.2	111	2.83	89.3	87.2	74	1.90	205.6	201.6
39	111	2.83	135.2	132.5	108	2.76	87.2	85.1	72	1.85	201.6	197.7
40	108	2.76	132.5	129.9	105	2.68	85.1	82.9	70	1.80	197.7	193.6
41	105	2.68	129.9	127.2	102	2.61	82.9	80.7	69	1.75	193.6	189.6
42	102	2.61	127.2	124.4	100	2.55	80.7	78.5	67	1.71	189.6	185.5
43	100	2.55	124.4	121.6	97	2.48	78.5	76.2	65	1.66	185.5	181.4
44	97	2.48	121.6	118.8	95	2.42	76.2	74.0	63	1.62	181.4	177.3
45	95	2.42	118.8	116.0	92	2.35	74.0	71.7	62	1.58	177.3	173.2
46	92	2.35	116.0	113.1	90	2.29	71.7	69.4	60	1.54	173.2	169.0
47	90	2.29	113.1	110.1	87	2.23	69.4	67.1	59	1.50	169.0	164.9
48	87	2.23	110.1	107.2	85	2.18	67.1	64.8	57	1.46	164.9	160.7
49	85	2.18	107.2	104.2	83	2.12	64.8	62.4	56	1.42	160.7	156.5

Table C.19 (continued)

Time	D_{in} =450 µm, FSHW+F				D_{in} = 60 µm, FSHW+F				D_{in} = 175 µm, FSHW+FE			
h	N	$L_{p(t=1)}$	$L_{(t=-1)}$	$L_{(t=t)}$	N	$L_{p(t=1)}$	$L_{(t=-1)}$	$L_{(t=t)}$	N	$L_{p(t=1)}$	$L_{(t=-1)}$	$L_{(t=t)}$
	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l
50	83	2.12	104.2	101.2	81	2.06	62.4	60.1	54	1.38	156.5	152.3
51	81	2.06	101.2	98.2	79	2.01	60.1	57.8	53	1.35	152.3	148.1
52	79	2.01	98.2	95.1	77	1.96	57.8	55.5	51	1.31	148.1	143.9
53	77	1.96	95.1	92.1	75	1.91	55.5	53.2	50	1.28	143.9	139.6
54	75	1.91	92.1	89.0	73	1.86	53.2	50.9	49	1.25	139.6	135.4
55	73	1.86	89.0	86.0	71	1.81	50.9	48.7	47	1.21	135.4	131.2
56	71	1.81	86.0	82.9	69	1.76	48.7	46.5	46	1.18	131.2	127.0
57	69	1.76	82.9	79.9	67	1.72	46.5	44.3	45	1.15	127.0	122.8
58	67	1.72	79.9	76.9	65	1.67	44.3	42.2	44	1.12	122.8	118.6
59	65	1.67	76.9	73.8	64	1.63	42.2	40.0	43	1.09	118.6	114.4
60	64	1.63	73.8	70.8	62	1.59	40.0	38.0	42	1.06	114.4	110.2
61	62	1.59	70.8	67.8	60	1.55	38.0	36.0	41	1.04	110.2	106.0
62	60	1.55	67.8	64.9	59	1.51	36.0	34.0	39	1.01	106.0	101.9
63	59	1.51	64.9	61.9	57	1.47	34.0	32.1	38	0.98	101.9	97.8
64	57	1.47	61.9	59.0	56	1.43	32.1	30.3	37	0.96	97.8	93.7
65	56	1.43	59.0	56.2	54	1.39	30.3	28.5	36	0.93	93.7	89.7
66	54	1.39	56.2	53.3	53	1.36	28.5	26.8	36	0.91	89.7	85.7
67	53	1.36	53.3	50.6	52	1.32	26.8	25.1	35	0.89	85.7	81.7
68	52	1.32	50.6	47.9	50	1.29	25.1	23.6	34	0.86	81.7	77.8
69	50	1.29	47.9	45.2	49	1.25	23.6	22.1	33	0.84	77.8	73.9

Time	D_{in} = 215 µm, FSHW+FE				D_{in} =450 µm, FSHW+FE				D_{in} = 60 µm, FSHW+FE			
h	N	$L_{p(t=1)}$	$L_{(t=-1)}$	$L_{(t=t)}$	N	$L_{p(t=1)}$	$L_{(t=-1)}$	$L_{(t=t)}$	N	$L_{p(t=1)}$	$L_{(t=-1)}$	$L_{(t=t)}$
	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l
0	183	4.68	163.0	162.0	145	3.71	351.0	348.5	247	6.32	154.0	154.7
1	178	4.56	162.0	161.0	141	3.61	348.5	345.9	241	6.15	154.7	155.3
2	174	4.44	161.0	159.8	138	3.52	345.9	343.2	234	5.99	155.3	155.7
3	169	4.32	159.8	158.5	134	3.43	343.2	340.4	228	5.84	155.7	156.0
4	165	4.21	158.5	157.1	131	3.34	340.4	337.5	222	5.69	156.0	156.1
5	160	4.10	157.1	155.6	127	3.25	337.5	334.6	217	5.54	156.1	156.0
6	156	4.00	155.6	154.0	124	3.17	334.6	331.6	211	5.40	156.0	155.8
7	152	3.89	154.0	152.4	121	3.09	331.6	328.5	206	5.26	155.8	155.5
8	148	3.79	152.4	150.6	118	3.01	328.5	325.3	200	5.12	155.5	155.0
9	144	3.69	150.6	148.7	114	2.93	325.3	322.0	195	4.99	155.0	154.5
10	141	3.60	148.7	146.8	112	2.85	322.0	318.7	190	4.86	154.5	153.7
11	137	3.51	146.8	144.8	109	2.78	318.7	315.3	185	4.73	153.7	152.9
12	134	3.41	144.8	142.7	106	2.71	315.3	311.9	180	4.61	152.9	151.9
13	130	3.33	142.7	140.5	103	2.64	311.9	308.4	176	4.49	151.9	150.9
14	127	3.24	140.5	138.3	100	2.57	308.4	304.8	171	4.37	150.9	149.7
15	123	3.16	138.3	136.0	98	2.50	304.8	301.2	167	4.26	149.7	148.4
16	120	3.07	136.0	133.6	95	2.44	301.2	297.5	162	4.15	148.4	147.0
17	117	2.99	133.6	131.2	93	2.37	297.5	293.7	158	4.04	147.0	145.5
18	114	2.92	131.2	128.7	90	2.31	293.7	289.9	154	3.94	145.5	144.0
19	111	2.84	128.7	126.2	88	2.25	289.9	286.1	150	3.84	144.0	142.3
20	108	2.77	126.2	123.6	86	2.19	286.1	282.2	146	3.74	142.3	140.5

Table C.19 (continued)

Time	D_{in} = 215 µm, FSHW+FE				D_{in} =450 µm, FSHW+FE				D_{in} = 60 µm, FSHW+FE			
h	N	$L_{p(t-1)}$	$L_{(t-1)}$	$L_{(t)}$	N	$L_{p(t-1)}$	$L_{(t-1)}$	$L_{(t)}$	N	$L_{p(t-1)}$	$L_{(t-1)}$	$L_{(t)}$
	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l
21	105	2.70	123.6	121.0	84	2.14	282.2	278.2	142	3.64	140.5	138.7
22	103	2.63	121.0	118.3	81	2.08	278.2	274.2	139	3.55	138.7	136.8
23	100	2.56	118.3	115.6	79	2.03	274.2	270.2	135	3.45	136.8	134.8
24	97	2.49	115.6	112.8	77	1.97	270.2	266.1	132	3.36	134.8	132.7
25	95	2.43	112.8	110.0	75	1.92	266.1	262.0	128	3.28	132.7	130.6
26	92	2.36	110.0	107.2	73	1.87	262.0	257.8	125	3.19	130.6	128.4
27	90	2.30	107.2	104.3	71	1.83	257.8	253.6	122	3.11	128.4	126.1
28	88	2.24	104.3	101.5	70	1.78	253.6	249.4	118	3.03	126.1	123.8
29	85	2.19	101.5	98.6	68	1.73	249.4	245.1	115	2.95	123.8	121.4
30	83	2.13	98.6	95.6	66	1.69	245.1	240.8	112	2.87	121.4	119.0
31	81	2.07	95.6	92.7	64	1.64	240.8	236.5	109	2.80	119.0	116.5
32	79	2.02	92.7	89.8	63	1.60	236.5	232.2	107	2.73	116.5	114.0
33	77	1.97	89.8	86.8	61	1.56	232.2	227.8	104	2.66	114.0	111.4
34	75	1.92	86.8	83.8	59	1.52	227.8	223.4	101	2.59	111.4	108.8
35	73	1.87	83.8	80.9	58	1.48	223.4	218.9	99	2.52	108.8	106.1
36	71	1.82	80.9	77.9	56	1.44	218.9	214.5	96	2.45	106.1	103.4
37	69	1.77	77.9	75.0	55	1.40	214.5	210.0	94	2.39	103.4	100.7
38	67	1.73	75.0	72.0	53	1.37	210.0	205.5	91	2.33	100.7	98.0
39	66	1.68	72.0	69.1	52	1.33	205.5	201.0	89	2.27	98.0	95.2
40	64	1.64	69.1	66.2	51	1.30	201.0	196.5	86	2.21	95.2	92.4
41	62	1.60	66.2	63.3	49	1.26	196.5	191.9	84	2.15	92.4	89.6
42	61	1.55	63.3	60.5	48	1.23	191.9	187.4	82	2.10	89.6	86.8
43	59	1.51	60.5	57.7	47	1.20	187.4	182.8	80	2.04	86.8	83.9
44	58	1.47	57.7	54.9	46	1.17	182.8	178.2	78	1.99	83.9	81.1
45	56	1.44	54.9	52.1	44	1.14	178.2	173.6	76	1.94	81.1	78.3
46	55	1.40	52.1	49.5	43	1.11	173.6	169.1	74	1.89	78.3	75.4
47	53	1.36	49.5	46.8	42	1.08	169.1	164.5	72	1.84	75.4	72.6
48	52	1.33	46.8	44.2	41	1.05	164.5	159.9	70	1.79	72.6	69.8
49	51	1.29	44.2	41.7	40	1.02	159.9	155.3	68	1.75	69.8	67.0
50	49	1.26	41.7	39.2	39	1.00	155.3	150.7	66	1.70	67.0	64.2
51	48	1.23	39.2	36.9	38	0.97	150.7	146.1	65	1.66	64.2	61.4
52	47	1.20	36.9	34.5	37	0.95	146.1	141.6	63	1.61	61.4	58.6
53	46	1.16	34.5	32.3	36	0.92	141.6	137.0	61	1.57	58.6	55.9
54	44	1.13	32.3	30.2	35	0.90	137.0	132.5	60	1.53	55.9	53.3
55	43	1.10	30.2	28.1	34	0.88	132.5	127.9	58	1.49	53.3	50.6
56	42	1.08	28.1	26.1	33	0.85	127.9	123.4	57	1.45	50.6	48.0
57	41	1.05	26.1	24.2	32	0.83	123.4	118.9	55	1.41	48.0	45.5
58	40	1.02	24.2	22.5	32	0.81	118.9	114.4	54	1.38	45.5	43.0
59	39	0.99	22.5	20.8	31	0.79	114.4	110.0	52	1.34	43.0	40.6
60	38	0.97	20.8	19.2	30	0.77	110.0	105.6	51	1.31	40.6	38.2
61	37	0.94	19.2	17.7	29	0.75	105.6	101.2	50	1.27	38.2	35.9
62	36	0.92	17.7	16.3	28	0.73	101.2	96.8	49	1.24	35.9	33.7
63	35	0.90	16.3	15.1	28	0.71	96.8	92.5	47	1.21	33.7	31.5
64	34	0.87	15.1	13.9	27	0.69	92.5	88.2	46	1.18	31.5	29.5
65	33	0.85	13.9	12.8	26	0.67	88.2	84.0	45	1.15	29.5	27.5
66	32	0.83	12.8	11.8	26	0.66	84.0	79.8	44	1.12	27.5	25.6
67	32	0.81	11.8	10.9	25	0.64	79.8	75.7	43	1.09	25.6	23.8
68	31	0.79	10.9	10.0	24	0.62	75.7	71.7	41	1.06	23.8	22.1
69	30	0.76	10.0	9.3	24	0.61	71.7	67.7	40	1.03	22.1	20.5

Table C. 20 Soluble COD concentration in the mixed-liquor of ASBRs fed FSHW, FSHW+F and FSHW+FE substrates and operated at 25°C

D_{in} µm	time h	FSHW 1	FSHW 2	FSHW+F 1	FSHW+F 2	FSHW+FE 1	FSHW+FE 2
		mg SCOD/l					
110	0	1374	1374	1471	1471		
110	0	1341	1373	1491	1462		
110	3	1136	1315	1312	1325		
110	23	268	275	298	292		
110	72	140	155	145	164		
215	0	1339	1339	1365	1365	1395	1395
215	0	738	613	733	897	828	864
215	3	672	521	695	682	710	713
215	7	675	454	676		636	558
215	18	296	190	296	149	117	122
215	42	139	87	97	102	85	98
215	67	152	124	162	131	125	199
450	0	1242	1242	1329	1329	1424	1424
450	0	1065	1006	1029	1036	1105	1141
450	6	683	456	624	673	658	662
450	18	164	153	217	311	211	250
450	42	136	115	134	134	133	153
450	72	154	115	174	168	155	162
60	0	1585	1585	1595	1595	1719	1719
60	0	1300	1161	1341	1146	1318	1401
60	6	903	859	927	824	938	967
60	24	229	233	246	259	267	274
60	44	220	215	218	223	233	214
60	72	195	194	187	174	195	197

Table C. 21 Volatile fatty acid concentration in the mixed-liquor of ASBRs fed FSHW, FSHW+F and FSHW+FE substrates and operated at 25°C

D_{in} µm	time h	FSHW 1	FSHW 2	FSHW+F 1	FSHW+F 2	FSHW+FE 1	FSHW+FE 2
		mg VFA/l					
215	0	228	174	217	205	233	239
215	3	222	169	230	225	219	233
215	7	242	138		208	160	187
215	18	37	9	37	23	23	10
215	42	22	24	26	23	22	9
215	67	10	10	10	9	17	20
60	0	769	684	686	614		621
60	0	238	175	213	168	244	269
60	7	79	69	84	53	88	113
60	23	12	15	17	27	25	14
60	47	19	14	21	25	58	
60	70	4	10	25	13	40	30
60	90	7	31	11	27	47	6

Table C.22 Methane content in biogas from reactors fed FSHW, FSHW+F, FSHW+FE substrates

D_{in} (µm)	FSHW 1	FSHW 2	FSHW+F 1	FSHW+F 2	FSHW+FE 1	FSHW+FE 2	FSHW+E 1	FSHW+E 2
	fraction of methane in biogaz							
110	0.767	0.769	0.760	0.757				
110	0.796	0.790	0.779	0.782				
175	0.803	0.798	0.798	0.798	0.786	0.789	0.798	0.797
215	0.796	0.796	0.785	0.784	0.781	0.785		
450	0.807	0.799	0.784	0.787	0.777	0.787		
60	0.796	0.797	0.783	0.784	0.835	0.783		
Average	0.794	0.791	0.781	0.782	0.795	0.786	0.798	0.797
STD	0.014	0.012	0.012	0.013	0.027	0.003		
Overall Avg.	0.789							
STD	0.014							

Table C. 23 Methane production from ASBRs fed FSHW, FSHW+F and FSHW+FE substrates and operated at 25°C

a) D_{in} = 110 µm

Cycle	Time h	FSHW 13	FSHW 15	FSHW+F 14	FSHW+F 16
		Cummulative L of methane			
1	0.0	0.00	0.00	0.00	0.00
	3.3	1.65	1.22	1.12	1.16
	6.8	4.09	3.25	3.04	3.16
	25.4	11.67	11.36	13.76	13.95
	48.5	13.84	13.69	18.07	18.11
	71.0	15.02	14.87	19.65	20.10

Cycle	Time h	FSHW 13	FSHW 15	FSHW+F 14	FSHW+F 16
		Cummulative L of methane			
2	0.0	0.00	0.00	0.00	0.00
	7.0	3.28	3.17	2.38	2.74
	22.7	10.77	10.51	11.95	12.20
	46.7	12.94	12.69	16.42	16.51
	54.7	13.58	13.33	17.43	17.39
	70.7	14.57	14.33	19.07	19.11

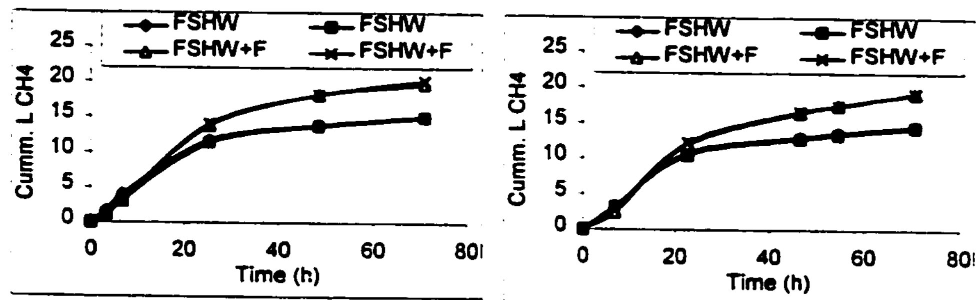


Figure C. 1 Cummulative methane production from ASBRs operated at 25°C and fed FSHW, FSHW+F and FSHW+FE substrates at a D_{in} of 110 µm

b) D_{in} = 175 µm

Cycle 1

Time h	FSHW 14	FSHW 16	FSHW+F 13	FSHW+F 21	FSHW+FE 15	FSHW+FE 20
	Cummulative L of methane					
0.0	0.00	0.00	0.00	0.00	0.00	0.00
2.7	1.28	1.10	0.96	1.21	1.00	1.04
6.8	3.66	3.69	3.04	3.64	3.45	3.55
20.5	10.05	10.05	10.80	10.80	11.85	11.92
43.6	12.68	12.88	15.10	14.86	15.87	15.91
66.5	13.60	13.94	16.85	16.85	17.61	17.67
69.0	13.68	14.02	17.01	17.00	17.77	17.83

Cycle 2

Time h	FSHW 13	FSHW 15	FSHW+F 14	FSHW+F 16	FSHW+FE 13	FSHW+FE 15
	Cummulative L of methane					
0.0	0.00	0.00	0.00	0.00	0.00	0.00
2.7	1.36	1.18	1.04	1.03	0.69	0.79
6.9	4.07	3.77	3.61	3.47	2.98	3.18
18.1	9.64	9.65	10.22	10.01	10.76	11.11
26.0	10.80	10.87	12.30	12.23	13.21	13.17
42.1	12.11	12.31	14.69	14.50	15.71	15.70
74.3	13.43	13.94	17.51	17.45	18.79	18.82

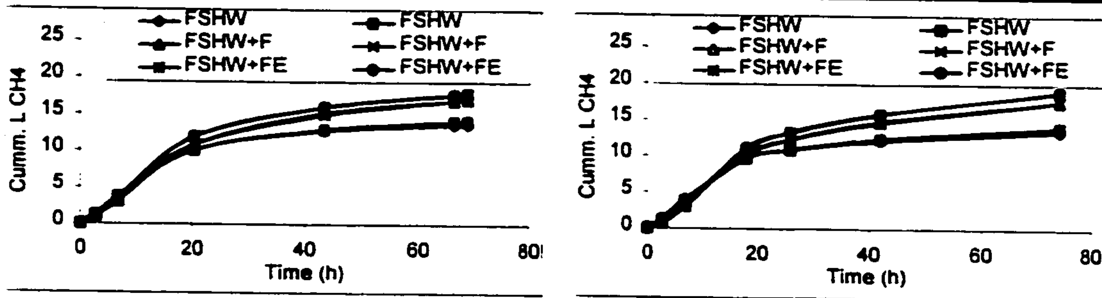
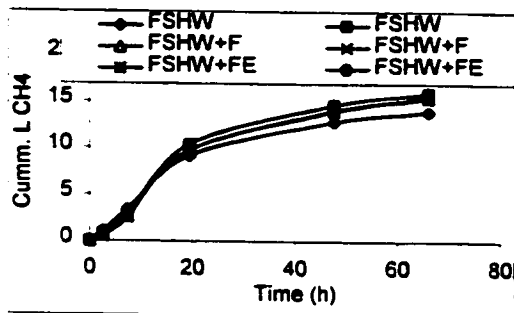


Figure C. 2 Cummulative methane production from ASBRs operated at 25°C and fed FSHW, FSHW+F and FSHW+FE substrates at a D_{in} of 175 µm

Table C.23 **(continued)**

c) D_{in} = 215 μm

Cycle 1						Cycle 2					
Time	FSHW	FSHW+F		FSHW+FE		Time	FSHW	FSHW+F		FSHW+FE	
h	21	13	20	15	16	h	21	13	20	15	16
	Cummulative L of methane						Cummulative L of methane				
0.0	0.00	0.00	0.00	0.00	0.00	0.0	0.00	0.00	0.00	0.00	0.00
2.6	1.07	0.70	0.79	0.61	0.62	3.1	1.53	1.25	1.44	0.92	0.95
7.3	3.39	2.77	2.77	2.51	2.81	7.7	4.68	4.26	4.56	3.75	3.47
19.3	9.15	9.64	9.64	10.29	10.29	18.8	9.07	9.69	9.56	10.23	9.70
47.7	12.79	13.90	13.99	14.56	14.56	26.5	9.89	11.03	10.97	12.04	11.74
66.0	13.85	15.34	15.39	15.86	15.86	45.8	11.35	13.29	13.25	14.22	13.83
						67.5	12.32	14.92	14.82	15.88	15.39



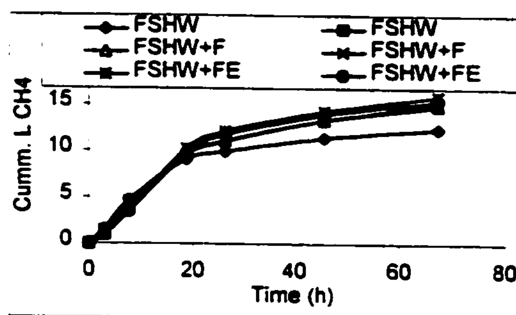
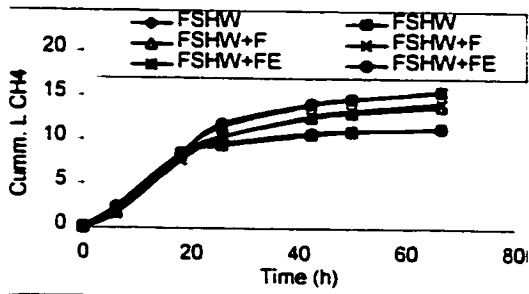


Figure C. 3 Cummulative methane production from ASBRs operated at 25°C and fed FSHW, FSHW+F and FSHW+FE substrates at a D_{in} of 215 μm

d) D_{in} = 450 μm

Cycle 1							Cycle 2					
Time	FSHW		FSHW+F		FSHW+FE		Time	FSHW	FSHW+F		FSHW+FE	
h	21		13	20	15	16	h	21	13	20	15	16
	Cummulative L of methane							Cummulative L of methane				
0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6.2	2.43	2.22	1.99	1.59	1.71	1.69	5.58	1.14	0.78	0.78	0.92	1.18
18.2	8.37	8.32	8.22	7.66	8.36	8.45	17.58	6.60	6.21	6.21	7.04	7.64
25.8	9.52	9.32	10.33	10.24	11.49	11.74	25.00	8.85	9.74	9.74	10.93	11.38
42.4	10.76	10.57	12.52	12.67	13.99	14.08	43.50	10.19	12.40	12.40	13.27	13.65
50.0	10.95	10.92	13.04	13.25	14.55	14.62	66.25	10.93	14.47	14.03	14.93	15.21
66.6	11.21	11.26	13.82	14.14	15.49	15.42						



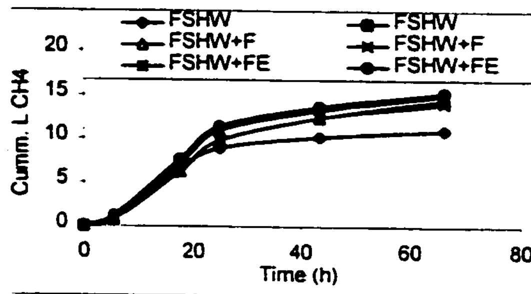


Figure C. 4 Cummulative methane production from ASBRs operated at 25°C and fed FSHW, FSHW+F and FSHW+FE substrates at a D_{in} of 450 μm

Table C.23 **(continued)**

e) D_{in} = 60 μm

Cycle 1

Time	FSHW		FSHW+F		FSHW+FE	
h	21		13	20	15	16
	Cummulative L of methane					
0.0	0.00	0.00	0.00	0.00	0.00	0.00
6.3	2.55	2.90	2.71	2.71	2.84	2.71
18.3	9.59	10.21	10.01	10.01	10.71	10.75
26.3	11.85	12.22	13.03	13.03	14.24	14.47
42.6	12.93	13.37	15.01	14.95	16.04	16.37
50.0	13.30	13.70	15.62	15.55	16.58	16.99
66.3	13.57	14.01	16.40	16.39	17.41	17.76

Cycle 2

Time	FSHW		FSHW+F		FSHW+FE	
h	21		13	20	15	16
	Cummulative L of methane					
0.0	0.00	0.00	0.00	0.00	0.00	0.00
7.0	3.45	3.67	3.22	3.64	3.59	3.23
22.5	10.76	10.82	11.99	12.51	12.65	12.65
47.0	12.21	12.30	15.01	15.37	15.66	15.76
66.0	12.49	12.63	16.14	16.56	16.73	16.81
74.0	12.67	12.78	16.48	16.97	17.11	17.24

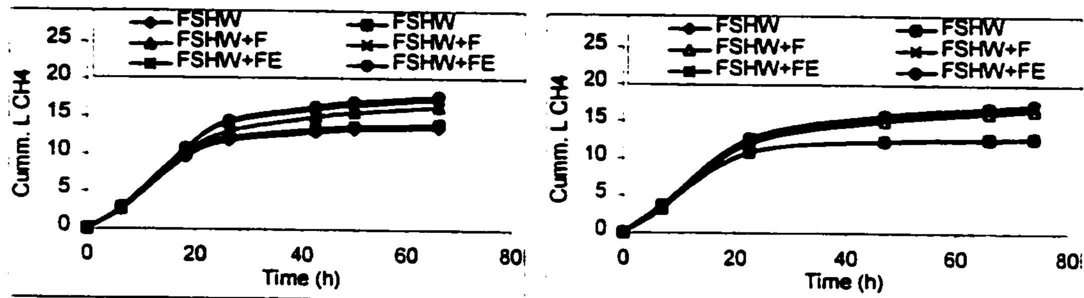


Figure C. 5 Cummulative methane production from ASBRs operated at 25°C and fed FSHW, FSHW+F and FSHW+FE substrates at a D_{in} of 60 μm

Table C.24 Transformation of input TCOD into methane during the 3-d ASBR treatment cycle

D_{in} µm	cycle	ASBR	CH_4-COD/$TCOD_{in}$ (g/g) FSHW	FSHW +F	FSHW +FE	TCODin (mg/l) FSHW	FSHW +F	FSHW +FE	total CH4-COD (g) FSHW	FSHW +F	FSHW +FE
60	2	1	0.86	0.76	0.77	3196	4682	4945	32.87	42.47	45.80
60	2	2	0.87	0.78	0.79	3196	4682	4945	33.23	43.57	46.74
60	3	1	0.84	0.79	0.81	3038	4386	4531	30.50	41.34	44.03
60	3	2	0.83	0.80	0.81	3038	4386	4531	30.41	42.25	44.25
110	2	1	0.84	0.76		3530	5229		35.51	47.59	
110	2	2	0.86	0.75		3530	5229		36.49	47.23	
110	3	1	0.97	0.81		3308	5132		38.35	50.19	
110	3	2	0.95	0.82		3308	5132		37.71	50.28	
175	2	1	0.87	0.76	0.80	3464	4899	4861	35.99	44.77	46.77
175	2	2	0.89	0.76	0.85	3464	4899	4861	36.89	44.74	49.45
175	3	1	0.85	0.78	0.80	3464	4899	4861	35.35	46.07	46.93
175	3	2	0.88	0.78	0.85	3464	4899	4861	36.68	45.92	49.53
215	2	1	0.94	0.80	0.83	3248	4201	4201	36.46	40.38	41.75
215	2	2	0.94	0.80	0.83	3248	4201	4201	36.46	40.50	41.75
215	3	1	0.93	0.78	0.83	2913	4201	4201	32.42	39.26	41.79
215	3	2	0.93	0.77	0.80	2913	4201	4201	32.42	39.00	40.49
450	2	1	0.88	0.76	0.85	2799	3987	4018	29.51	36.36	40.77
450	2	2	0.88	0.78	0.84	2799	3987	4018	29.62	37.20	40.59
450	3	1	0.86	0.80	0.81	2799	3987	4018	28.77	38.07	39.30
450	3	2	0.86	0.77	0.83	2799	3987	4018	28.77	36.93	40.03
60 Average			0.85	0.78	0.80						
110			0.90	0.79							
175			0.872	0.77	0.826						
215			0.931	0.79	0.822						
450			0.869	0.78	0.833						
overall			0.885	0.781	0.819						
60 STD			0.02	0.02	0.02						
110			0.06	0.03							
175			0.02	0.01	0.03						
215			0.00	0.02	0.01						
450			0.01	0.01	0.01						
overall			0.041	0.020	0.022						

Table C. 25 Methane production from ASBRs fed PL-250 dissolved in tap water

Cycle	Time h	Enzyme dose (mg/l) 0	0	75	75	250	250	1000	1000
		cummulative l of methane							
2	0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	6.3	0.00	0.00	0.00	0.00	0.17	0.00	0.74	0.58
	22.5	0.00	0.00	0.18	0.15	0.43	0.15	1.48	1.17
	29.8	0.17	0.17	0.36	0.31	0.78	0.52	1.92	1.92
	46.0	0.34	0.26	0.63	0.61	1.12	0.90	2.37	2.34
	53.9	0.52	0.52	0.90	0.84	1.29	1.12	2.59	2.67
	70.0	0.94	0.95	1.35	1.23	1.81	1.57	3.18	3.17
3	0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	5.6	0.00	0.00	0.19	0.15	0.34	0.22	1.22	1.42
	22.2	0.17	0.00	0.38	0.23	0.77	0.59	2.21	2.17
	29.6	0.26	0.17	0.67	0.46	1.03	0.88	2.59	2.42
	46.1	0.44	0.43	0.95	0.69	1.37	1.18	2.97	2.84
	53.8	0.61	0.60	1.15	0.92	1.54	1.32	3.28	3.01
	69.8	0.61	0.60	1.24	1.00	1.62	1.32	3.35	3.09
		l of methane corrected for reactors receiving 0 mg/l of enzyme							
2	0.0			0.00	0.00	0.00	0.00	0.00	0.00
	6.3			0.00	0.00	0.17	0.00	0.74	0.58
	22.5			0.18	0.15	0.43	0.15	1.48	1.17
	29.8			0.19	0.13	0.60	0.35	1.75	1.75
	46.0			0.33	0.31	0.82	0.60	2.06	2.04
	53.9			0.38	0.33	0.78	0.60	2.07	2.16
	70.0			0.40	0.28	0.86	0.62	2.23	2.23
3	0.0			0.00	0.00	0.00	0.00	0.00	0.00
	5.6			0.19	0.15	0.34	0.22	1.22	1.42
	22.2			0.29	0.14	0.68	0.50	2.12	2.09
	29.6			0.45	0.24	0.81	0.67	2.38	2.21
	46.1			0.52	0.26	0.93	0.74	2.54	2.41
	53.8			0.54	0.31	0.93	0.72	2.67	2.40
	69.8			0.63	0.39	1.02	0.72	2.75	2.48
70-h		Average l CH_4		0.43		0.81		2.42	
		STD		0.15		0.17		0.25	
		Average g COD		1.12		2.11		6.34	
		STD		0.39		0.45		0.65	

Table C. 26 Methane production from ASBRs fed PL-250 dissolved in tap water

Cycle	Time h	Enzyme dose (mg/l) 0	0	70	70
		cummulative l of methane			
2	0.00	0.00	0.00	0.00	0.00
	2.75	0.98	1.17	0.80	1.30
	6.75	3.26	3.92	3.29	4.19
	20.50	9.55	9.59	9.55	10.06
	43.58	12.08	11.92	12.28	12.43
	66.50	12.90	12.59	13.24	13.11
	69.00	12.98	12.68	13.32	13.19
3	0.00	0.00	0.00	0.00	0.00
	2.75	1.06	0.75	0.88	0.99
	6.92	3.67	3.50	3.37	3.96
	18.08	9.14	9.34	9.15	9.53
	26.00	10.20	10.34	10.27	10.52
	42.08	11.51	11.59	11.71	11.74
	74.33	12.73	12.76	13.24	12.96
		corrected l of methane			
2	0.00			0.00	0.00
	2.75			0.22	-0.27
	6.75			0.60	-0.30
	20.50			0.49	-0.02
	43.58			0.42	0.27
	66.50			0.37	0.49
	69.00			0.36	0.49
3	0.00			0.00	0.00
	2.75			0.09	-0.02
	6.92			0.38	-0.22
	18.08			0.29	-0.09
	26.00			0.25	0.00
	42.08			0.19	0.16
	74.33			0.21	0.49
72-h		Average l CH_4		0.39	
		STD		0.13	
		Average g COD		1.02	
		STD		0.35	