Anaerobic hydrolysis during digestion of complex substrates

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Voorwoord

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Abstract - Sanders, W.T.M. (2001). Anaerobic hydrolysis during digestion of complex substrate. Doctoral Thesis, Wageningen University, The Netherlands.

Complex waste(water) such as, raw sewage, dairy wastewater, slaughterhouse wastewater, fish processing wastewater, primary sludge and the organic fraction of municipal solid waste have been proven to be degradable under anaerobic conditions. However, during the digestion process the conversion of the complex organic molecules into mono- and dimer components, also called the hydrolysis, is often the rate-limiting step. For design and optimization of the anaerobic conversion of complex waste(water) a good knowledge of the hydrolysis kinetics is therefore essential. The scope of this thesis was therefore to clarify the hydrolysis kinetics during the anaerobic digestion of complex waste(water), with emphasis on the hydrolysis of particles, dissolved macromolecules and lipids in coherence with the process conditions during the digestion. The mechanisms of the hydrolysis were elucidated by lab experiments and simulations with mechanistic hydrolysis models. For the hydrolysis of particulate substrates the results presented in this thesis revealed that, at constant pH and digestion temperature, the amount of surface available for the hydrolysis is the most important parameter for the hydrolysis rate and all other parameters are of minor importance.

With respect to dissolved polymers, such as gelatine and dissolved starch, the results indicate that the mechanism of the enzymatic hydrolysis in batch experiments can be described as a random polymerisation process. Moreover, the hydrolysis rate of dissolved components is linearly related to the sludge concentration in the batch experiment. The hydrolysis of neutral lipids under acidogenic conditions is slower as compared to the hydrolysis under methanogenic conditions. Based on the results presented in this thesis it was hypothesised that this is due to positive effect of the methane production on maintaining the lipid-water interface and subsequent higher volumetric hydrolysis rate.

In practice the hydrolysis rate is most commonly described by an empirical first order relation, in which the hydrolysis rate is linearly related to the amount of biodegradable substrate that is available (Eastman and Ferguson, 1981).

The identification of the essential parameters of the hydrolysis mechanisms in this thesis made it possible to evaluate the first order approach and designate the limitations of the relation. The evaluation revealed that the hydrolysis only proceeds according to first order kinetics if no changes in the rate limiting step or the biodegradability occur during the degradation of a substrate. Moreover, the first order hydrolysis constant seems system and substrate specific and the use of literature values for the hydrolysis constant is therefore not advised.

For assessment of a hydrolysis constant in a lab experiment the following guidelines were presented: (1) For waste(water) containing mainly protein and carbohydrates, first order kinetics can be established under acidic and methanogenic conditions in batch or completely stirred tank reactor (CSTR) system. (2) For waste(water) that contains high concentrations of lipids the assessment of the hydrolysis constant for neutral lipids under acid conditions is impossible due to coagulation of the lipid. Under methanogenic conditions the hydrolysis constant can be assessed in a 'multiple flask' batch system. However as (gas) mixing can differ between a laboratory batch and a full-scale CSTR-system, the subsequent effect on the lipid-water interface might cause a difference in the prevailing k_h value of the two systems.

CONTENTS

1 GENERAL INTRODUCTION

Scope of this thesis – Complex waste(water) such as, raw sewage, dairy wastewater, slaughterhouse wastewater, fish processing wastewater, primary sludge and the organic fraction of municipal solid waste have been proven to be degradable under anaerobic conditions. However, during the digestion process the conversion of the complex organic molecules into mono- and dimer components, also called the hydrolysis, is often the rate-limiting step. For design and optimization of the anaerobic conversion of complex waste(water) a good knowledge of the hydrolysis kinetics is therefore essential.
The scope of this thesis is to clarify the hydrolysis kinetics during the anaerobic digestion of complex waste(water), with emphasis on the hydrolysis of particles, dissolved macromolecules and lipids in coherence with the process conditions during the digestion. Moreover, a practical approach for calculations on the hydrolysis process when designing reactors for the anaerobic digestion of complex waste(water) is presented.

1.1 introduction

Aerobic and anaerobic (including anoxic) digestion are the two major biological treatment methods for waste (water). Under aerobic conditions organic components are oxidized to carbon dioxide and under anaerobic to carbon dioxide and methane. When comparing the two treatment methods with respect to sustainability, anaerobic digestion by far is the favorite because hardly if any energy input is needed, the methane produced can be used as a substitute for fossil fuels and the production of excess sludge is much lower. Moreover the technology of anaerobic treatment is much less complex and anaerobic systems are applicable at any site and any size.
Complex waste(water) which can be (pre)treated anaerobically are amongst others; slaughterhouse wastewater (Sayed 1987, Batstone, 2000), fish processing wastewater (Palenzuella-Rollon, 1999), domestic sewage (Elmitwalli, 2000), primary sludge (Miron et al. 2000), dairy wastewater (Zeeman et al. 1997), waste activated sludge (Zeeman et al. 1997), manure (Zeeman, 1991) and the organic fraction of municipal solid waste (Ten Brummeler, 1987).
Although they differ greatly in origin these complex waste(waters) all consist of protein, lipids, carbohydrates and sometimes lignin.

1.2 complex substrates

Most of the substrate in complex waste(water) is present as particulate matter, e.g. 45-75% of domestic sewage (Levine et al. 1991), ~80% of fish processing wastewater (Pallenzuella-Rollon 1999), ~80% in primary sludge (Miron et al. 2000) and 45-55% of slaughterhouse wastewater (Sayed et al. 1988). Although less common, some of the complex wastewater's contain a significant amount of dissolved substrate requiring hydrolysis. For instance, slaughterhouse wastewater contains a significant amount of dissolved protein in the form of gelatine (Batstone, 2000).

Most natural carbohydrates are macromolecules like polysaccharides. These polysaccharides are, predominantly simple and derived sugars linked together by glycosidic bonds. Most polysaccharides are insoluble in water and they can form colloidal suspensions (Gaudy and Gaudy, 1980). Polysaccharides found in complex organic waste(water) are cellulose, hemi-cellulose, pectin and starch.
Cellulose is the most abundant polysaccharide in complex organic waste. Cellulose is a linear polymer that consist of D-glucose units linked together through β-1,4 bonds. A considerable fraction of the cellulose in organic household waste is incorporated in a lignocellulosic complex with lignin.
Starch consist of two types of polysaccharides, viz. the linear amylose (~20%) and the branched amylopectin (~80%). In amylose the glucose units are linked together through α-1,4 bonds. In amylopectin the glucose units are linked together through

α-1,4 bonds, but also through α-1,6 bonds. Amylose is soluble in water where as amylopectin is not (Engbersen and de Groot,1988). In some research, soluble starch is used (Goel et al. 1997,1998) in which case the substrate only consists of amylose.

Proteins can be divided into two general groups i.e. globular and fibroid proteins. Fibroid proteins have a fibrous structure and are the most important building material for animal tissue. Collagen and elastin (in connective tissue, ligaments and tendons), keratin (in skin, hair, feathers, horns and hoofs) and myosin (in muscles) are fibroid proteins. Due to their structure and biological function, fibroid proteins are water-insoluble and rather stable at changing pH and temperature. Globular proteins are water-soluble or form colloidal suspensions. These proteins have a more regulatory function (enzymes, hormones, antibody's) and are rather sensitive to changes in pH and temperature. Gelatine is a not naturally occurring protein, but it origins from collagen. When collagen is treated in boiling water, its tertiary structure is destroyed and the soluble gelatine is set free (Engbersen and de Groot,1988).

The bulk of the fats in complex waste(water) are triglyceride esters also called triacylglycerols or neutral lipid. About 90% of these triglycerides are composed of glycerol and myristic (C14: 0), palmitic (C16: 0), stearic (C18: 0), oleic (C18: 1) and linoleic (C18: 2) acids (Viswanathan et al., 1962). Lipids are water insoluble and due to their hydrophobic nature they will easily attach to particles in the waste(water).

1.3 anaerobic digestion of complex substrates

The anaerobic digestion process of complex organic waste can be subdivided in four stages: hydrolysis, acidification, acidogenesis and methanogenesis (fig 1.1). In the hydrolysis step, complex suspended compounds and colloidal matter are converted into their monomer or dimeric components, such as amino acids, single sugars and long chain fatty acids (LCFA). During the acidogenesis, the hydrolysis products are converted into acetic acid and other volatile fatty acids and alcohol's. Acetic acid, CO_2 plus H_2 and methanol constitute the main substrate for the methanogenesis to form methane and carbon dioxide. All other fatty acids and alcohol's first have to be converted to acetic acid or hydrogen and carbon dioxide during the acetogenesis.
The main intermediary and end-products of the anaerobic digestion process are volatile fatty acids, hydrogen and biogas, respectively. Because the methanogenic bacteria are very sensitive to a drop in pH, that could be caused by accumulation of volatile fatty acids, the digestion of complex waste obviously is a delicate balance between the rate of hydrolysis, acidogenesis and methanogenesis.

According to Batstone (2000), three main mechanisms exist for the release of enzymes and the subsequent hydrolysis of the complex substrate during anaerobic digestion, viz.

1. The organism secretes enzymes to the bulk liquid, where they will either adsorb to a particle or react with a soluble substrate (Jain et al. 1992)
2. The organism attaches to the particle, secretes enzymes into the vicinity of the particle and next the organism will benefit from the released dissolved substrates (Vavilin et al., 1996).
3. The organism has an attached enzyme that may double as a transport receptor to the interior of the cell. This method requires the organism to absorb onto the surface of the particle.

Because, mechanisms 2 and 3 both require attachment of the organism to the substrate particle and no literature references could be found with respect to mechanism 3, the latter mechanism will not be considered.

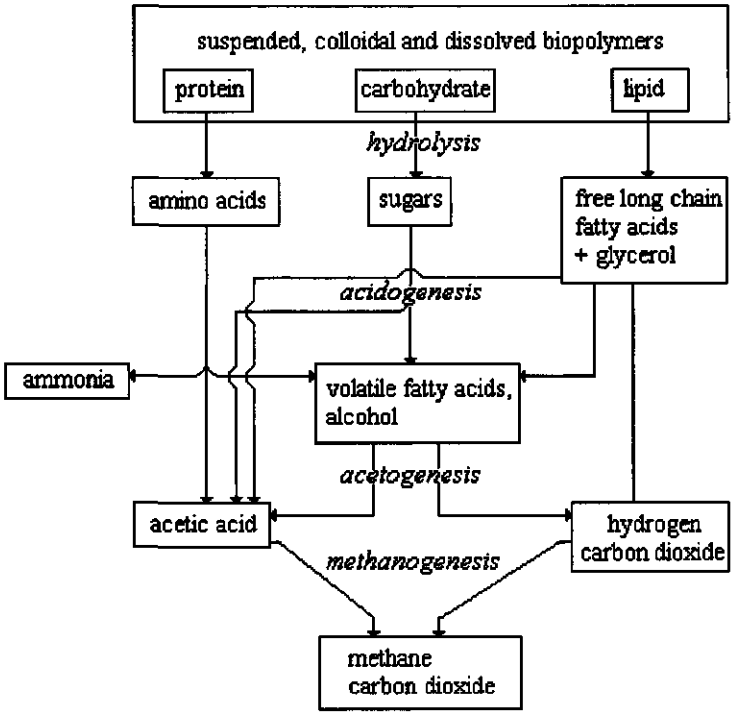

Figure 1.1: *Simplified Schematic representation of the anaerobic degradation process of complex waste(water).*

Besides the actual enzymatic hydrolysis reaction, the mechanisms as proposed by Batstone (2000) also involve other processes such as the production of enzymes and

several diffusion processes such as the diffusion of enzymes, organisms or hydrolysis products. Batstone (2000) argued that when treating dilute wastewater, such as slaughterhouse wastewater, the diffusion processes would not be rate limiting. However, when digesting a concentrated substrate such as vegetable, fruit and yard waste at substrate concentrations of 35-40% Total Solids (TS) the diffusion rates may become the rate-limiting step (Veeken and Hamelers 2000, Kalyuzhnyi et al. 2000).

Goel et al. (1998) and Confer and Logan (1997a,b, 1998a,b) investigated the hydrolysis kinetics of dissolved polymeric substrates. The results of their investigations show that most of the enzyme activity is located on the sludge and the macromolecules have to diffuse to the sludge in order to be hydrolysed. This approach is opposite to mechanism 1 proposed by Batstone (2000). However, as the enzymes are produced by the sludge, the concentration and activity of this sludge is equally important in both mechanisms.

Investigations on the hydrolysis process of particulate substrates have shown that the hydrolysis rate is related to the particle size of the particles or the number of adsorption sites at the particle surface (Hills and Nakano 1984, Chyi and Dague 1994, Veeken and Hamelers, 1999). Moreover, microscopic observations showed that the substrate particles in a digester are immediately colonised by bacteria (Hobson, 1987) which secrete extra-cellular enzymes. In experiments that Philip et al. (1993) conducted with septic tank sludge the lipase, protease and cellulase activity in the sludge and the bulk liquid was assessed. No activity could be detected in the bulk liquid. Moreover, measurements of the activities in washed sludge samples lead to the conclusion that 50% of the enzyme activity remained strongly bound to the sludge regardless if the sludge had been washed with distilled water, tris-buffer, or Triton X100 (dissolves cell walls and membranes).
The results above lead to the idea that mechanism 2 prevails for particulate substrate. Moreover, as enzymes are believed to be present in excess (Hobson, 1987) it seems that the surface of the substrate plays an important role in the hydrolysis of particulate substrates.

1.4 Enzymatic hydrolysis of proteins

Proteins are hydrolysed during anaerobic digestion by two groups of enzymes, i.e. protease and peptidases. The joined groups of peptidases and proteases are also referred to as proteinases. The general scheme for the enzymatic breakdown of proteins is (Stryer, 1988):

Protein → polypeptides → peptides → amino acids

From results of experiments with pure cultures it appears that micro-organisms start producing proteinases when the concentration of amino acids and inorganic nutrients in the water-phase are low or protein and peptides concentrations are high (Glenn, 1976). The production of proteinase by bacterial genera can be inhibited by components, such as amino acids, high inorganic phosphate levels and glucose (Glenn, 1976).
This implies that to attain a high level of proteinase activity, amino acids are not allowed to accumulate during the digestion. The amino acids can be degraded either through anaerobic oxidation linked to hydrogen production or through fermentation according to the Stickland reaction. The degradation of some important amino acids through anaerobic oxidation and the corresponding change in Gibb's free energy are given in table 1.1. The coupled oxidation-reduction reactions between pairs of amino acids is referred to as the Stickland reaction. An example is given in equation 1.1.

Table 1.1: The degradation of some amino-acids through interspecies hydrogen transfer (Örlygsson et al (1994).

Reaction	*G° (KJ/mole)*
Valine + 3 H_2O → isobutyrate + HCO_3^- + H^+ + NH_4^+ + 2H_2	*+9.7*
Leucine + 3 H_2O → isovalerate + HCO_3^- + H^+ + NH_4^+ + 2H_2	*+4.2*
Isoleucine + 3 H_2O → 2-methylbutyrate + HCO_3^- + H^+ + NH_4^+ + 2H_2	*+5.0*

$$2\ CH_2(NH_2)\text{-}COOH + 4H \rightarrow 2\ CH_3COOH + 2NH_3$$

$$CH_3\text{-}CH(NH_2)\text{-}COOH + 2H_2O \rightarrow CH_3COOH + CO_2 + NH_3 + 4H \quad + \quad (1.1)$$

$$CH_3\text{-}CH(NH_2)\text{-}COOH + 2\ CH_2(NH_2)\text{-}COOH + 2H_2O \rightarrow 3\ CH_3COOH + CO_2 + 3\ NH_3$$

Thus, a complex type of competition may occur between interspecies hydrogen transfer and the Stickland reaction, which will vary depending on the organisms involved and the amino acids present. This is illustrated by the results of Örlygsson et al. (1994) who investigated the anaerobic protein degradation in a steady state thermophilic enrichment culture. They found that the degradation of amino acids and the hydrolysis of protein were inhibited when the recovery of carbon in methane was only 4-9%. At higher methane recovery, no inhibition was observed.

The results indicated that the organisms in the enrichment culture mainly degraded the amino acids through interspecies hydrogen transfer.

Also during anaerobic digestion the proteolysis can be inhibited by glucose (Breure et al. 1986, Sarada and Joseph 1993). Sarada and Joseph (1993) found during semi-continuous digestion experiments with tomato solid waste only 14% protein degradation at a HRT of 8 days. They concluded that this low level of proteolysis should be due to the presence of easily degradable substrate such as glucose, which could have evolved in the degradation of cellulose and hemi-cellulose. However, another reason could have been the adaptation procedure that was applied in these experiments. According to the results of Breure et al. (1986) dealing with the digestion of mixtures of gelatine and glucose in an anaerobic chemostat at 30°C and different pH values, the ability of a bacterial culture to convert protein depends on the adaptation procedure. The ability of a culture adapted to glucose to degrade protein was distinctly lower than a culture adapted to protein to degrade glucose.

1.5 Enzymatic hydrolysis of carbohydrates

The hydrolysis of cellulose is performed by a mixture of cellulolytic enzymes, viz. exo-glucanases, endo-glucanases and cellobiases (Fig 1.2). The mixture of these enzymes is usually referred to as, cellulase.
The hydrolysis of starch is performed by a mixture of amylases that is able to hydrolyse the α-1,4 bonds and α-1,6 bonds of the amylose and amylopectin.

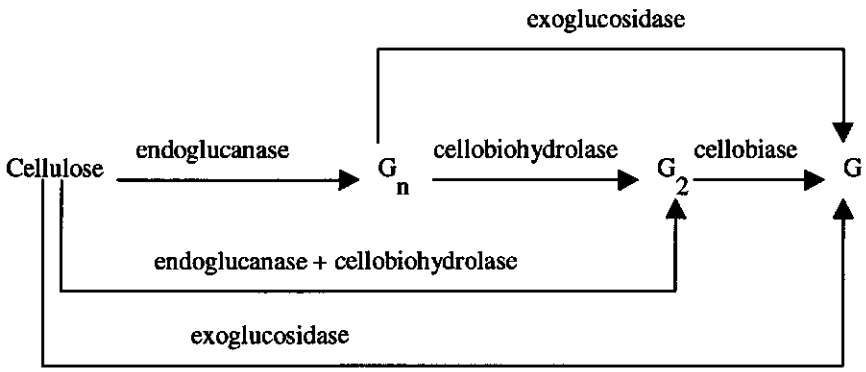

Figure 1.2: *Schematic representation of the enzymatic hydrolysis of cellulose according to Klyosov (Beldman, 1986) (G=glucose).*

With respect to the production of cellulase similar findings were made as for proteinase. The production of cellulase becomes inhibited by high glucose levels

but is stimulated by low glucose levels. However, no effect of the concentration of free amino acid on the production of cellulase were reported (Glenn, 1976).
Also NH_4^+ can inhibit the hydrolysis of cellulose. The results of Zeeman (1991) reveal that during the anaerobic digestion of cow slurry the hydrolysis of the suspended solids (mainly cellulose) is inhibited at high NH_4^+ concentrations. The mechanism for this inhibition however remained unclear.

1.6 Enzymatic hydrolysis of lipids

As mentioned in paragraph 1.2 most lipids in waste(water) are present as triacylglycerides, a glycerol ester with three long chain fatty acids (LCFA). During hydrolysis, these compounds are hydrolysed to glycerol and the three free long chain fatty acids (Fig. 1.3). This enzymatic reaction is executed by triglyceride lipases.

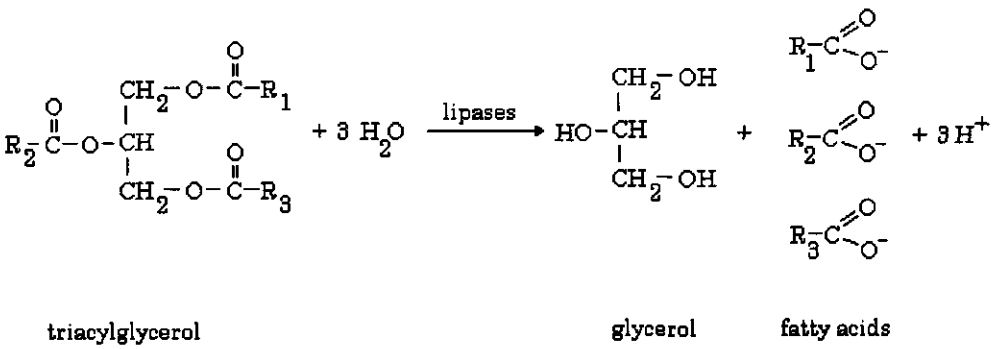

Figure 1.3: *The hydrolysis of neutral lipids by lipase (reproduced from Stryer, 1988)*

The lipases are more active towards insoluble than soluble substrates. Sarda and Desnuelle (1958) showed that the activity of lipases increases when the concentration of the substrate reaches its maximum solubility and starts forming a second phase. The lipases adsorb to the lipid-water interface of the second phase, according to a Langmuir adsorption isotherm and the amount of adsorbed lipase strongly correlates to the measured enzyme activity.
During anaerobic digestion of complex waste(water) not only lipids, but also other substrate and sludge are present in the digester. Because the lipases have a relatively poor substrate specificity, they easily attach to the sludge. Once adsorbed to the sludge it is difficult to establish good contact between lipase and the water-fat surface (Pronk et al. 1987).
Hydrolysis of most lipids does not lead to the formation of soluble monomers. In the case of lipids the poorly soluble LCFA represent the major portion of the

reduction equivalents. The free LCFA will partially adsorb to the biomass present in the reactor, to the reactor wall, and/or accumulate at the surface of the lipid emulsion or form micelles. These LCFA micelles have a maximum diameter of approximately 200 Å (Stryer, 1988), which makes it difficult to distinguish them from the dissolved fraction.
Verger and de Haas (1976), Rietsch et al. (1977) and Verger (1980) proposed a kinetic model for the hydrolysis of insoluble lipids. In their model the lipases successively adsorb to the lipid surface, then they bind with the substrate and next they perform the hydrolysis. Accumulation of hydrolysis products at the lipid-water interface can cause product inhibition and physical-chemical changes of the interface, e.g. the surface tension. The model therefore also accounts for inactivation of the lipase caused by these changes in the surface tension.

When the lipid-water interface is, because of the small particle size, very large, such as with milk, the hydrolysis is sometimes not the rate-limiting step in the anaerobic lipid degradation process. Instead, the degradation of the free LCFA's is rate-limiting (Hanaki et al. 1981). β-Oxidation is the mechanism of anaerobic oxidation of long chain fatty acids with as products hydrogen and acetate. The bacteria catalysing this reaction are obligate hydrogen producers and their metabolism is inhibited by hydrogen (Novak and Carlson, 1970) and LCFA (Hanaki, 1981). The Standard Gibbs free energy for the complete ß-oxidation of palmitate to acetate is +345.6 kJ/mol (Sayed, 1987). This implies the prevalence of a synthropic association with hydrogen oxidising bacteria is required for anaerobic degradation of LCFA, because only an extreme low partial hydrogen pressure makes the degradation of LCFA energetically favourable. Free LCFA may cause severe inhibition of methanogenic and though in lesser extent to the acetogenic bacteria and consequently the degradation of the LCFA themselves (Hanaki et al 1981, Rinzema 1988, Angelidaki and Ahring 1992).

1.7 Effect of temperature on the hydrolysis

The overall effect of the digestion temperature on the hydrolysis originates from the combined temperature effect on the enzyme kinetics, bacterial growth and solubility of the substrate.
For instance, the Gibbs-Helmoltz equation gives the relation between the change of temperature and the value of pKa of the enzymes. When assuming an average heat of ionisation of -32 kJ/mol for the ionising groups in the enzymes a shift of 0.5 units towards a lower pH for pKa can be calculated on increasing the temperature with 25°Celcius. The change in charge will have consequences for the structure of the enzyme resulting in changes of catalytic efficiency, amount of active enzyme and binding of the substrate (Chaplin and Bucke, 1990).

In general, the rate of all reactions vary with temperature in accordance with the Arrhenius equation:

$$k = A.e^{-\Delta G^*/RT} \qquad (1.2)$$

With:

k = kinetic rate constant, in this case the hydrolysis constant,
A = the Arrhenius constant,
G* = the standard free energy of activation ($J.mol^{-1}$),
Typical standard free energies of activation are 15-70 kJ mol^{-1} (Chaplin and Bucke, 1990),
R = the gas law constant ($J.mol^{-1}.K^{-1}$),
T = the absolute temperature (K).

Veeken and Hamelers (1999) digested several biowaste components, such as orange peels, bark, leafs and grass at 20°, 30° and 40°C. The biodegradability of the biowaste components did not show a significant difference with an increase of the temperature. Moreover, a good Arrhenius relation between the first order hydrolysis constant for the total substrate and the digestion temperature (R^2 0.984-0.999) with an average standard free energy of activation of 46±14 kJ mol^{-1} was found for the biowaste components.

The solubility of neutral lipids and LCFA increases with temperature. This implies that with increase of temperature the lipid-water interface will increase, moreover the accumulation of LCFA at the surface will be less.

1.8 Effect of the structure of the substrate

An important factor for the hydrolysis is the structure of the substrate and its accessibility for hydrolytic enzymes. It is therefore obvious that, due to their structural differences, the soluble globular proteins are far more susceptible to hydrolysis than fibrous proteins.
The accessibility of a substrate can also be altered by formation of complexes with other compounds. For example, cellulose itself is easily degradable, but once it is incorporated in a lignocellulosic complex, the biodegradability of the cellulose is distinctly lower (Tong et al. 1990). When a substrate contains more than 25% lignine it even becomes non-biodegradable (Chandler et al. 1980).

The lignocelllosic complex is already formed in nature, but also during the digestion itself complexes can be formed which have an effect on the accessibility of the substrate. For instance, indications have been obtained (Brons, 1984) that the structure of protein and cellulose can be affected by humic acids. Brons (1984)

found an influence of humic acids on the hydrolysis of potato protein during anaerobic digestion, with an inhibition of the proteolysis at humic acid concentrations exceeding 1000 mg/l. This inhibition probably originats from the bonding of the humic acid to the protein. Due to this the accessibility of the protein to protease becomes poorer. Addition of Ca^{2+} reduced the inhibition by humic acids. The degradation of single amino acids was not influenced by the presence of humic acids.

1.9 Effect of the pH

The simplest relation between pH and activity of an enzyme is the 'bell shaped' curve (Fig 1.4) which has its optimum pH at:

$$pH_{optimum}=(pKa_1+pKa_2)/2 \qquad (1.3)$$

With:
pKa_n = the pKa of the n^{th} dissociation form of the enzymes.

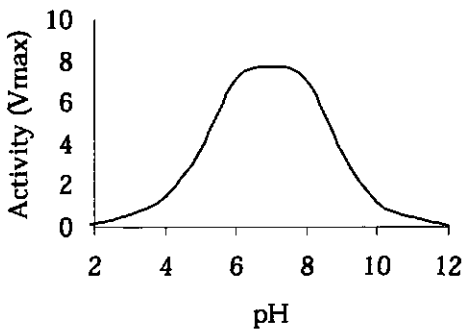

Figure 1.4: *'bell shaped' curve of the relation between enzyme activity and pH (Chaplin and Brucke, 1990)*

This relation is simplified as compared to the real situation as it is assumed that only one charged form of the enzyme is active and the enzyme is a single ionised species, while it could contain a mixture of different ionised groups. Despite its simplifications the 'bell-shaped' relation is commonly encountered in single enzyme substrate reactions (Fersht, 1999).
However, during anaerobic digestion it is very likely that several enzymes all with different pH optima are present. Moreover, the 'bell-shaped' relation ignores ionisation of the substrate, products and enzyme-substrate complexes (Chaplin and Bucke, 1990). Obviously, the effect of the pH on the anaerobic digestion is

much more complex. The net effect of pH on the hydrolysis rate is specified by the pH optima of the different enzymes present in the digester and the effect of pH on the charge/solubility of the substrate. The latter especially applies to the digestion of substrates that contain proteins.

1.10 Hydrolysis related problems during the digestion of complex substrate.

With the treatment of complex waste(water) several problems related to the hydrolysis process can arise. The more concentrated complex waste, e.g. primary sludge, the organic fraction of municipal solid waste and manure are usually digested in completely stirred tank reactors (CSTR's) or accumulation systems (Zeeman, 1991). A problem that occurs in these types of reactors is the formation of scum layers (Elzen and Koppes, 2000). Moreover, because of the high substrate concentration in the CSTR, the concentration of free LCFA that can cause LCFA toxicity is easily reached (Angelidaki and Ahring, 1992).
Due to the high solid retention time needed to complete the hydrolysis of the suspended solids, the CSTR's can not be applied for the treatment of dilute complex waste(water) such as slaughterhouse wastewater, domestic sewage, fish processing wastewater or dairy wastewater. For this type of wastewater an upflow system, like the UASB reactor, is more suitable. However, the high amounts of suspended solids in the wastewater make the application of high volumetric loading rates in one stage anaerobic upflow reactors, especially in the case of low temperature conditions, virtually impossible. This is due to very slow hydrolysis of the solids entrapped in the sludge bed (Man et al. 1986, Sayed 1987).

The treatment of complex wastewater with a high fraction of suspended solids in a two-step system with a high loaded upflow reactor as first step, could lead to more stable process conditions (Zeeman and Lettinga 1999, Palenzuela-Rollon 1999, Elmitwalli 2000). The proposed system could consist of two sequential UASB systems. In the first reactor little if any gas production will occur because of the high loading rate and the short sludge retention time, here a high suspended solids removal can be achieved (Wang, 1994). The effluent of the first step will be treated in the second UASB. Elmitwalli (2000) proposed to use an anaerobic filter reactor packed with polyurethane vertical sheets as first step of this system to improve the suspended solids removal. Moreover; the anaerobic filter might prevent the formation of scum layers.
As similar approach can be used, when the wastewater contains a high fraction of lipids, e.g. slaughterhouse wastewater or palm oil mill effluent (Borja et al. 1996, Perot and Amar (1989)). However as results of several authors indicate that under acid conditions the hydrolysis is slower than under methanogenic

conditions (Heukelian and Mueller 1957, Palenzuella-Rollon 1999, Miron et al. 2000) only a very low hydrolysis of lipids can be expected. Moreover, because the specific density of lipid particles is lower than water and they easily attach to the sludge, then the formation of scum layers or washout of biomass can occur when some gas production is provided (Rinzema, 1988).

A special case is the two-step treatment of dairy wastewater. The lipids in dairy wastewater are emulsified and surrounded by a membrane of proteins, casein. Casein has an iso-electric point of 4.6; below this pH the protein will destabilise and precipitate. The lipids will be entrapped in the precipitate and settle with the proteins. Establishment of such a low pH can be achieved by acidification of the easy biodegradable substrate in the wastewater, e.g. lactose (Zeeman et al. 1997) or by addition of HNO_3 (MTI, 1995).

1.11 Mathematical description of the hydrolysis kinetics during anaerobic digestion of complex substrates

Regarding the need to design and optimise anaerobic reactors treating complex organic waste(water), it is necessary to describe the process of hydrolysis adequately. In literature, two types of mathematical relations describing the hydrolysis process are available i.e. empirical and more mechanistic relations.

The simplest and commonly used relation is an empirical first order relation (Eastman and Ferguson, 1981). In this first order hydrolysis kinetic relation it is assumed that the hydrolysis rate is linearly related (at constant pH and temperature) to the amount of biodegradable substrate in the digester (eq 1.4).

$$\frac{dX_{degr.}}{dt} = -kh.X_{degr.} \qquad (1.4)$$

With:
kh = first order hydrolysis constant (day^{-1})
$X_{degr.}$ = biodegradable substrate (kg COD. m^{-3})
t = time (day)
Since all physical and enzymatic aspects of the hydrolysis process are lumped together in the first order hydrolysis constant, the mathematical description of the hydrolysis rate remains simple. On the other hand, such an empirical relation doesn't contribute to an improvement of the insight in the actual hydrolysis process and the optimisation of the hydrolysis process.

To study the hydrolysis process in more detail more mechanistic hydrolysis models were introduced. These mechanistic models can be divided into two groups: growth-related and surface-based models.

In the surface based hydrolysis models it is assumed that enzyme activity is present in excess for the digestion of particulate substrates and that the hydrolysis rate depends on the amount of surface available for the hydrolytic enzymes (Hobson 1987, Negri et al 1993, Vavilin 1996). The particles are believed to be peeled layer by layer. For this reason, these models are sometimes addressed to as shrinking-core models.

In contrast to the surface-based models, the growth-related models are based on the assumption that there is a deficiency in enzyme activity. In the growth-related models the rate of hydrolysis therefore depends on the concentration and activity of the hydrolytic enzymes or the hydrolytic biomass (Chen and Hashimoto 1980, Jain et al. 1992, Negri et al.1993, Vavilin et al 1996, Goel et al. 1998, Munch et al. 1999, Batstone 2000). These models are mainly used to desribe the hydrolysis of dissolved polymeric substrates.

Many growth-related models for the hydrolysis are very similar to the Michaelis-Menten kinetics.

$$\frac{dS}{dt} = V_{max}\frac{S}{k_m + S} \quad \text{with } V_{max} = k.S_{enzyme} \tag{1.5}$$

with:
S = concentration of dissolved substrate (g/l),
S_{enzyme} = enzyme concentration (g/l),
V_{max} = maximum conversion rate (g/l/h),
k_m = half velocity constant (g/l).

Jain et al (1992) developed a model for the hydrolysis of cattle dung in which the hydrolysis was considered as proceeding as a multi-step process, i.e. including enzyme production, enzyme diffusion, adsorption and reaction with the substrate. Although cattle dung is particulate, Jain et al (1992) assumed that the particles were very porous and therefore completely accessible to the hydrolytic enzymes. Based on this assumption, the substrate could be observed as a solution of single macromolecules and the actual hydrolysis process therefore could be described by Michaelis-Menten kinetics.

The model proposed by Goel et al. (1998) for the hydrolysis of soluble starch is based on the sludge concentration instead of the enzyme concentration. However, as it was observed that the enzymes are bound to the sludge, the sludge concentration and enzyme activity can be considered as interchangeable parameters. The same applies to the Contois model, which has been used by Chen and Hashimoto (1980) and Vavilin et al (1996).

Negri et al.(1993) and Munch et al. (1999) applied a modified first order kinetics model in which the hydrolysis rate not only is regulated by the hydrolysis constant but also by the concentration of enzymes. They also assumed that the enzymatic yield is directly related to the amount of volatile solids consumed.

1.12 Conclusion

From the literature survey presented in this chapter it can be concluded that for the hydrolysis of particles the enzymes seem to be present in excess and therefore the amount of surface that is available for the hydrolysis is most important. Moreover, since with respect to the hydrolysis of insoluble particles consisting of proteins and carbohydrates the enzymes are in excess a possible inhibition of the enzyme production due to temporary accumulation of hydrolysis products seems to be less important as in the case of dissolved substrates. Because, in the latter case the enzyme activity is the most important factor for the hydrolysis. The hydrolysis of neutral lipids is also a surface related process but then it seems that prevention of accumulation of hydrolysis products seems to be of crucial importance, as a relative small amount of accumulated LCFA could cause severe inhibition.
Therefore regarding the hydrolysis kinetics a differentiation needs to be made between hydrolysis of particles, dissolved substrates and neutral lipids.

1.13 Outline of this thesis

Chapters 2 to 4 deal with the mechanisms of hydrolysis of particulate substrates, dissolved polymeric substrates and neutral lipids, respectively. The mechanisms are elucidated by results of lab experiments and simulations with mechanistic hydrolysis models.
Chapter 5 evaluates the empirical first order model, most used for the design and optimisation of anaerobic reactors, based on the results presented in previous chapters. Moreover, in this chapter also guidelines are presented for the determination of the first order constant in a batch experiment.
Chapter 6 summarises the results of the investigations in this thesis and the implications for the process lay-out of anaerobic reactors treating complex waste(water) is discussed.

2 HYDROLYSIS KINETICS OF PARTICULATE SUBSTRATES

abstract- A mathematical description of the surface related hydrolysis kinetics for spherical particles in a batch digestion is presented as well as a verification of this model with particulate starch as a substrate. Three substrates containing starch with different particle size distributions (PSD) were used. Two were obtained from fresh potatoes by wet sieving and for the third substrate, a commercially available starch was used. The substrates were batch digested at 30°C with granular sludge as inoculum and the hydrolysis efficiency was measured and fitted with the model. The results revealed that the hydrolysis rates for the three substrates were equal, viz. 0.4±0.1 g starch/m^2/hour. Moreover, for the commercial starch not only the hydrolysis efficiency but also the changes within the PSD of the starch was determined several times with the use of light microscopy and image analysis. The obtained experimental PSD showed good similarity with the theoretical PSD from model calculations. This shows that the surface of the particulate substrate is the essential factor for the hydrolysis process.

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2.1 Introduction

Hydrolysis is the first step in the process of anaerobic digestion of organic waste. During hydrolysis, complex particulate compounds are converted into soluble monomeric or dimeric substrates.
Most authors describe the hydrolysis with first order kinetics based on biodegradable substrate and constant pH and temperature (Pavlostathis and Giraldo-Gomez, 1991):

$$\frac{dX_{degr.}}{dt} = -kh.X_{degr.} \quad , \tag{2.1}$$

With:
X_{degr} = concentration degradable substrate (kg/m^3),
t = time (days),
k_h = first order hydrolysis constant (1/day).

This is however an empirical relation and even if the reactor conditions and substrate type are kept constant different k_h's can be found due to changes in the particle size distribution of the substrate (Hills and Nakano 1984, Chyi and Dague 1994).
To gain more insight in the hydrolysis process some authors have tried to develop a deterministic model for the anaerobic hydrolysis (Hills en Nakano 1984, Hobson 1987, Vavilin 1996). In this model, it is assumed that the substrate particles are completely covered with bacteria that secrete the hydrolytical exo-enzymes during digestion. As it is assumed that enzymes are excessively present, the hydrolysis rate is constant per unit area available for hydrolysis. Advantage of this approach is that the hydrolysis constant in this model is not affected by the particle size of the substrate. This model will further be referred to as the Surface Based Kinetics (SBK) model:

$$\frac{dM}{dt} = -K_{sbk} * A \tag{2.2}$$

With:
M = mass of substrate (kg),
t = time (days)
K_{sbk} = surface based hydrolysis constant (kg/m^2 day),
A = surface available for hydrolysis (m^2).

Hobson (1987) investigated the sensitivity of the SBK model for particle size, shape, and degradation pattern by calculating them. From the calculations it can be concluded that if the *loss of radius* with time of the cylindrical or spherical

particles is linear, the curve of the calculated *loss in weight* could also be approximated by the first order relation as given in Eq. 2.1.

Vavilin et al. (1994) tried to verify the SBK model by comparing it with data obtained from literature. The model matched the data quite well, but no quantification of the hydrolysis constants was given. Moreover, Vavilin et al. (1994) found that not only first order or surface related kinetics, but also Contois kinetics were very suitable for describing the hydrolysis process.
By assuming that hydrolysis is the rate limiting step during anaerobic digestion, Hills and Nakano (1984) derived from the SBK model that at a constant initial mass of the substrate the gas production rate is linearly proportional to the inverse particle size. To verify this, they blended 'tomato solid waste' (peels, seeds, stems) and divided it into several fractions with average particle diameters of 2.0 to 0.13 cm. The separate fractions were digested in completely stirred tank reactors at 30°C and 10 days SRT. By introducing a shape factor for the larger particles to correct for the deviation from sphericity, a linear relationship between the gas production rate and the inverse particle diameter was found indeed.

This chapter presents a mathematical description of the surface related hydrolysis kinetics for spherical particles in a batch digestion and a verification of this model with particulate starch as a substrate. Starch was used for verification and validation of the model because it consists of almost spherical particles and is easily biodegradable. For the verification of the model two different experimental approaches were used. The first approach is similar to the approach of Hills and Nakano (1984). From fresh potatoes, two starch fractions were obtained by wet sieving. One fraction consisted of starch particles smaller than 45 μm and the second fraction consisted of particles between 45 and 125 μm diameter. Both fractions were batch digested separately at 30°C with granular sludge as inoculum and the obtained CH_4 production was fitted with the model. In the second approach the entire procedure was repeated but also the particle size distribution (PSD) of the starch was determined before and several times during the digestion with the use of light microscopy and image analysis.

2.2 Materials and Methods

2.2.1 The model

For calculations, most authors assume that particles of a substrate are spherical and are degraded from the outside. If the total mass ($4\pi R^3 n\rho/3$, with n= number of particles and ρ= particle density) and total surface ($4\pi n R^2$) of spherical

particles in a digester are substituted in equation 2.2, the decrease of the average particle radius with time can be written as follows:

$$R_t = R_0 - \frac{K_{sbk} * t}{\rho}, \qquad (2.3)$$

with:
ρ = density of the substrate (kg/m^3),
R_t = average particle radius at time=t (m),
R_0 = average radius at time=0 (m),
t= time (days),
K_{sbk}= surface based hydrolysis constant (kg/m^2.day).

2.2.2 Batch experiments

Two of the three starch containing substrates originated from fresh potatoes. The fresh potatoes were peeled and blended with addition of some destilled water, leaving yellowish slurry containing starch granules and fibres. The fibres were rather large and could be separated from the starch by means of a 250 µm sieve. By wet sieving through a 125 and a 45 µm stainless steel sieve, the starch was divided in 2 fractions containing different particle sizes. A third starch substrate was obtained from Merck (p.a.).

The PSD (table 2.1, figure 2.1) of the three different starches were determined as follows. From the starch a sample was taken and the starch particles were photographed with a light microscope (Olympus BH2, C-35). The lower detection limit for the particle size was 10 µm. The photographs were digitalized and by image analysis (WAU, version 1.0) the total projected surface of each particle in the photographs was determined. By subsequently assuming that the starch particles were spherical, the radius of each particle could be calculated. For the determination of the particle size distribution, 380 particles were taken into account.

The anaerobic hydrolysis of the starch fractions was observed in 3 batch experiments at 30°C. The experiments were performed in 0.25 litre serum-flasks with septum containing screw caps, but they were only filled up to 100ml. The contents of the flasks are given in table 2.1.
The experiments were carried out with granular sludge to simplify the sampling for the benefit of the microscopic and image analysis of the amount of surface. The anaerobic granular sludges used for inoculations in the batch experiments were from different origin but were all adapted to starch (Merck, p.a.) for at least 5 weeks in a continuously fed UASB reactor (30°C, 1 kg COD/m^3.day). Prior to each experiment, some sludge was taken from the reactor and washed once with

destilled water. After filling serum flasks with sludge and starch as stated in table 2.1, also nutrients, trace elements, yeast extract, and 3 g bicarbonate was added. After this, the serum-flasks were filled up to 100 ml with distilled water, flushed with N2, closed and incubated at 30°C. Experiments 1 and 2 were performed 3 times in a period of 2 weeks in duplicate. Twice with whole granular sludge and once with crushed sludge. Because the results of the experiment with the crushed sludge were similar to the whole sludge, the experiment was treated as a duplicate of the experiment with the whole granular sludge. Experiment 3 was performed once in duplicate.

The amount of hydrolysis during the digestion was monitored in two different ways.
In the fresh potato experiments 1 and 2, two flasks with identical filling were used. One flask was attached to a marriotte flask to measure the gas production by liquid (3% NaOH sol., T=30°C) displacement. From the other flask, regularly samples were drawn to analyse the VFA (GC-analysis) and glucose (phenol-sulfuric acid method (Bardley et al. 1971) after 0.45 µm membrane filtration (Schleicher & Schuell).
In experiment 3, multiple flasks with identical filling were used. In these flasks, the produced gas was allowed to accumulate in the headspace. For each sample, one whole bottle was taken, and the accumulated gas was measured by attaching the flask to a marriotte flask. The measurements were done at 20°C. After measurement of the gas, the flask was opened and the water phase was analysed for the VFA and glucose concentration. In the experiments, blanks were included without starch to account for the gas production from the sludge.

In experiment 3, also the PSD of the starch was analysed. During digestion, the starch particles were overgrown with sludge. To visually separate the starch and the sludge for the image analysis the starch was stained purple with a drop of Iodine (Betadine®, Asta Medica).

Table 2.1: *Characteristics of starch and sludges used in the batch experiments*

	origin of sludge	amount of sludge (g TS)	origin of starch	Average diameter (µm)	starch in reactor (mg COD/l)
1	Johma	0.25	Fresh potato	17.9	6600
2	Johma	0.25	Fresh potato	46.6	6600
3	aviko/nedalco mixture	1.6	Merck p.a.	32.1	6800

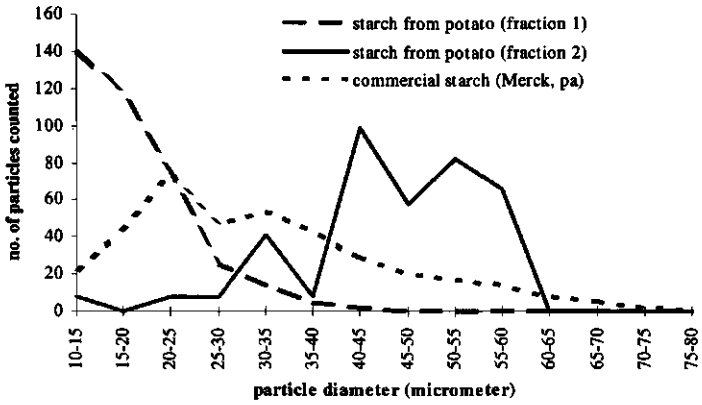

Figure 2.1: *The initial particle size distribution of the three starch fractions used in the batch experiments.*

2.2.3 calculations

In all experiments the efficiency (η) of the degradation at time t was calculated as follows:

$$\eta = \frac{M_0 - M_t}{M_0} * 100\% , \qquad (2.4)$$

With:
M_0 = Total mass of starch added at t=0 (mg COD),
M_0-M_t = Total mass of starch hydrolysed at time t (mg COD),
Mo-Mt = cumulative CH_4 production(t) + (VFA(t)*total volume) + Glucose(t)*total volume),
VFA = volatile fatty acids (mg COD/l),
Glucose= all reducing sugars < 0.45 μm (mg COD/l),
CH_4 production = displaced $NaOH_{(sol.)}$ (g) x conversion factor,
conversion factor: at 20°C 2.67 mg COD/g $NaOH_{sol.}$, at 30°C 2.58 mg COD/g $NaOH_{sol.}$

Substituting the total mass ($4\pi R^3 n\rho/3$) and equation 3 in equation 4 results in:

$$\eta = 1 - \frac{(R_{0,} - \frac{K_{sbk} * t}{\rho})^3}{R_{0,}^3} * 100\%, \qquad (2.5)$$

If the PSD of the substrate has a normal distribution the average particle radius of the substrate can be used in equation 2.5. However, if the substrate does not have a normal distributed PSD each particle has to be considered separately and equation 5 has to be adapted to account for the contribution for each particle:

$$\eta = \sum_{0}^{n} \left(1 - \frac{(R_{0,} - \frac{K_{sbk} * t}{\rho})^3}{R_{0,}^3} \frac{M_0^{particle}}{M_o} \right) * 100\%, \qquad (2.6)$$

with $M_0^{particle}$ = Initial mass of a separate starch particle.

2.3 Results and discussion

The anaerobic hydrolysis of the three starch substrates is depicted in Figures 2.2 to 2.4. As stated before, the experiments 1 (Fig. 2.2) and 2 (Fig. 2.3) were performed three times within a period of two weeks. In that period, the UASB reactor from which the sludge was taken for the experiments was in steady state. Moreover, the substrates and experimental conditions were equal for the three separate experiments. Therefore, the data gathered from the three separate experiments were handled as being obtained from one experiment and presented in Figures 2.2 and 2.3. The figures also show the fit of the Surface Based Kinetics Model to the experimental data. As Fig. 2.1 revealed that the substrates had no normal distributed PSD; equation 2.6 was used for the calculations. K_{sbk} was used as the fit parameter and the initial radius of the particles as depicted in Fig.2.1 was used for the calculations. Furthermore it was assumed that the density of the starch particles was 1.58 kg/dm^3 (Gallant et al., 1997).

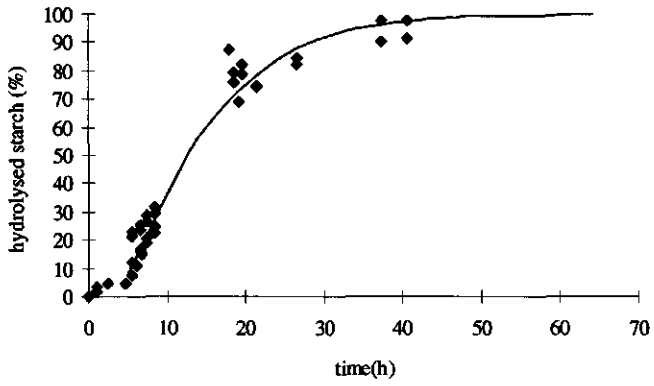

Figure 2.2: *Hydrolysis of the smaller starch particles from fresh potato (fraction 1). Experimental results (◆) and the curve of the model fit. Equation 2.6 was fitted with the experimental results with the least squares method and assuming a lag phase of 4 hours resulting in a surface based hydrolysis constant (K_{sbk}) of 0.4±0.1 g/m²/h.*

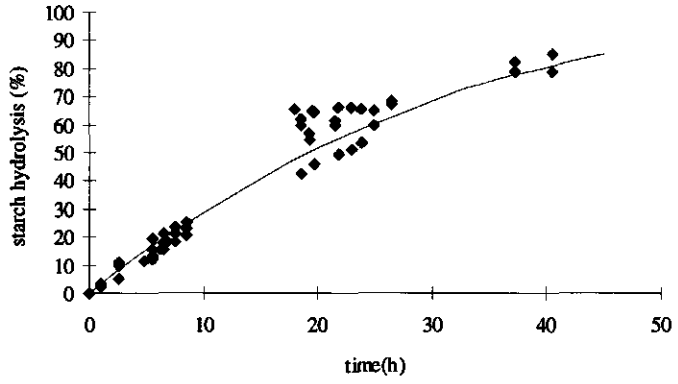

Figure 2.3: *Hydrolysis of the larger starch particles from fresh potato (fraction 2). Experimental results (◆) and the curve of the model fit. Equation 2.6 was fitted with the experimental results with the least squares method resulting in a surface based hydrolysis constant (K_{sbk}) of 0.45±0.05 g/m²/h.*

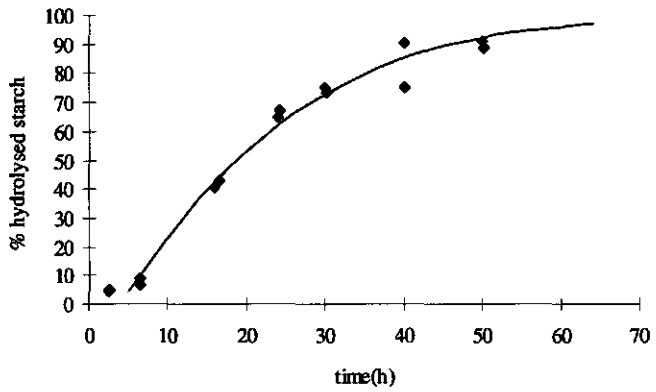

Figure 2.4: *Hydrolysis of the starch particles from the commercial starch (Merck, pa). Experimental results (◆) and the curve of the model fit. Equation 2.6 was fitted with the experimental results with the least squares method and assuming a lag phase of 4 hours resulting in a surface based hydrolysis constant (K_{skh}) of 0.5±0.05 g/m²/h.*

In the experiments with the small starch particles from potatoes a lag phase was observed, while it was not in the experiment with the large starch particles from potatoes. However, in the experiment with the small particles about 2.5 times more substrate surface was available than in the experiment with the larger particles. This implies that already enough enzymes were present in the inoculum sludge to cover the substrate surface in the experiment with the larger particles, but in the experiment with the small particles still some enzymes had to be produced which resulted in a slower hydrolysis during the first few hours of the experiment.

In the experiment with the commercially available starch, also a lag phase was observed although more inoculum sludge was used as compared to the fresh potatoes starch experiments. The experiment with the commercially available starch was performed a few months later than those with the fresh potatoes starch. In the months between the experiments, the sludge in the UASB had to be replaced due to some technical problems. Although the performance of the reactor was similar as before the enzyme activity of the sludge seemed to be less.

The calculated surface based hydrolysis constants that resulted from the fit of equation 2.6 to the experimental data in figures 2.2 to 2.4 were 0.4±0.1 g/m²/h, 0.45±0.05 g/m²/h, and 0.5±0.05 g/m²/h for the small particles from potatoes, the large particles from potatoes, and the commercially available starch, respectively. When these values are compared, considering the experimental error, it has to be concluded that the hydrolysis constants for all three substrates are equal. Therefore the average K_{skh} during anaerobic digestion of particulate starch at 30°C is 0.4±0.1 g/m²/h. From the experimental data also the first order hydrolysis constants were calculated. They were 2.1±0.08, 1.0±0.02, and 1.2±0.05 d^{-1} for the

small particles from potatoes, the large particles from potatoes, and the commercially available starch, respectively. This shows that when the SBK model is used the hydrolysis constant is indeed independent of the particle size of the substrate where as the first order hydrolysis constant is not constant.

For the commercial starch (Fig.2.4) not only the efficiency of the hydrolysis was measured (see Fig. 2.4) but also the changes in the PSD during the experiment. This *experimental* PSD was compared with the *theoretical* PSD. The *theoretical* PSD at t=6.5, 16, 24, and 40 hours after incubation were calculated with equation 3. Here the initial particle sizes as depicted in Fig. 2.1 were used and the K_{sbk} obtained from figure 2.4 (5.0 mg/μm^2/h). The results of both the theoretical and experimental PSD at t=6.5, 16, 24, and 40 hours after incubation are revealed in Fig 2.5. The obtained experimental PSD shows good similarity with the theoretical PSD from the model calculations. This again shows that the SBK model is very suitable for describing the anaerobic hydrolysis of particulate substrates.
The here presented method for PSD has shown a suitable method for the determination of spherical starch particles. The results of the present research show that the particles are fully covered with biomass after a few hours of batch digestion. This implies that the changes within the particle size distribution can only be used for verification of the model if the substrate can be separated from the biomass to determine the particle size distribution. For starch, this can easily be done by staining the starch with iodide. When the changes within the particle size distribution for different types of substrates are to be followed new methods for separation of particles and biomass have to be developed.

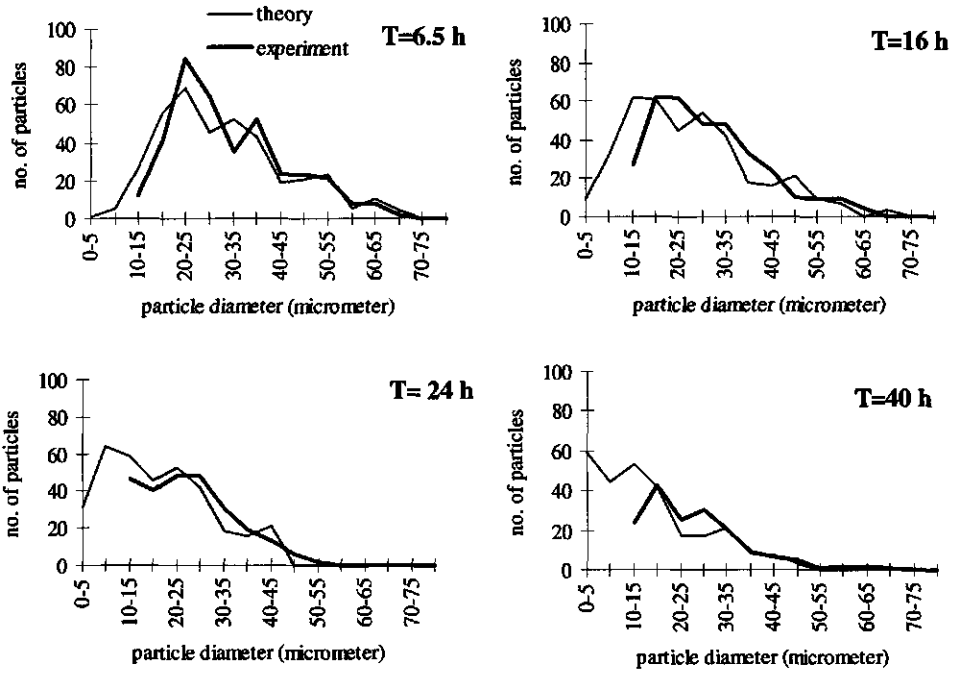

Figure 2.5: *The theoretical and experimental PSD of the commercial starch after 6.5, 16, 24 and 40 hour of digestion. The theoretical PSD was calculated with equation 2.3 and* K_{skb}*=0.5 g/m²/h starting from the initial PSD as given in Fig 2.1. The experimental PSD was determined by image analysis as described earlier.*

2.4 Final discussion

In the SBK model it is assumed that the substrate particles are completely covered with bacteria and the hydrolytical enzymes are excessively present and cover the whole surface of the substrate. Hobson (1987) reported that microscopical observations showed that for continuous digesters the substrate is indeed covered with bacteria and presumably also with enzymes. Microscopical observations in this research show that the substrate in batch reactors becomes also covered with bacteria within a few hours after inoculation. Results of recent research on enzyme activity measurements during anaerobic digestion (not yet published) confirm that enzymes are excessively present. The model also assumes the particles to be spherical and completely biodegradable and considers a constant number of particles. It is therefore only completely valid when used with a substrate such as particulate starch that is not susceptible to breaking up during the digestion, has spherical particles and is completely degradable. The results of the experiments reveal that the surface based hydrolysis constant for starch is constant even when the starch is from different origin and different

inocula are used. This shows that at constant pH and temperature, the amount of surface available for the hydrolysis is the most important parameter in the hydrolysis rate and all other parameters are of minor importance. With substrates that are more complex the particles are not only "peeled" from the surface, but also broken into fine particles. When the breaking of the particles could be included in the present model, breaking should result in a larger surface and therefore an increase of hydrolysis rate (g/l/day). However in practice it has been shown that the hydrolysis can be described by a first order relation (eq. 2.1). Which means that the hydrolysis rate (g/l/day) is not increasing but decreasing with prolonged digestion time. This implies that the break up of the particles does not increase the surface available to hydrolysis. The total amount of surface is apparently not equal to the amount of surface which is available to hydrolysis which might indicate that the fine particles formed during the digestion consist of less biodegradable material.
Because the empirical First Order model does not take the substrate surface into account the first order hydrolysis constants (k_h) obtained from literature can not be extrapolated to similar substrates if the particle size distributions are not comparable. This implies, since the PSD of a substrate is usually unknown, that when using the First Order model the hydrolysis constant has to be determined experimentally for each substrate.

2.5 Conclusions

In this study, the relationship between the particle size distribution of a particulate substrate (starch) and the anaerobic hydrolysis was investigated. From the results is has to be concluded that the hydrolysis rate is directly related to the amount of substrate surface available. For particulate starch the hydrolysis was constant per unit area available viz. 0.4±0.1 $g/m^2/h$.

Moreover, comparison of the particle size distribution obtained from model calculations and experiments showed that the Surface Based Kinetics model is capable to describe the changes within the particle size distribution during anaerobic digestion of particulate starch.

The Surface Based Kinetics model gives a good description of the anaerobic hydrolysis of simple spherical particulate substrates and shows that the amount of substrate surface available for hydrolysis is the essential factor in the hydrolysis rate.

The empirical First Order model does not consider the surface of the substrate. This implies that the use of k_h values from literature for the design of anaerobic reactors for complex substrates is only legitimate when both the biopolymer

composition of the substrate as well as the particle size distribution are comparable to the applied substrate.

3 HYDROLYSIS KINETICS OF DISSOLVED POLYMER SUBSTRATES

Abstract - In this chapter, the relation between the hydrolysis rate of dissolved polymer substrates and sludge concentration was investigated in two ways, viz. by laboratory experiments and by computer simulations.
In the simulations, the hydrolysis of dissolved polymer components was regarded as a general depolymerization process in which the bonds of the parent molecule break randomly until only monomer and dimer components remain.
The results illustrate that for the hydrolysis of dissolved polymer substrates the enzyme activity is the rate-limiting factor. Moreover, a general depolymerisation process can describe the enzymatic hydrolysis of these components.

3.1 Introduction

The previous chapter describes the results of research on the hydrolysis of particulate substrates. The results reveal that the rate of hydrolysis of particles is limited by the amount surface available to the hydrolytic enzymes. When dealing with dissolved substrates the available amount of surface is much larger and corresponds to the total amount of substrate. Therefore it is very likely that in the case of dissolved polymer substrates the amount of active enzymes plays a role. As research dealing with the kinetics of the hydrolysis of dissolved compounds has indicated that the hydrolytic enzymes are located on the sludge (Goel et al. 1998, Confer and Logan 1998a) the sludge concentration could affect the hydrolysis rate of dissolved polymer components. Some authors (Negri et al. 1993, Munch et al. 1999) have therefore included the biomass concentration in their models for the hydrolysis of dissolved compounds, but their approaches remain merely empirical.

San Pedro (1994) investigated the hydrolysis of dissolved starch and azocasein by *Bacillus Amyloliquefaciens* and *Aeromonas hydrophila*. The results reveal that the hydrolysis of these dissolved polymers can be described by empirical first order kinetics (Eastman and Ferguson, 1981). For dissolved starch a linear relationship between the first order hydrolysis constant and the biomass concentration was established. However for azocasein no relation between the biomass concentration and the first order hydrolysis constant seemed to exist. Goel et al. (1998) investigated the hydrolysis of dissolved starch by waste activated sludge. They suggested a type of Michaelis-Menten kinetics for the hydrolysis of dissolved components instead of the first order kinetics.

Confer and Logan (1997a, 1997b, and 1998a) found that during the hydrolysis of bovine serum albumin and dextran, fragments of these polymers accumulated in the water phase. Based on their results they presented a conceptual model for the hydrolysis of dissolved polymer substrates. In this model, it is proposed that the hydrolysis of the macromolecules can be divided into three steps:
1 Large macromolecules diffuse to the surface of the cell.
2 Next, hydrolysis proceeds on the cell-surface resulting in release of polymer fragments.
3 The polymer fragments diffuse away from the cell, either to the bulk solution or to other cells.
These three steps repeat until the polymer fragments are sufficiently small to be converted by bacteria. Because the hydrolysis is assumed to take place at the cell surface also in this concept the sludge concentration might play an important role in the hydrolysis rate.

In this chapter, the relation between the hydrolysis rate and sludge concentration was investigated in two ways, viz. by laboratory experiments and by computer simulations. In the simulations, the hydrolysis of dissolved polymer components was regarded as a general depolymerisation process. Chemical engineers are already engaged for more than 60 years in research on this

depolymerisation process of long chain molecules. Several authors have attempted to predict the evolution of the size distribution of the macromolecules during the degradation process (Montroll and Shima 1940, Ziff and McGrady 1986, Kostouglo 2000). This approach not only allowed for investigation into the effect of the enzyme activity, but also on the occurrence of polymer fragments during the degradation process as was observed by Confer and Logan (1997a, 1997b, and 1998a).

3.2 Methods

3.2.1 Lab experiments.

Goel et al. (1998) carried out research into the relation between the hydrolysis rate, substrate concentration and biomass concentration by using batch experiments. Two batch experiments were conducted with dissolved starch as substrate and activated sludge as the inoculum. The activated sludge was harvested from a sequencing batch reactor fed with dissolved starch. In the first experiment, the relation between the hydrolysis rate and the biomass concentration was assessed. In this experiment, enzyme assays were conducted at different sludge concentrations and a constant starch concentration. In the second experiment, the sludge concentration was kept constant at different concentrations of starch in order to assess the relation between the hydrolysis rate and the initial substrate concentration.
In the here presented research similar experiments were carried out, but with a protein, gelatine (Merck, pa), as a substrate.
The sludge used in the experiments was fresh waste activated sludge taken from a pilot plant for biological nitrogen and phosphorus removal. This pilot plant was fed with domestic wastewater at a loading rate of 0.1 kg COD/kg TSS/day.
The relation between the initial substrate concentration and the hydrolysis rate was assessed in 250 ml serum-flasks all filled with 10 g TSS/l of fresh activated sludge. The initial gelatine concentration in the serum-flasks ranged from 1 to 4 g gelatine/l. The effect of the sludge concentration on the hydrolysis rate was determined in a second experiment, using activated sludge diluted with demi-water to make a concentration range between 3 and 11 g/l TSS. At the start of the experiment a gelatine stock solution was added to a final concentration of 2 g/l gelatine. Both experiments were performed in duplicate. After filling the flasks they were flushed with N_2 for 3 minutes and incubated at 30°C on a wrist action shaker. Paper filtered samples (Schleicher & Schuell 595½) were taken 2.4, 3.3 and 6.0 hours after incubation and immediately after sampling the protein concentration of the samples was determined by Biuret method (Herbert et al. 1971) with gelatine as standard. Goel et al. (1996) gave no definition of the initial hydrolysis rate. In this research the initial hydrolysis rate was calculated according to equation 3.1.

$$initial\ hydrolysis\ rate = \frac{(S_0 - S_1)}{\Delta t} \tag{3.1}$$

With;

S_0 = gelatine concentration at the start of the experiment (g/l),

S_1 = gelatine concentration in the first sample that is taken from the serum-flask (g/l),

Δt = amount of time between the start of the experiment and the time at which sample S_1 is taken (h).

3.2.2 The depolymerisation model

As mentioned earlier several authors have derived equations to describe the depolymerisation of polymers. In the approach of Ziff and McGrady (1986) the breaking of the polymer bonds does not occur randomly but the bonds close to the centre of the chains break preferentially. The equation presented by Ziff and McGrady can therefore be used to describe the hydrolysis of macromolecules by endo-enzymes, which prefer to cut the bonds towards the middle of the molecule. In contrast, Kostoglou (2000) derived equations to describe the degradation of polymers by chain-end scission, in which the bonds on the end of the chain break more easily. This approach can be used to describe the hydrolysis of macromolecules by exo-enzymes, which prefer to cut the bonds near to the edges of the macromolecule. However, during anaerobic digestion a mixture of different enzymes are present and both endo and exo enzymes will perform the hydrolysis. As the ratio between the endo and exo enzymes during the digestion is unknown it is assumed that the activity of both enzymes is equal. Consequently, all bonds in the macromolecule have the same chance of being cut. Summation of the model of Ziff and McGrady (1986) and Kostoglou (2000) will result in a model in which the polymer bonds break randomly and independently of their position in the chain. Montroll and Shima already presented this model in 1940.

In their theory of depolymerization of long chain molecules Montroll and Shima (1940) made the following assumptions:

1. All molecules originally have the same molecular weight.
2. The accessibility to reaction of a bond in a given chain is independent of the position in the chain and independent of the length of its parent chain.
3. All chains in the mixture are equally accessible to reaction.

By applying statistical calculations they derived the following equations for the fraction of monomers present in chains with a length q when an average number of cuts (r_0) has been made to each macromolecule with a length of p+1 monomers.

$$\Phi_q(p,\alpha) = \frac{\alpha q(1-\alpha)^{q-1}}{(p+1)}[2+(p-q)\alpha] \qquad q \le p, \alpha \le 1 \tag{3.2}$$

$$\Phi_{p+1}(p,\alpha) = (1-\alpha)^p \tag{3.3}$$

$$\alpha = \frac{r_0}{p} \qquad \alpha \le 1 \tag{3.4}$$

with
p = number of bonds in the parent macromolecule,
α = average degree of polymerization, $\alpha \in [0;1]$,
r_0 = average number of scissions made to a macromolecule,
q = number of monomers in the macromolecule under consideration,
Φ_q = fraction of monomers involved in chains with a length of q monomers,
Φ_{p+1} = fraction of monomers involved in chains with a length p+1.

So, for instance for a solution of macromolecules with an initial length of 500 monomers, calculations using equation 3.2 show that when an average of 5 scissions have been made to these molecules still 0.66% of the initial number of monomers is present in chains with a length of 500 (eq 3.3, p=499, α=5/499) and 0.44% of the monomers is present in chains with a length of 100 monomers (eq 3.2, p=499, α=5/499, q=100).

Hydrolysis is defined in this thesis as the conversion of polymer substrates to merely monomer and dimer components. The hydrolysed fraction ($\Phi_{hydr.}$) can be calculated by the sum of $\Phi(p,\alpha)$ at q=1 and q=2 (equation 3.5).

$$\Phi_{hydr.}(p,\alpha) = \frac{2\alpha + \alpha^2(p-1) + 4\alpha(1-\alpha) + 2\alpha^2(p-2)(1-\alpha)}{(p+1)} \tag{3.5}$$

For introducing the aspect of time it is assumed that the enzymes executing the hydrolysis have a constant activity (E). This activity can be expressed as the number of scissions to each macromolecule per unit of time and is similar to V (or dS/dt) in the Michaelis-Menten kinetics (eq 1.5). By introducing the enzyme activity in Equation 3.4, α becomes time dependent according to equation 3.6:

$$\alpha = \frac{E.t}{p} \qquad \alpha \leq 1 \tag{3.6}$$

with
E = enzyme activity (scissions per macromolecule(spm)/h),
t = time (h).

So far, it was assumed that all polymers have the same initial chain length. However, in wastewater the initial length of the molecules varies over a certain range. The evolution in the chain lengths of a distribution with molecules between 3 and n monomers can be calculated by the sum of each separate chain length. Moreover, each chain length has to be multiplied by the factor n_p, comprising the fraction that each chain length contributes to the total distribution range (Eq. 3.7). The hydrolysed fraction is calculated in a similar way (Eq. 3.8). Equation 3.7 and 3.8 are further in this paper referred to as the depolymersisation model.

$$\Phi_{q,distribution}(p,\alpha) = \sum_{p=3}^{p=n} n_p \frac{\alpha q(1-\alpha)^{q-1}}{(p+1)}[2+(p-q)\alpha] \qquad q \leq p, \alpha \leq 1 \tag{3.7}$$

$$\Phi_{hydr.,distribution}(p,\alpha) = \sum_{p=3}^{p=n} n_p \frac{2\alpha + \alpha^2(p-1) + 4\alpha(1-\alpha) + 2\alpha^2(p-2)(1-\alpha)}{(p+1)} \qquad \alpha \leq 1 \tag{3.8}$$

with:

$$n_p = \frac{\text{no. of monomers in chains with length p + 1}}{\text{total no. of monomers in distribution range}} \quad \text{and}$$

$$\alpha = \frac{E.t}{p} \qquad \alpha \leq 1$$

3.2.2 The substrate for the model calculations

To simulate the hydrolysis of a wastewater containing polymer components, with equation 3.7 and 3.8 a substrate with a known polymer size distribution was needed. The polymer size distribution of the gelatine used in the batch experiments described in section 3.2.1 was unknown. Therefore another

substrate had to be searched. Confer and Logan (1997b) and Carlson and Silverstein (1998) both used dextran 70k as a model substrate in their laboratory experiments. Moreover, Carlson and Silverstein gave a rough estimation of the polymer size distribution of this compound. For the model simulations, it was therefore assumed that the model substrate resembled dextran 70k. A suitable distribution (Table 3.2, Fig 3.1) was calculated based on the following assumptions:

- 1.0k is approximately 7 monomeric units (Confer and Logan, 1997b).
- the chain length distribution resembles a Normal Distribution.
- the dissolved organic carbon (DOC) is distributed as depicted in table 3.1 (Carlson and Silverstein, 1998).
- chains smaller than 0.5k are considered to be hydrolysed.
- chain lengths are packed together in groups at intervals of 10 monomeric units.

The results of the simulations were divided in three fractions to allow comparison with the results from the laboratory experiments and those obtained by Confer and Logan (1997a, b).

- hydrolysed components (Φ_{hydr}), which is the fraction of monomeric components present in chains of length 1-2 monomeric units.
- intermediate size components ($\Phi_{7\text{-}70}$), which is the fraction of monomeric components present in chains of length 7-70 monomeric units or 1-10k (Confer and Logan 1997b).
- small size components ($\Phi_{3\text{-}6}$), which is the fraction of monomeric components present in chains of length 3-6 monomeric units or 0.5-1k.

Table 3.1: *Size distribution of Dextran 70k (Carlson and Silverstein, 1998)*

molecular size	% of total DOC
<0.5 k	3
0.5-1 k	-
1 - 3 k	-
3-10 k	12
10-30 k	80
30-100 k	5

Table 3.2: *The chain length distribution, n_p and p of the model substrate used for simulation of the hydrolysis of dissolved polymer substrate with equation 3.7 & 3.8.*

k	% of DOC	chain length	% of monomers	n_p	p
3		20	0,75	0,0075	19
4		30	1	0,01	29
6	12,0	40	1,5	0,015	39
7		50	2	0,02	49
9		60	3	0,03	59
10		70	3,75	0,0375	69
11		80	4,5	0,045	79
13		90	5,5	0,055	89
14		100	6,5	0,065	99
16		110	7	0,07	109
17		120	7,5	0,075	119
19		130	7,75	0,0775	129
20		140	8	0,08	139
21	80,0	150	7,75	0,0775	149
23		160	7,25	0,0725	159
24		170	6,5	0,065	169
26		180	5,05	0,0505	179
27		190	3,45	0,0345	189
29		200	2	0,02	199
30		210	1,25	0,0125	209
31		220	1	0,01	219
33		230	1	0,01	229
34		240	1	0,01	239
36		250	1	0,01	249
37	5,0	260	1	0,01	259
			$\Sigma = 100$	$\Sigma = 1$	

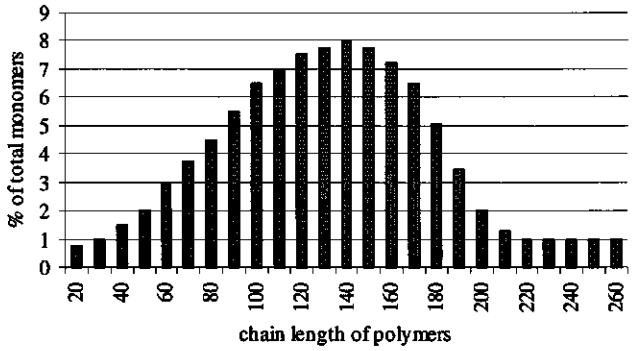

Figure 3.1: *Graphical reproduction of the chain length distribution of the model substrate in table 3.2.*

3.3 Results and discussion

3.3.1 The relation between the hydrolysis rate and the sludge concentration

Results of the lab experiments

Goel et al. (1998) investigated the hydrolysis rate of dissolved starch at several sludge and substrate concentrations in a batch experiment (methods described in 3.2.1). From the results obtained, it appeared that the initial hydrolysis rate was linear related to the sludge concentration (R^2 =0.9288). The relationship found between the initial hydrolysis rate and the substrate concentration is of a saturation type, in which the inverse initial rate was linear to the inverse substrate concentration (R^2 =0.9998). Goel et al. (1998) proposed a Michaelis-menten type of equation for the hydrolysis of dissolved polymers:

$$\frac{dS}{dt} = k_h \frac{S}{k_x + S} X \qquad (3.9)$$

With:
S= substrate concentration (g/l),
kh =maximum hydrolysis rate (l/g/h),
kx = half rate constant (g/l),
X = sludge concentration (g/l),
t = time (h).

The results of the here presented research reveal that similar relations prevail for the hydrolysis of gelatine (Fig 3.2 and 3.3). Results of the Lineweaver-Burk plot (Fig 3.3) show that the values for the k_h and k_x for the anaerobic hydrolysis of gelatine by the fresh activated sludge were 0.212 l/g TSS/h and 25.95 g/l (X=10 g/l), respectively.

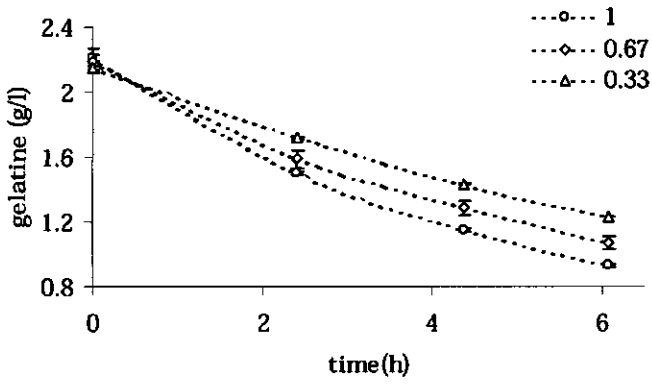

Figure 3.2: *The course of the gelatine concentration during the batch digestion experiment at several relative sludge concentrations.*

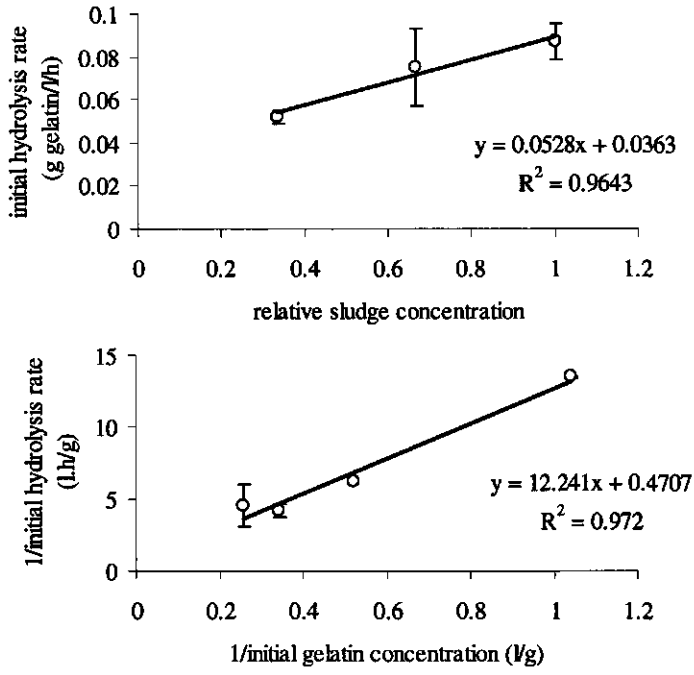

Figure 3.3: *Graphical presentation of the results of the anaerobic hydrolysis of gelatine in the batch experiments at 30°C. The upper graph presents the relation between the sludge concentration and the initial hydrolysis rate. The bottom graph is the Lineweaver-Burk plot for the initial hydrolysis rate and the gelatine concentration.*

Results of the model simulations

The results of the simulation of the hydrolysis (eq 3.8) of the model substrate during batch digestion at several enzyme activities (Fig 3.4) show that obviously the hydrolysis is faster at higher enzyme activity. Moreover, the relation between the initial hydrolysis rate and the enzyme activity (Fig 3.5) is linear.

In the depolymerisation model (eq 3.7 & 3.8) presented in this chapter the enzyme activity (E) depends on the enzyme concentration. As the enzymes that perform the hydrolysis are attached to the cells (Confer and Logan 1998a, Goel et al.1998), the enzyme activity will be directly related to the sludge concentration. Consequently the initial hydrolysis rate calculated with the depolymersiation model is also linearly related to the sludge concentration. The depolymerisation model is therefore capable to describe the relationship between the initial hydrolysis rate and the sludge concentration as it was assessed in batch experiments (Goel et al. 1998 & Fig 3.3).

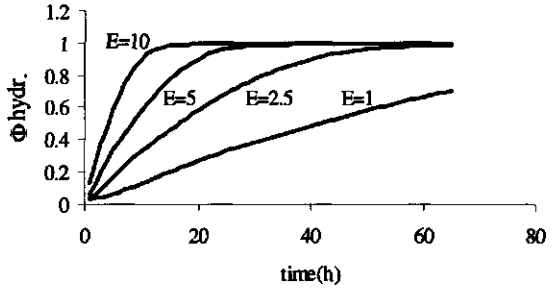

Figure 3.4: *Hydrolysis of the model substrate at increasing enzyme activity(E in spm/h) as simulated using equation 3.8*

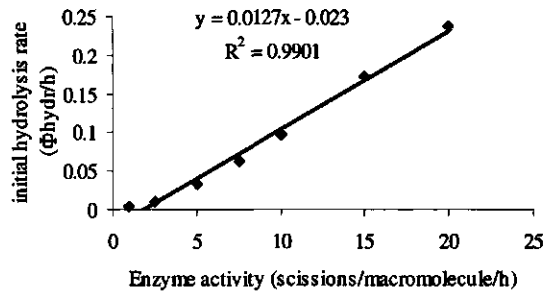

Figure 3.5: *Relation between initial hydrolysis rate and enzyme activity as calculated from the results of the model simulations as depicted in Fig .3.4*

The relation between the substrate concentration and the hydrolysis rate during the simulated degradation of 1-g/l of the model substrate at different enzyme activities is presented in Figure 3.6. The results illustrate that in accordance with the results of Goel et al.(1998) and the gelatine experiments (Fig 3.3) as described in this paper, the inverse hydrolysis rate as it was calculated with the depolymerisation model is linear related to the inverse substrate concentration.

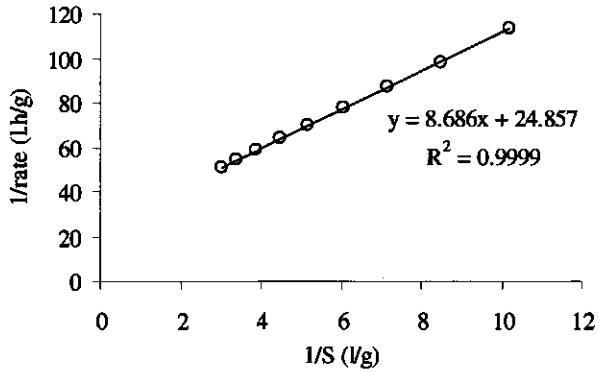

Figure 3.6: *Lineweaver-Burk plot of relation between substrate concentration and the hydrolysis rate during the degradation of 1 g/l of the model compound (Table 3.2, Fig 3.1) as simulated by the depolymerisation model (Eq 3.7) at E=2.5 spm/h.*

3.3.2 accumulation of hydrolysis intermediates

Confer and Logan (1997a,b) reported accumulation of hydrolysis intermediates during the degradation of dextran, dextrin and bovine serum albumin. Especially an increase of components smaller than 1k (1-6 monomers) was observed. The changes in the intermediate size fraction (7-70k) were only small. The latter was attributed to the fact that the substrate already contained a significant amount of intermediate components.
Figures 3.7 and 3.8 reveal the accumulation of small size (3-6 monomers) and intermediates size (7-70 monomers) hydrolysis intermediates as they appeared from simulations with the depolymerisation model. The figures show accumulation of intermediate and small size components at both enzyme conditions. The maximum amounts that accumulated are 0.736 and 0.322 for the intermediate and small size fraction, respectively. Although the height of the peaks is equal for both conditions the maximum is reached within a shorter time at higher enzyme activity. Moreover, the small size fraction remains longer detectable than the intermediate size.

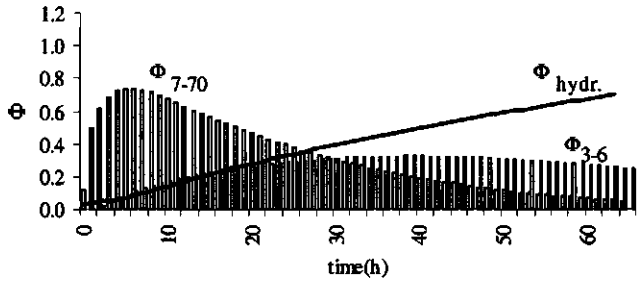

Figure 3.7: *Hydrolysed ($\Phi_{hydr.}$), intermediate (Φ_{7-70}) and small size fraction (Φ_{3-6}) during the degradation of dissolved polymers with initial chain length distribution as given in table 3.2 and Fig 3.1. At an average enzyme activity (E) of 1 spm/h*

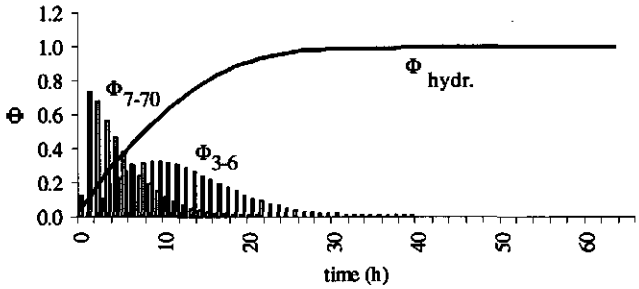

Figure 3.8: *Hydrolysed ($\Phi_{hydr.}$), intermediate (Φ_{7-70}) and small size fraction (Φ_{3-6}) during the degradation of dissolved polymers with initial chain length distribution as given in table 3.2 and Fig 3.1. At an average enzyme activity (E) of 5 spm/h*

3.4 General discussion

Results of the present batch experiments and those of Goel et al. (1997) with starch and gelatine reveal that for the hydrolysis of dissolved polymer components:

- the initial hydrolysis rate and sludge concentration are linearly related,
- the inverse initial hydrolysis rate and inverse initial substrate concentration are linearly related.

The here presented depolymerisation model describes the enzymatic hydrolysis of dissolved polymer compounds as a general depolymerisation process. The results of the simulations with the model show similar relations between the hydrolysis rate, sludge concentration and substrate concentration as shown from the experimental results. Moreover, accumulation of hydrolytic fragments as obtained by Confer and Logan (1997a,b) during the degradation of dextran, dextrin and bovine serum albumin could also be simulated by the model. From these results it is clearly illustrated that unlike particulate substrate, the hydrolysis of dissolved polymers is limited by the enzyme activity. Moreover, it is shown that the mechanism of the enzymatic hydrolysis can be described by a random depolymerisation process.

The depolymerisation model proved to be a good tool to clarify the mechanism of the hydrolysis of dissolved polymer compounds. However, the depolymerisation model requires a detailed knowledge on the composition of the substrate. The single compounds in the substrate as well as their chain length distribution have to be known. This limits the applicability of the model to wastewater with simple substrates. The model as presented by Goel et al. (1998) seems more feasible for a practical application, as it requires a less detailed knowledge on the influent. However, the model has so far only been used for batch experiments and the hydrolysis kinetics in continuously fed systems, especially the relation between the loading rate of the reactor and the enzyme activity, still has to be investigated. The latter also applies to the use of the first order kinetics and the probability model.

'Real' complex wastewater often contains, besides dissolved polymers, also particulate substrate. The hydrolysis of the dissolved polymers is much faster than the hydrolysis of the particles. For example, for a complete hydrolysis of gelatine in a UASB reactor a HRT of 0.5h (at pH=7 and 35°C) seems enough (Breure et al., 1985) as for particulate protein under the same conditions a retention time of several days is needed (Palenzuella-Rollon, 1999). Because the degradation of the particles is the rate-limiting factor, the reactor design should be based on the hydrolysis rate of the particles.

3.5 Conlusions

- The initial hydrolysis rate of dissolved polymers is linearly related to the sludge concentration.
- The inverse hydrolysis rate and inverse initial concentration of dissolved polymers are linearly related.
- The hydrolysis kinetics of dissolved polymer macromolecules appears to proceed as follows:
  1) Large macromolecules diffuse to the surface of the cells. 2) At the surface of the cells the bonds of the macromolecules are cut and 3) hydrolysis fragments are released into the bulk liquid. This process follows the general principles for depolymerization of macromolecules in which the bonds of the parent molecule break randomly until only monomer and dimeric components remain.

4 THE HYDROLYSIS OF LIPIDS

Abstract - The effect of the absence of methane production with respect to the rate of hydrolysis of neutral lipids was studied. The results show that under acidogenic conditions the hydrolysis of neutral lipids was lower as compared to methanogenic conditions. This lower volumetric hydrolysis rate under acidogenic conditions could not be ascribed to a low pH, accumulation of long chain fatty acids or accumulation of di- or mono glyceride. The results indicate that there is a relation between the hydrogen concentration and the volumetric hydrolysis rate. However, as the mechanism of this relation remains unexplained, it could be an indirect effect. Moreover, calculations showed that with a constant surface based hydrolysis rate of 0.19 mg COD/m^2/day the decrease of the available lipid-water interface was less under methanogenic conditions. This indicated that the production of biogas could have a positive effect on maintaining the lipid-water interface and subsequently cause a higher volumetric hydrolysis rate.

Submitted for publication in Water Research as: Sanders, W.T.M, Bosch, M. van den, Leenders, H., Zeeman, G., Lettinga, G., Hydrolysis of neutral lipids during anaerobic sludge digestion.

4.1 Introduction

Approximately 15-20% of the total solids in sewage sludge consist of fat (Zeeman, 1991). The bulk of these fats are triglyceride esters also called triacylglycerols or neutral lipid (Viswanathan et al., 1962). During hydrolysis, a triglyceride is converted to glycerol and three long chain fatty acids (LCFA).
Hanaki et al (1981) digested whole milk at 37°C in batch reactors with seed sludge, previously adapted to the whole milk. In their experiments 90% (950-mg lipid/l) of the triglycerol in the milk was hydrolysed to LCFA within one day. The reactor was at acidogenic conditions because almost all of the lactose in the milk was acidified, but less than 20% of the produced volatile fatty acids (VFA) was converted into CH_4. Moreover, it took 7 days before most of the LCFA was converted into VFA and CH_4. This suggests that during the anaerobic degradation of triglycerols the conversion of LCFA to VFA is the rate limiting step and the hydrolysis of triglycerols proceeds quickly.
However, some authors have reported problems with hydrolysis of neutral fats especially at acidogenic conditions. Heukelian and Mueller (1958) followed the composition of the lipids in seeded and unseeded batch digestion experiments with sewage sludge. In the unseeded batch experiment, the pH remained below 5.8 during the experiment and gas production was poor. Only 28% of the esters of fatty acids were hydrolysed within 20 days at 20°C. The long chain fatty acids (LCFA) were not degraded. In the seeded batch digestion experiment (with digested sewage sludge), the pH remained always above 6.2 and gas production was good. 50% of the esters of fatty acids were hydrolysed in the first 5 days and 60% in 40 days. The LCFA were further degraded to CH_4. In both the seeded and unseeded batches, synthesis of esters of fatty acids was observed.
Angeldaki and Ahring (1992) observed poor hydrolysis of glycerol trioleate under acidogenic conditions. They inoculated a suspension of 4.4-g/l glycerol trioleate in a nutrient medium with 5% (v/v) digested cattle manure at 55°C for 2 months. Only 30% of the glycerol trioleate was hydrolysed to LCFA and glycerol and 10% was converted into methane. LCFA and VFA increased up to 6mM and 10mM, respectively.
Miron et al. (2000) conducted digestion experiments with primary sludge at 25°C in completely stirred tank reactors at different SRT's. Their results revealed that under acidogenic conditions the hydrolysis of the neutral lipids present in the sludge proceeded relatively fast at an SRT of 3 days but at higher SRT started to decline (SRT 3-8 days). However, the rate of liquefaction increased once methanogenic conditions (SRT 8-15 days) established in the system.

Based on information from literature two hypothesis can be formulated to elucidate the lower volumetric hydrolysis rate of neutral lipids under acidogenic conditions as compared to methanogenic conditions. Firstly, the lipase activity at acidogenic conditions could be lower than at methanogenic conditions due to a low pH or

accumulation of hydrolysis products or intermediates, such as LCFA or mono-glycerides. Secondly, a lower bioavailability could exist under acidogenic conditions. The latter can be amplified by observing the physical chemistry of lipid hydrolysis. Neutral lipids are hydrophobic and consequently will therefore stay in the original emulsion or adsorb to the sludge during the digestion. The hydrolysis of the neutral lipids only takes place at the lipid-water surface (Deeth and Fitz-Gerald, 1983). The free LCFA produced from the neutral lipids consist of a hydrophobic chain of 12 to 16 carbon atoms with a more hydrophilic carboxylic acid group on the end. Because of this physical property, the free LCFA will prefer the lipid-water interface instead of moving into the bulk solution. If the conversion of this free LCFA is slow, the LCFA could form a physical barrier between the neutral lipids and the hydrolytic enzymes or alter the surface tension of the lipid (Verger and de Haas,1976, Rietsch et al. 1977, Verger 1980). The hydrolysis rate will then be affected by covering a part or even the total surface of the emulsion (Fig 4.1).

The gas production itself could also effect the hydrolysis rate. By analogy with the homogenisation of lipids in milk (Walstra, 1983), where the lipid particles are decreased in size by aeration, the gas production could cause smaller emulsion particles to occur. In the presence of an excess amount of enzymes the hydrolysis rate is determined by the amount of surface, that is available (Hills and Nakano, 1984). Smaller particles and thus a larger surface area could therefore result in a higher hydrolysis rate under methanogenic conditions.

In the research, performing batch digestion experiments, the presented hypotheses are tested. For the conversion of neutral lipid at acidogenic and methanogenic conditions, the hydrolysis of palm oil, butter and tripalmitin, was observed under both conditions. Moreover, the effect of different initial palmitic acid concentrations, the composition of the triglyceride, and enzyme activity, during the digestion of tripalmitin was observed.

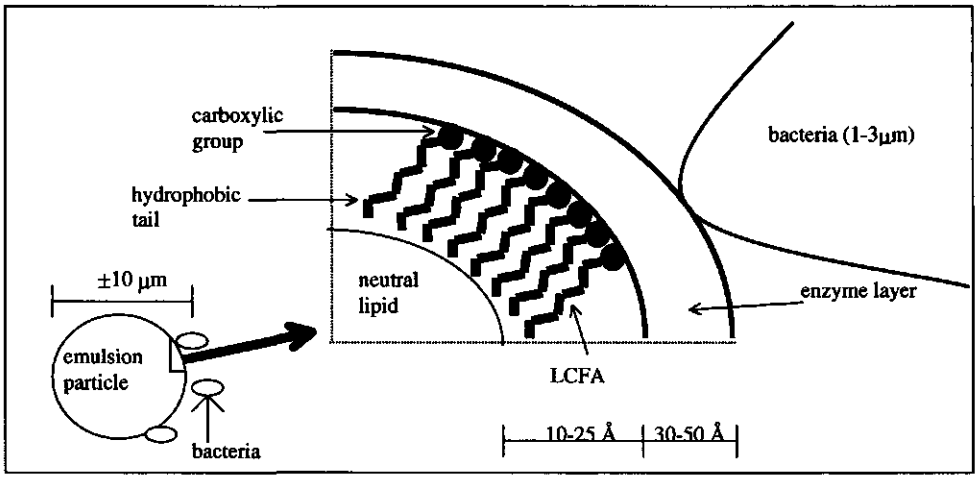

Figure 4.1: *A schematic presentation of the possible physical barrier between the neutral lipid and the lipolytic enzymes due to accumulation of LCFA at the emulsion surface (dimensions are merely indicative).*

4.2 Materials and Methods

4.2.1 Set up of the batch experiments

In experiments with lipids, it is difficult to take a representative sample due to the fact that part of the lipid attaches to the reactor wall. Therefore, in the present experiment a procedure was selected in which a set of serum-flasks with identical contents were used as batch reactor instead of employing one large reactor. Each serum-flask therefore represented the equivalent of a single batch reactor. The flask were incubated statically in an incubator at 30°C. At different time intervals after the start of the experiment, one of the serum-flasks was analysed for neutral lipid, LCFA, and VFA. One of the serum flasks of a specific experiment remained closed throughout the whole experiment. From this flask, the gas headspace was regularly sampled through the rubber septum in the cap by means of a gas chromatographic syringe to determine the percentage of CH_4 and H_2.

The experiments conducted concerned:
1. The effect of acidogenic conditions on the hydrolysis of emulsified palm oil, butter and tripalmitin in batch reactors. The composition of the contents of the batches is shown in Table 4.1. The palm oil and butter were emulsified as

described by Rinzema (1988). Microscopy showed that the average radius of the emulsion droplets was 5 μm. All batches were seeded with a flocculent type of sludge from a UASB reactor treating domestic wastewater (TSS 42.5 g/l, VSS 26.1 g/l). To induce and maintain acidogenic or methanogenic conditions, one set of serum flasks received 2-bromoethane-sulfonic acid sodium salt (BESA), an inhibitor of methanogenic activity, in order to impose acidogenic conditions. The other set of batches was supplied with extra methanogenic activity in the form of 1.5-g VS/l granular sludge that originated from a UASB reactor treating wastewater from the paper industry. All batches received nutrients and trace elements according to Rinzema (1988). The analysis for the CH_4 production and VFA concentration were made after time intervals of 1, 2, 3, 4, 7, 9, 11, 14 and 18 days. The LCFA and triglycerol concentrations in the experiment with the butter were analysed at day 0, 1, 7, and 18 and for the experiment with palm oil at day 0, 1, 3, 7, 14 and 26. In the latter experiment, the lipase activity was also measured, viz. at day 0, 2, 6, 12 and 16. In the experiment with tripalmitin, the amount of tri, di and monoglycerids was analysed at day 2, 7, 10, 16, 26, 33 and 36.

2. Effect of palmitic acid on the hydrolysis of tripalmitin. In this experiment tripalmitin and/or palmitic acid was digested using digested primary sludge as a seed sludge (WWTP Ede (NL), 30°C, CSTR with HRT approx. 30 days, TSS 24.5 g/l, VSS 15.3 g/l). For this purpose, eight sets of serum flasks were used. In four of these sets tripalmitin was batch digested in the presence of different amounts of palmitic acid. In the other four sets only palmitic acid was used (Table 4.2). In addition to the digested primary sludge, also nutrients and trace elements were added to the batches (Rinzema, 1988). The experiment was performed in duplicate. During the experiment the CH_4 production, LCFA, triglycerol and VFA concentration were analysed after 1, 3, 5, 7, 14, 22 and 36 days of digestion.

Table 4.1: *contents of the batches for assessment of the effect of acidogenic and methanogenic conditions on the digestion of emulsified palm oil and butter. All batches were seeded with 400-ml UASB sludge, nutrients, and trace elements. (Experiment 1)*

Batch no.	neutral fat	Extra additives
1	butter, 5.2 g COD/l	25 mmol/l BESA
2	butter, 5.2 g COD/l	1.5 g VS/l granular sludge
3	palm oil, 6.1 g COD/l	25 mmol/l BESA
4	palm oil, 6.1 g COD/l	1.5 g VS/l granular sludge
5	tripalmitin 12 g COD/l	25 mmol/l BESA
6	tripalmitin 12 g COD/l	1.5 g VS/l granular sludge

Table 4.2: *Contents of the batches for assessment of the effect of LCFA on the digestion of tripalmitin. All batches were seeded with 200ml digested primary sludge. All batches received Also nutrients and trace elements. (Experiment 2)*

Batch no.	tripalmitin (g COD)	palmitic acid (g COD)
1	-	-
2	-	0.04 (200 mg COD/l)
3	-	0.12 (600 mg COD/l)
4	-	0.24 (1200 mg COD/l)
5	2.45	-
6	2.45	0.04 (200 mg COD/l)
7	2.45	0.12 (600 mg COD/l)
8	2.45	0.24 (1200 mg COD/l)

4.2.2 Analytical methods.

LCFA and VFA analysis

As mentioned before each sample was prepared from one serum flask. For analysis of the amount of neutral lipid, LCFA and VFA the content of each serum flask was centrifuged for 15min at 2000rpm. As the liquid phase still contained some suspended material it was subsequently filtered (Schleicher &Schuell 595½) after which 1 ml of the liquid phase was used for VFA analysis. The filter with solids and solid phase from the centrifuge tube were quantitatively transferred into a soxhlett extraction thimble (Schleicher & Schuell, extraction thimbles 33x118 mm) which was dried for 14 h at 80°C. The empty serum flask was also dried. The dry flask was rinsed with 150 ml of petroleum ether (40-60, Baker) to extract the fat that was adsorbed to glass wall. This petroleum ether was subsequently used to extract the fat from the dried solids in the soxhlett extraction thimble. After 4 hours of soxhlett extraction (APHA, 1992) and evaporation of the petroleum ether, the total amount of fat was determined gravimetrically. The amount of LCFA in the extracted fat was analysed as proposed by Kaluzny et al. (1985). According to their method the extracted fat was dissolved in chloroform and then transferred on a solid phase extraction column (Varian Bond Elute, bonded phase NH_2). By elution using chloroform-2-propanol (2:1) first, the neutral lipids were removed from the column. Next, the free LCFA were eluted using a 2% acetic acid solution in diethylether. After the elute was evaporated from the collected sample the LCFA were esterified by 14% BF_3 in methanol. After extraction with petroleum ether, the samples were ready for chromatographic analysis. For determination of the LCFA content of the samples, a Hewlett Packard 5890 series II gaschromatograph was used. The chromatograph was equipped with a CP-WAX 58 CB column (initial temperature 140°C increased at 5°C/min to 240°C) and a FID detector (250°C). Helium was used as a carrier gas.

The composition of the petroleum ether extracted fat in batch 5 and 6 of experiment 1 was determined by gas chromatography according to Dutch Standard no. NEN 6348.

VFA samples were diluted and acidified with formic acid. The gas chromatograph used was a Hewlett Packard 5890A equipped with a 2m x 4mm glass column with supelcoport (11-20mesh) coated with 10% Fluorrad FC 431. The temperature of the injector, column and FID were 130, 200 and 280°C respectively. N_2 at 20°C saturated with formic acid was used as carrier gas.

Gas analysis

The CH_4 and H_2 content of the gas samples from the gas headspace and the VFA concentration of the sample from the liquid phase were determined by gas chromatography. The CH_4 was analysed on a Packard-Becker gas chromatograph with a 2mx2mm stainless steel column (200°C) packed with Poropack Q (80-100 mesh) and a FID detector (220°C). The H_2 content of the gas sample was analysed on a Hewlett-Packard gas chromatograph with a 1.5 m 1/8" stainless steel column (125°C) packed with mol. sieve (60-80 mesh). The temperature of the thermal conductivity detector was 110°C. For both the CH_4 and H_2 gas chromatographs N_2 was used as a carrier gas.

Lipase activity

Adding 1.0 ml of tributyrin emulsion (5.24-ml tributyrin, 0.25-g oleic acid and 1.16 ml 1M NaOH) and 0.25 g BESA to 5-ml sludge sample from the serum flasks assessed the lipase activity of the sludge. After 10 minutes incubation at 30°C the sludge was centrifuged and the amount of butyrate production was determined by gas chromatography as described above. BESA was added to inhibit conversion of the produced VFA to CH_4.

4.2.3 Calculations

Calculation of the surface based hydrolysis rate

If lipolysis only proceeds at the lipid-water interface, the relative size of this interface plays an important role in the hydrolysis rate. Calculations based on microscopic observations and experimental results (Hobson, 1987, Sanders et al. 2000) have shown that the hydrolysis rate of particulate substrates is constant per unit of surface area:

$$\frac{dM}{dt} = -K_{sbk} \cdot A \qquad (4.1)$$

With:

M =	Mass of substrate (g COD/l),
A =	Surface available for hydrolysis (m^2/l),
K_{sbk} =	Hydrolysis constant (g COD/m^2/day),
t =	time (days).

calculation of the amount of surface available to hydrolysis

The initial amount of surface available for the hydrolysis is directly related to the particle size and consequently the number of particles present in the emulsion. The amount of surface in the emulsion can be calculated using equation 4.2:

$$A = 4\pi r^2 n \quad with \quad n = \frac{3 \cdot X}{4\pi \cdot \rho \cdot f \cdot r^3} \qquad (4.2)$$

With:

n =	number of lipid particles (m^{-3}),
X =	fat (=LCFA+ neutral lipids) COD (kg COD/m^3),
ρ =	specific density of the fat (860 kg/m^3),
f =	COD conversion factor (2.8 kg COD/ kg fat),
r =	initial particle radius (m),

calculation of the amount of surface occupied by LCFA

To calculate the amount of lipid surface that is covered by LCFA it was assumed that the LCFA molecules at the surface of the dispersed lipids are arranged with their carboxylic group directed to the water phase and their hydrophobic chain directed to the core of the emulsion droplets (Fig 4.1). The bond angle of the O=C-OH group is 122° and the O=C and C-OH bond lengths are 1.25 Å and 1.31 Å, respectively (McMurry, 1988). Therefore the distance between the double bonded and the single bonded oxygen atom is 2.25 Å. Moreover, if it assumed that this carboxylic group can still spin around the C-COOH axis, the minimum amount of surface area that one LCFA molecule occupies is 2.25 Å x 2.25 Å. Using an average molecular weight for LCFA of 228 g/mol LCFA (C12, C14, C16 average) and taking into account that 1 g LCFA is 2.8 g COD, it can be calculated that the estimated surface area occupied by LCFA is 47580 m^2/kg COD. This value approximates the minimal surface that is occupied by LCFA since different orientations of the hydrophobic chain are possible (Myers, 1991).

4.3 Results and discussion

4.3.1 Hydrolysis of neutral lipids under methanogenic and acidogenic conditions

The results in Figure 4.2 show the time course of the assessed concentration of neutral lipids in experiment 1, conducted with butter and palm oil. The results clearly reveal a lower hydrolysis rate under acidogenic conditions compared to methanogenic conditions, particularly in the case of palm oil. The results in Table 4.3 depict the assessed accompanying free LCFA and VFA concentrations, and the amount of CH_4 produced.

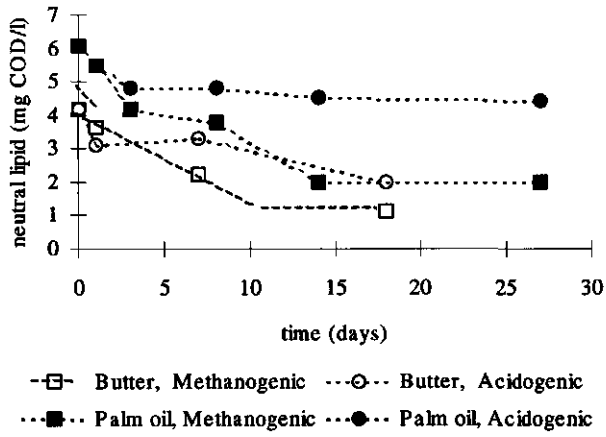

Figure 4.2: *The degradation of neutral lipids in emulsified butter and palm oil under methanogenic and acidogenic conditions (Experiment 1).*

Table 4.3: *The assessed LCFA and VFA concentrations, the cumulative methane production, and the calculated surface area possibly occupied by the LCFA (eq 4.3) under the methanogenic and acidogenic degradation of emulsified butter fat and palm oil. (Experiment 1).*

	digestion time (d)	methanogenic conditions (with addition of granular sludge) LCFA (g COD/l)	VFA (g COD/l)	CH4 (g COD/l)	acidogenic conditions (with addition of BESA) LCFA (g COD/l)	VFA (g COD/l)	CH4 (g COD/l)
butter	0	0.26	0.10	0.00	0.26	0.10	0.00
	1	0.78	0.08	0.10	1.50	0.50	0.10
	7	0.34	0.07	2.50	0.80	0.80	0.25
	18	0.08	0.04	4.00	0.70	1.90	0.50
palm oil	0	0.18	0.09	0.00	0.21	0.09	0.00
	1	0.10	0.16	0.30	0.22	0.20	0.12
	3	0.85	0.20	0.85	0.45	0.75	0.13
	8	0.20	0.90	2.00	0.20	0.88	0.20
	14	0.35	0.04	4.00	0.37	0.90	0.25
	27	0.15	0.03	4.00	0.23	1.20	0.30

4.3.2 The course of H_2, propionic acid concentration and pH

In the experiment with palm oil as well as the experiment with butter, the pH varied between 6.1 and 6.5 under both acidogenic and methanogenic conditions. The partial hydrogen pressure in the gas phase during the experiment as well as the volumetric hydrolysis rate is presented in Figure 4.3. The results show that in general under acid conditions the partial hydrogen pressure in the gas phase was higher than under methanogenic conditions. In the experiment with palm oil the partial hydrogen pressure in the gas phase was high after 1 day of digestion (approx. 90 ppm) but decreased during the experiment. When butter was used as a substrate the conditions where the other way around. After one day of digestion the hydrogen pressure was low (0-40 ppm) but increased during the experiment. All serum-flasks where flushed with nitrogen gas before inoculation therefore the difference in partial hydrogen pressure at day 1 between the palm oil and butter experiment has to be caused by the conversions during the first day. However, due to the limited analyses done on the first day the nature of these conversions remains unclear.

When observing the assessed partial hydrogen pressure in the gas phase and the calculated hydrolysis rate in Figure 4.3 it could be concluded that apparently the hydrogen concentration has an effect on the volumetric hydrolysis rate. Salminen et al (2000) concluded from model calculations on the digestion of poultry slaughterhouse waste that propionic acid could cause inhibition of the lipid

hydrolysis. Although a high propionic acid concentration is usually accompanied by a high hydrogen concentration, Salminen et al. (2000) reported no effect of the hydrogen concentration on the hydrolysis of lipids. Moreover, when the propionic acid concentrations as they were assessed in this research (Fig. 4.4) are compared to the hydrolysis rates a relation is difficult to establish.

As neither hydrogen nor propionic acid plays a direct role in the enzymatic hydrolysis reaction of neutral lipid, it has to be concluded that the apparent effect of the hydrogen concentration might be an indirect effect. A high hydrogen concentration could inhibit the growth of lipase producing microorganisms and thus inhibit the enzyme activity.

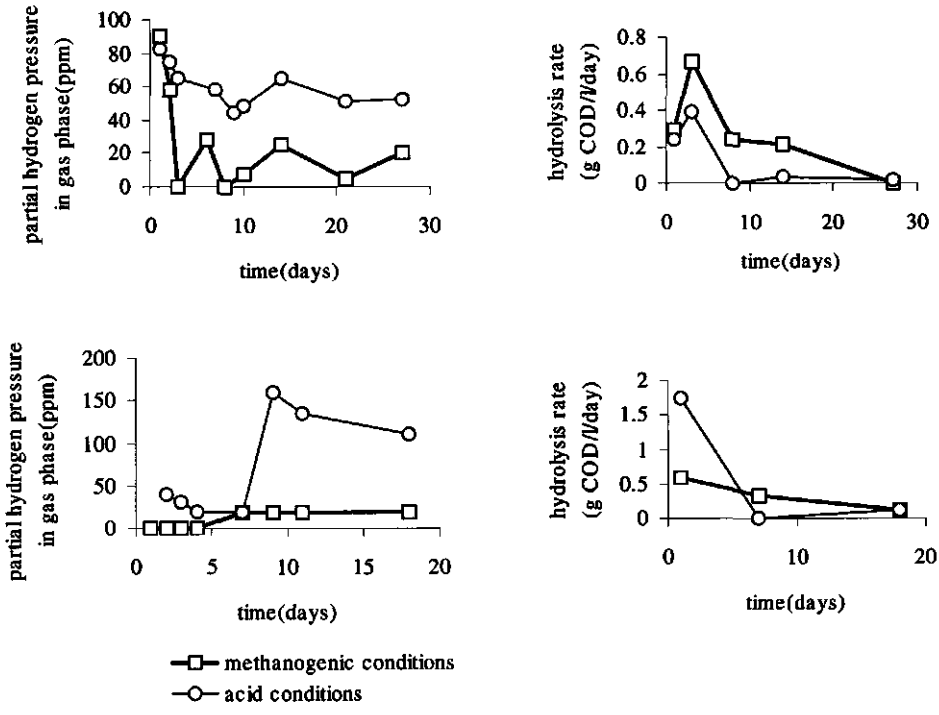

Figure 4.3: *The partial hydrogen pressure in the gas phase and the calculated volumetric hydrolysis rates (dM/dt) during the degradation of neutral lipids in emulsified butter (bottom graphs) and palm oil (upper graphs) under methanogenic and acidogenic conditions (experiment 1).*

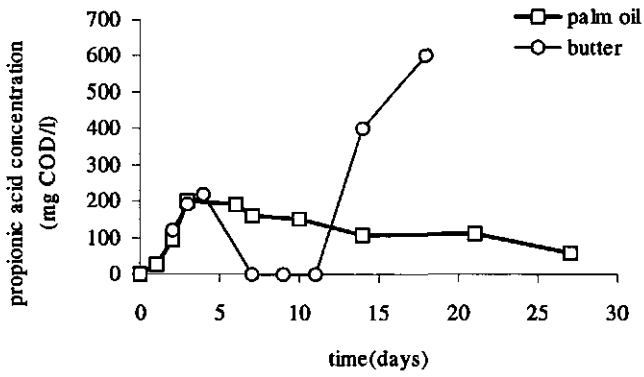

Figure 4.4: *The propionic acid concentration during the acid phase degradation of palm oil and butter as assessed in experiment 1.*

4.3.3 accumulation of hydrolysis intermediates

In experiment 1 the composition of the neutral lipids was analysed in order to assess any occurrence of hydrolysis intermediates such as di- and mono glycerides. The first 16 days of the experiment the hydrolysis of tripalmitin proceeded at a similar rate under acidogenic and methanogenic conditions. In both batches, LCFA increased gradually up to 550 mg COD/l at day 16, while only 4.6% of the added tripalmitin were hydrolysed in total. After this period, the batch experiment that was conducted at acidogenic conditions was terminated. The experiment with addition of granular sludge was continued until day 36. During this period, the concentration of LCFA dropped to 340 mg COD/l, while 36% of the tripalmitin was converted to methane at day 36. At day 2, 7, 10, 16, 26, 33 and 36 the amount of triglyceride, diglyceride, monoglyceride and LCFA was analysed in the fat that was extracted from the sludge and wall of the serum flasks with petroleum ether. The results revealed that this fat contained only triglycerides and LCFA at any time during the experiment at both acidogenic and methanogenic conditions, implying that any accumulation of mono- or diglycerides did not prevail.

4.3.4 Course of the lipase activity

The measurements of the lipase activity during the digestion of palm oil revealed that under both acidogenic and methanogenic conditions the lipase activity varied between 300 and 450 mg butyrate/g VSS/hour during the whole

experiment. Consequently, the lower volumetric hydrolysis rate under acidogenic conditions can not be attributed to a lower lipase activity based on enzyme activities determined with the model substrate tributyrin. This is contradictory to the suggestion made above that under acidogenic conditions a high hydrogen concentration can inhibit the lipase production.

The hydrolysis rate of tributyrin under both conditions was far higher than the observed hydrolysis rate of palm oil in the reactors. It therefore has to be concluded that although the lipase enzymes were excessively present during the anaerobic degradation, the triglycerids in palm oil are more difficult to hydrolyse than tributyrin. This is due to the steric hindrance of the LCFA in the triglycerids. The lower hydrolysis rate during acidogenic conditions indicates that under these conditions the hydrolysis experiences more hindrance than under methanogenic conditions. The hypothesis suggesting that a lower hydrolysis rate under acidogenic conditions is only due to a low enzyme activity therefore is likely to be dismissed.

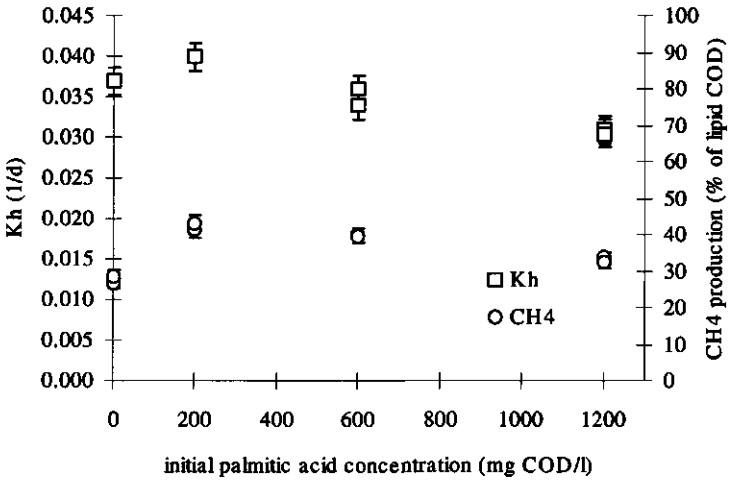

Figure 4.5: *The first order hydrolysis constant for tripalmitin as a function of the initial palmitic acid concentration and tripalmitin converted to CH_4 after 22 days of digestion.*

4.3.5 Effect of the LCFA concentration

From the results presented in Table 4.3 and Figure 4.3 any effect of the LCFA concentration on the rate of hydrolysis can not be deduced because again the experiments with palm oil and butter show contradicting results. In the experiment with butter the LCFA accumulate to 1.5g COD/l (Table 4.3) under

acidogenic conditions which coincides with an inhibition of the hydrolysis (Fig 4.3). Under methanogenic conditions the LCFA concentration only reaches to 0.78g COD/l and the inhibition of the hydrolysis is less severe. In the experiment with palm oil, however the situation is reverse. The highest concentration of LCFA is reached under methanogenic conditions and the inhibition of the hydrolysis is less than under acidogenic conditions.
Therefore, in a second experiment we investigated the methanogenic digestion of tripalmitin in the presence of initial palmitic acid concentrations in the range of 0-1200 mg COD/l. To compare different conditions the first order hydrolysis constant was calculated assuming 100% biodegradability for the tripalmitin (Gujer and Zehnder, 1983). The results in Figure 4.5 indicate only a small difference between 0-200 and 1200 mg COD/l of palmitic acid. However, because the LCFA and tripalmitin were added as separate components it is likely that they remained physically separated during the digestion. This could have reduced the effect of the LCFA on the hydrolysis. The assessed lipase activity in this experiment ranged between 400 and 700 mg butyrate/g VSS/hour, which indicates that also in this experiment lipase enzymes were excessively present.

4.3.6 Physical effects

From the results and discussion as presented above a low lipase activity and/or high hydrogen concentration could not be designated as the only cause for a lower hydrolysis rate under acidogenic conditions when compared to methanogenic conditions. Apparently, to find the cause for the lower volumetric hydrolysis rate there also has to be looked for the amount of substrate surface that is available for the hydrolysis.
The amount of surface that is actually available for hydrolysis can be calculated using equation 4.1 and 4.2. In the experiment with palm oil, 6.1 g COD/l of fat was used. The average diameter of the fat particles in the emulsion was 5 μm. With equation 4.2 the initial number of particles in the emulsion and the initial size of surface were calculated to be $4.8.10^{12}$ m^{-3} and 1520 m^2, respectively.
When enzymes are excessively present and pH and temperature remain constant throughout the experiment it can be assumed that the surface based hydrolysis constant (K_{sbk}) is constant (Hobson, 1987, Sanders et al. 2000, Eq. 4.1). In that case, any changes in the volumetric hydrolysis rate (dM/dt) are the result of changes in the size of the lipid surface. The initial volumetric hydrolysis rate for methanogenic conditions was 0.29 g COD/l/day (dM/dt, Fig 4.3). This resulted in a surface based hydrolysis rate of 0.19-mg COD/m^2/day (Eq .1). With K=0.19 mg COD/m^2/day and the volumetric hydrolysis rates from Figure 4.3 the time course of the surface that is available for the hydrolysis was calculated using Equation 1. In Figure 4.6, it can be observed that the amount of available surface under methanogenic conditions is larger than under acidogenic conditions. In both cases

the amount of available surface during the first day increases but after that decreases sharply. The increase of available surface in the first day probably is due to the addition of the UASB sludge (absorption surface) and the incubation at 30° Celsius. The subsequent decrease of the available lipid surface can be ascribed to three factors:

- decrease of the amount of palm oil due to conversion to VFA and CH_4,
- coagulation or emulsion of the lipid spheres,
- accumulation of LCFA at the lipid water interface.

Although the decrease of the available surface due to degradation of the palm oil and the decrease of the available surface due to coagulation of the palm oil emulsion can not be studied separately still some considerations can be made. Under methanogenic conditions, 60% of the palm oil was converted to CH_4. It is therefore plausible that the major part of the decrease of the available surface will be due to this degradation and the minor part will be due to coagulation of the remaining lipid. At acidogenic conditions, almost no conversion of palm oil occurs so it has to be concluded that for acidogenic conditions the decrease of the available surface mainly has to be ascribed to coagulation of the lipid emulsion. Figure 4.6 shows the minimal amount of surface that could be occupied by LCFA in the experiment with palm oil. From this figure, it can be concluded that the amount of lipid surface that will be shielded from the lipase enzymes by LCFA is very small as compared to the available surface. On the other hand, the surface that is calculated is the minimal surface that is occupied by free LCFA and other orientations of the hydrophobic chain (Myers, 1991) would increase the surface occupied by LCFA. Moreover, the effect of the LCFA on the surface tension of the lipids during anaerobic digestion is unclear. However, the decrease of surface available for the hydrolysis under acidogenic conditions on day 3 (Fig 4.6, left) is accompanied by a reduction of the amount of surface occupied by LCFA (Fig 4.6, right). This indicates that the decrease in available surface is not caused by the accumulated LCFA. These considerations suggest that the observed difference in available surface between acidogenic and methanogenic conditions can probably be attributed to the emulsifying effect of the biogas production that reduced coagulation of the lipid at methanogenic conditions.

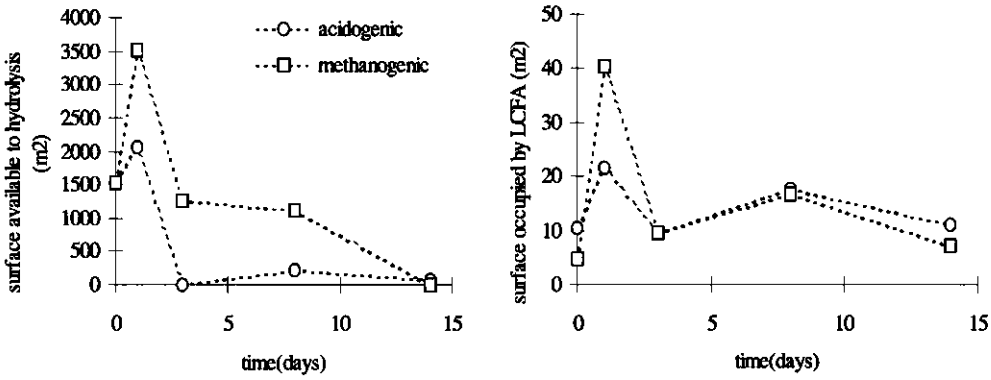

Figure 4.6: *The amount of surface available for hydrolysis during the anaerobic hydrolysis of palm oil, calculated with equation 1 when assuming K=0.19 mg COD/m²/day and an initial surface area of 1520 m²/l (left). The amount of surface occupied by LCFA as calculated with equation 4.2 (right).*

In this research, the maximum volumetric hydrolysis rates for butter and palm oil were 1.4 g COD/l/day and 0.67 g COD/l/day respectively. From the results of Hanaki et al. (1981), a volumetric hydrolysis rate of 2.66-g COD/l/day could be calculated, which is far higher than the observed volumetric hydrolysis rates in this research. However, the average particle radius of the lipid emulsion in milk is 0.3 µm (Walstra, 1983). This is much smaller then the particle sizes of the butter and palm oil emulsion. Moreover, the lipid emulsion in milk is very stable so that coagulation of the particles is far less even under acidogenic conditions. When assuming an average radius of 0.3 µm for the lipid particles in milk and an influent concentration of 2.8-g COD/l the total amount of surface in the experiment of Hanaki et al. (1981) was calculated to be 11649 m^2. This resulted in a surface based hydrolysis constant of 0.23 $mg/m^2/day$, which is comparable with the rate as calculated for palm oil. The latter supports the indications that differences in volumetric hydrolysis rates are governed by the available surface rather than the enzyme activity.

Komatsu et al. (1991) suggested a two-phase system for lipid digestion to prevent LCFA inhibition. However, their experiments were conducted with milk as a substrate, which has a very stable lipid emulsion. Our research implies that using a two-phase system for lipid digestion is not favourable when using a lipid substrate which has a less stable emulsion. In the acidogenic stage of the system, coagulation of the lipid will not be discouraged by gas production and consequently the lipid will enter the methanogenic stage with a reduced lipid

water interface as compared to the raw substrate. This could result in a lower volumetric hydrolysis rate than when using a one-stage system.

4.4 Conclusions

- The volumetric hydrolysis rate of neutral lipids in palm oil and butter was higher under methanogenic conditions as compared to acidogenic conditions.
- The lower volumetric hydrolysis rate under acidogenic conditions could not be ascribed to a low pH, accumulation of LCFA or accumulation of di or mono glyceride.
- The results indicate a possible non-causal relation between the hydrolysis rate and the hydrogen concentration.
- Calculations indicate that the amount of surface available for the hydrolysis is an important factor determining the difference in the volumetric hydrolysis rates between acidogenic and methanogenic lipid digestion.
- The larger available surface for the hydrolysis under methanogenic conditions as compared to acidogenic conditions could be caused by the emulsifying effect of the gas production.
- Practical implication of this research is that one-stage digestion is favourable over two-stage digestion when digesting lipid substrate, except for milk lipid, to prevent coagulation under acidogenic conditions.

Acknowledgements - The author would like to thank the Dutch Food Inspection Department for performing the analysis on the composition of the petroleum ether extracted lipids.

nomenclature
LCFA = Long Chain Fatty Acids (free or bound to glycerol)
Neutral lipids = Triacylglycerols, consisting of LCFA and glycerol.
Fat = Neutral lipid + Free LCFA
Volumetric hydrolysis rate = hydrolysis rate in g COD/l/day
Surface based hydrolysis rate = hydrolysis rate in g COD/m^2/day

5 EVALUATION OF THE FIRST ORDER KINETICS AND THE HYDROLYSIS CONSTANT.

abstract – This chapter provides a survey concerning the limitations of the first order relation for hydrolysis and about the effects of digestion conditions on the first order hydrolysis constant. From the results of the evaluations it can be concluded that the hydrolysis process only proceeds according to first order kinetics if no changes in the rate limiting step or the biodegradability occur during the degradation of the substrate. Moreover evidence was obtained that the first order hydrolysis constant is system and substrate specific and the use of literature values for the hydrolysis constant is rather questionable because it is difficult to correct for differences in pH and particle size distribution. For waste(water) containing mainly protein and carbohydrates, the value for the first order hydrolysis constant can be assessed under acidic and methanogenic conditions in a batch experiment. For lipids, the hydrolysis constant can be assessed under methanogenic conditions in a batch experiment. As (gas) mixing conditions in a full scale CSTR digester might differ from laboratory batch systems, herewith the kh value might differ also.

A modified version of this chapter will be published as part of : Sanders, W.T.M., Vecken, A., Zeeman, G., Lier van, J.B., Analysis and optimisation of the anaerobic digestion process of the organic fraction of municipal solid waste, In: Biomethanisation of the organic fraction of municipal solid waste, Mata-Alvarez eds.

5.1 Introduction

Although first order kinetics does not reflect the actual kinetics of the hydrolysis process (Hobson 1987, Sanders et al., 2000) it is commonly used to describe the hydrolysis of particulate substrates during anaerobic digestion (Eastman and Ferguson, 1981,Pavlostathis and Giraldo-Gomez, 1991). Provided the underlying microbiological and physical processes are well understood, the first order kinetics represent a good tool for reactor design.

$$dX_{degr}/dt = -k_h.X_{degr}, \qquad (5.1)$$

Whit:
X_{degr}: concentration biodegradable substrate (kg/m3),
t : time (days),
k_h : first order hydrolysis constant (1/day).

The first order kinetics originally were used to describe the degradation of particulate substrate (Eastman and Ferguson 1981) but recently it is also used to describe the degradation of dissolved polymeric substrates (San Pedro 1994, San Pedro et al. 1994).
Several researchers showed that the hydrolysis mechanism of particulate substrates is surface related (Hills and Nakano 1984, Sanders et al. 2000). In the digestion of particulate substrates the amount of enzymes usually is present in excess relative to the available surface area (Hobson, 1987), the hydrolysis constant in fact is determined by the latter factor. Such surface limited kinetics can very well be described with a first order relation (Vavilin et al. 1996, Veeken and Hamelers 1999, Chapter 2).

For dissolved substrates the available substrate surface area virtually is infinite. This obviously implies that not the amount of surface but the amount of active enzymes is the rate-limiting factor to the hydrolysis. Moreover, as the enzymes are produced by the biomass, the concentration of this biomass evidently could also play a role in the hydrolysis rate of dissolved polymeric components. Some researchers therefore have introduced a more sophisticated model for the hydrolysis of dissolved polymeric substrates. Goel et al. (1998) proposed a saturation type of relationship for the hydrolysis of dissolved starch in which the hydrolysis rate is directly proportional to the biomass concentration. Negri et al. (1993) included the enzyme concentration in the first order kinetics equation to describe the production of VFA from dissolved polymeric substrates in the OFMSW. Munch et al. (1999) used the same kinetics in a model for prefermentation of dissolved polymeric components in primary sludge. Chapter 3 of this thesis discusses the effect of biomass or enzyme activity on the hydrolysis

of dissolved polymers. It was argued that at this stage the simple first order kinetics still offer the best alternative for reactor design and process optimisation, provided that possible effects of changes in the biomass concentration or composition are accounted for.

This chapter surveys the limitations of the first order relation and the effects of digestion conditions on the first order hydrolysis constant. Moreover the applicability of kh values from literature will be evaluated, while also guidelines for the experimental assessment of the hydrolysis constant will be provided.

5.2 Methods

5.2.1 experimental set ups for the assessment of the hydrolysis constant

When evaluating literature it is clear that commonly two different experimental set ups are used to investigate the hydrolysis of particulate substrates, i.e. batch (Veeken and Hamelers, 1999) or continuous (Miron et al. 2000) experiments. In the batch approach, the selected substrate is incubated at a specific temperature with or without an excess amount of methanogenic sludge. The continuous set up uses completely stirred tank reactors (CSTR) operated at a specific temperature and at several hydraulic retention times (HRT). Analyses are made from the effluent once steady state has established.

To monitor the hydrolysis process during the digestion two methods are used, viz.:

1. measurement of the amount of methane, and possibly also the concentration of soluble COD and volatile fatty acids (VFA) produced (Veeken and Hamelers, 1999).
2. measurement of the concentration of the individual relevant components (Miron et al. 2000).

In order to be able to follow the degree of hydrolysis just by means of the methane production, the hydrolysis needs to be the rate-limiting step in the degradation process. This in fact implies that the methanogenic activity present during the test is sufficient to prevent accumulation of volatile fatty acids, and obviously it means that this method can only be applied to determine the hydrolysis constant under methanogenic conditions. Although for a batch experiment the required concentration of methanogenic inoculum can be calculated, measurement of the concentration of dissolved COD and volatile fatty acid in addition to the methane production is advised. In that case the degree of hydrolysis is calculated from the methane production, and the production of dissolved COD during the digestion.

The second method to follow the degree of hydrolysis, viz. by measurement of the individual components, requires sampling of the reactor contents during the test for assessment of the suspended COD, proteins, carbohydrates or lipids in the system. This method is not always applicable, especially in tests that are conducted under methanogenic conditions, as with addition of inoculum sludge extra protein and carbohydrates are introduced. Because it is not possible to distinguish between proteins and carbohydrates originating from the biomass and the waste(water) this method is insufficiently accurate for waste(water) that contains only a small amount of hydrolysable proteins and carbohydrates.

If the hydrolysis constant is assessed in a batch experiment, two types of systems can be used. The first is a large stirred batch reactor that is regularly sampled during the degradation process and will further be addressed to as the 'classic' batch reactor. The classic batch reactor is best used when the degree of hydrolysis is determined via measurement of the methane production and of dissolved products and acids in the reactor mixture. This reactor type is less suitable for the direct measurement of suspended COD or individual particulate components in the reactor mixture as possible floating or settling layers and lipids attached to the reactor wall make representative sampling difficult. In that case, a system where several small reactors or flasks represent one large reactor, is more suited. At the start of the experiment, all flasks have the same content and it is assumed that the progress of the hydrolysis is similar in all flasks. Each time the reactor is sampled one whole flask is sacrificed and its contents is analysed. This type of batch reactor will be further addressed to as the 'multiple flask' reactor. The 'multiple flasks' procedure allows thorough homogenisation of the flasks contents before sampling. Moreover, after emptying the flask can be rinsed with an organic solvent to remove the lipids that where attached to the wall.

In this chapter, the results of several investigations into the hydrolysis of complex waste(water) were used to evaluate the first order kinetics for hydrolysis. Table 5.1 provides a list of the applied experimental set up and analyses that were used in the research in question.

Table 5.1: *Experimental set up and performed analysis of the research used in this chapter.*

Authors	Experimental set up	Analysis	Kh (re)calculated*
Veeken and Hamelers (1999)	Batch (classic)	CH_4 and VFA	No
Canalis (1999)	Batch (classic)	CH_4 and VFA	No
O'Rourke (1968)	CSTR	Grease free LCFA Cellulose Organic-N	Yes
Boon (1991)	Batch (classic)	NH_4^+-N	Yes
Palenzuela-Rollon (1999)	Batch (multiple flask)	NH_4^+-N	No
Eastman and Ferguson (1981)	CSTR	Kjeldahl-N Cellulose Grease	Yes
Miron et al. (2000)	CSTR	Kjeldahl-N Carbohydrates Grease free LCFA	Yes
Hills and Nakano (1984)	CSTR	CH_4	Yes
Sanders et al. (2000)	Batch (Classic)	CH_4 VFA dissolved carbohydrates	No

* Hydrolysis constant was (re)calculated from the results presented in the concerning literature reference

5.2.2 Calculations

From equation 5.1 the relation between the hydrolysis constant, digestion time and effluent concentration for a batch (eq 5.2) and CSTR (eq 5.3) can be derived.

$$X_{ss,effluent} = X_{ss,influent}(1 - f_h) + f_h\ X_{ss,influent} e^{-k_h.t} \quad (5.2)$$

$$X_{ss,effluent} = \frac{X_{ss,influent}\, f_h}{1 + \theta\, k_h} + X_{ss,influent}(1 - f_h) \quad (5.3)$$

With:

$X_{ss,effluent}$: concentration of total substrate in the effluent (biodegradable + non biodegradable part) (g/l),

$X_{ss,influent}$: concentration of total substrate in the influent (biodegradable + non biodegradable part) (g/l),

f_h : biodegradable fraction of substrate, fh $\in$ [0;1],

k_h : first order hydrolysis constant (d^{-1}),

θ : hydraulic retention time of reactor (d),

t : digestion time of batch (d).

From results of several investigations reported in literature (table 5.1) the hydrolysis constant was (re) calculated in order to assess its dependency on the digestion conditions. For the estimation of the first order hydrolysis constant from the two set up's, equation 5.2 and 5.3 can be linearised which results in equation 5.4 and 5.5, respectively.

$$\ln\left(\frac{X_{ss,effluent} - X_{ss,influent}(1 - f_h)}{X_{ss,influent} f_h}\right) = -k_h.t \quad (5.4)$$

$$\theta = (f_h.X_{ss,influent})\left(\frac{\theta}{X_{ss,influent} - X_{ss,effluent}}\right) - \frac{1}{k_h} \quad (5.5)$$

For CSTR systems (eq 5.5) 'θ' (y-axis) can be plotted against θ /($X_{ss,influent}$-$X_{ss,effluent}$). The hydrolysis constant then follows from the intercept of the line with the y-axis. The biodegradability of the substrate follows from the slope of the line

(Eastman and Ferguson, 1981). In batch experiments, the hydrolysis constant is determined by plotting equation 5.4. The biodegradability can not be derived from the graph, but has to be assessed directly from the experimental results. The biodegradability can be calculated from the maximum methane yield of the substrate (Veeken and Hamelers, 1999) or the final effluent concentration. The hydrolysis constant is calculated from the slope of the line.

A more direct and accurate method for assessing the hydrolysis constant and biodegradability from batch and continuous experiments is the non-linear least squares fit on the assessed effluent concentration. This method should be assessed whenever possible. However, with the linear presentation of the results according to equation 5.4 and 5.5 more insight is gained in possible deviations from the first order relation. Therefore, for the evaluation of the first order kinetics the linearised equations are used in this chapter.

Additionally, it should be emphasised that the biodegradability in equations 5.2 to 5.5 refers to the biodegradability under the applied 'steady state' conditions and may change with the imposed reactor conditions.

5.2.3 Possible errors

The effluent values for estimation of the hydrolysis constant obviously are important, especially in the batch digestion set up. By determining the derivative of the hydrolysis function, the sensitivity of the hydrolysis constant to the effluent concentration can be assessed. The equations, $dk_h/dX_{ss,effluent}$,, are presented in equations 5.6 and 5.7, for batch and CSTR systems, respectively. From figure 5.1 it can be seen that the sensitivity of the calculated hydrolysis constant increases when the effluent concentration approaches the minimum value of $X_{ss,influent}(1\text{-}f_h)$. Since at very low biodegradable substrate levels a small error in the effluent concentration has a large impact on the value of the calculated hydrolysis constant it is advised to use only effluent values, obtained before 50% of the biodegradable substrate is converted.

$$\frac{dk_h}{dX_{ss,effluent}} = \frac{1}{t} * \frac{X_{ss,influent}\, f_h}{X_{ss,effluent} - X_{ss,influent}(1-f_h)} \tag{5.6}$$

$$\frac{dk_h}{dX_{ss,effluent}} = \frac{\theta - 1}{(f_h + \theta\, X_{ss,effluent})^2} \tag{5.7}$$

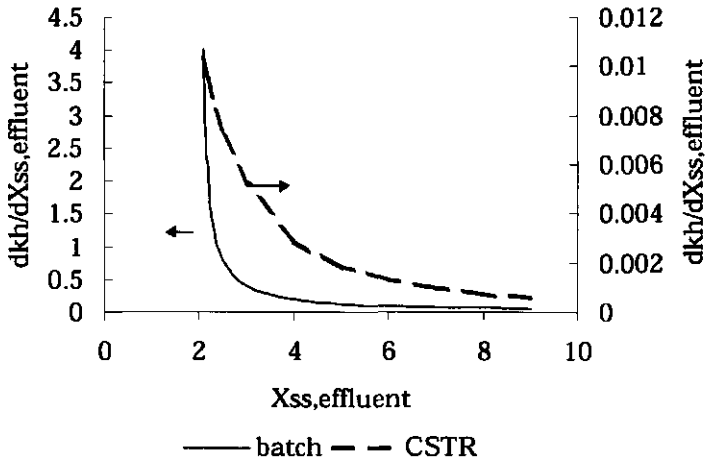

Figure 5.1: *The sensitivity of the value of the first order constant to the assessed effluent values. When digesting the substrate at* $X_{ss,influent}$ *= 10 g/l,* f_h *= 0.8, and HRT = 20 days*

Another concern for errors in the estimation of the hydrolysis constant can be found in the formation of new biomass during the digestion. The growth of biomass, in both analytical methods discussed in 5.2.1, leads to an underestimation of the hydrolysis constant. However, the biomass yield during anaerobic digestion is relatively low and it can be calculated that a biomass yield of 0.1-0.2 g/g only results in a 0.5-2.5 % error in the value of the hydrolysis constant. The effect of biomass growth on the value of the hydrolysis constant can therefore be neglected.

When waste(water) contains sulphate or nitrate and the degree of hydrolysis is measured via methane production the hydrolysis obviously will also be underestimated due to consumption of volatile fatty acids for the formation of H_2S and N_2. Direct measurement of the individual components is advised for this type of waste(water).

5.3 The effect of temperature on hydrolysis constant and biodegradability

In general, the rate of all reactions varies with temperature in accordance with the Arrhenius equation (eq 1.2). Veeken and Hamelers (1999) found a Arrhenius relation (R^2 0.984-0.999) between the first order hydrolysis constant for several organic wastes and the digestion temperature in the range of 20°-40°C. They

calculated an average standard free energy of activation of 46±14 kJ mol^{-1} for the organic waste components. This is within the range of 15-70 kJ mol^{-1}, which is the typical range for standard free energies of activation as given by Chaplin and Bucke (1990). Moreover the results showed no significant difference in the biodegradability of the organic waste components within that temperature range.

Canalis (1999) digested particulate starch at 15°, 20°C, 25°C and 30°C. Most of the batch experiments were terminated before the starch hydrolysis had reached its asymptote but it was assumed that the biodegradability (fh) of the starch was 1.0 at all temperatures. The calculated hydrolysis constants amounted to 1.24±0.57 d^{-1}, 1.24±0.29 d^{-1}, 0.13±0.05 d^{-1} and 0.03±0.01 d^{-1} for 30°C, 25°C, 20°C, 15°C, respectively. From the results a Arrhenius relation (R^2 0.91) was derived between the hydrolysis constants and the temperature, but the calculated standard free energy of activation of 190 kJ mol^{-1}, was beyond the range of 15-70 kJ mol^{-1}. A reason for this high standard free energy of activation can not be given.

O'Rourke (1968) digested primary sludge in CSTR's at 15°, 20°, 25° and 35°C and assessed the concentrations of grease, cellulose and protein in the influent and effluent. From his results, the first order hydrolysis constant and biodegradability of the sludge at the different digestion conditions was calculated using equation 5.5. Table 5.2 summarises the results of our calculations and figure 5.2 presents them graphically. Table 5.3 provides the pH values and whether acid or methanogenic conditions prevailed in the digesters.
It appears that only the hydrolysis of protein followed first order kinetics over the whole temperature range. Cellulose was degraded according to first order kinetics at 20° to 35°C. At 15°C the first order constant could only be calculated for cellulose in the HRT range of 30-60 days. Below 30 days HRT the hydrolysis of cellulose was not in accordance with first order kinetics. Cause for this could be a lower enzyme activity at HRT<30 days due to which the enzyme activity instead of the surface of the substrate became rate limiting.
The Arrhenius relation of the calculated hydrolysis constants for proteins and cellulose in Table 5.2 was poor. For protein a standard free energy of activation of 53 kJ/mol was calculated with R^2 0.79. For cellulose it was 173 kJ/mol with R^2 0.88. This again is above the range of 15-70 kJ mol^{-1} as was specified by Chaplin and Bucke (1990).
The amount of neutral lipid (total grease minus free long chain fatty acids) was only ~25% of the total amount of grease in the sewage sludge. The hydrolysis did not proceed according to first order kinetics at any temperatures investigated. The assessed values of the biodegradability (fh) presented in Table 5.2, appear to be hardly effected by the temperature.

Table 5.2: *The calculated values of the first order hydrolysis constant and of the biodegradability calculated with equation 5.5 from the results of the digestion of raw domestic sewage sludge as conducted by O'Rourke (1968), values between brackets are the calculated standard errors (in %).*

	kh (d^{-1})				f_h			
	15°C	20°C	25°C	35°C	15°C	20°C	25°C	35°C
Cellulose	0.14[1]	0.13	0.38	12.0	0.92[1]	0.97	0.95	0.92
					(3.6)	(3.6)	(1.6)	(0.5)
Protein	0.14	0.39	0.33	0.67	0.37	0.35	0.41	0.40
					(2.5)	(1.3)	(2.2)	(2.7)

(1) calculated for the range HRT 30-60 days.

Table 5.3: *The prevalence of acidic or methanogenic conditions and measured values of the pH during the digestion experiments conducted by O'Rourke (1968) at different temperatures.*

	15°C	20°C	25°C	35°C
Acid conditions	HRT 5-60 days	HRT 5-15 days	HRT 3.75-10 days	HRT 0-5 days
Methanogenic conditions		HRT 30-60 days	HRT 15-60 days	HRT 7.5-60 days
pH[(1)]	6.95±0.35	7.05±0.35	7.1±0.3	7.1±0.3

(1) variations in pH showed little if any relation to the HRT.

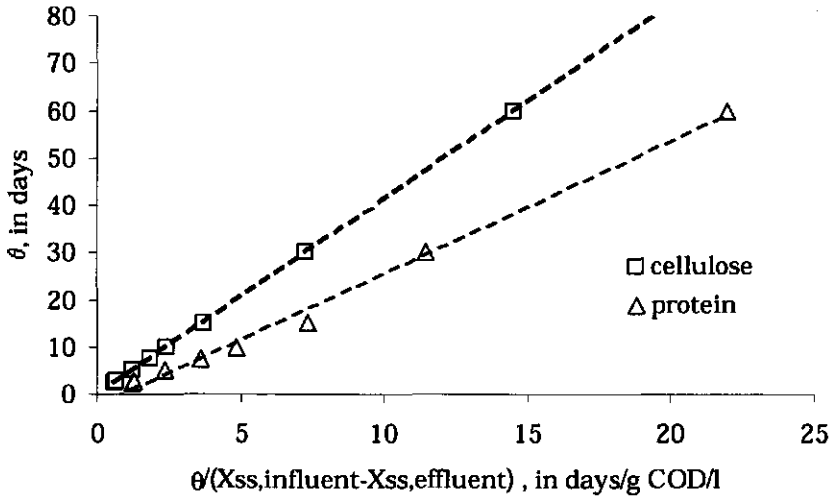

Figure 5.2: *Presentation of the linearised equation (eq 5.5) of the hydrolysis of cellulose and protein in primary sludge at 35°C as calculated from O'Rourke(1968) (Slope=$f_h \cdot X_{ss,influent}$, y-intercept=$-k_h^{-1}$).*

5.4 The effect of pH on hydrolysis constant and biodegradability.

Boon (1991) and Palenzuela Rollón (1999) performed batch digestion experiments at controlled pH between 5 and 8 with primary sludge (35°C) and fish processing wastewater (30°C), respectively.
Figure 5.3 show the assessed values of the first order hydrolysis constant and biodegradability of protein COD and total COD in primary sludge in relation to the pH. It appears that the biodegradability (f_h) slightly increases with increasing pH, while the relation found between the hydrolysis constant and the pH resembles the 'bell shaped' curve (Fig. 1.4) with an optimum at pH 6.5 for both the total COD and the proteineous COD.

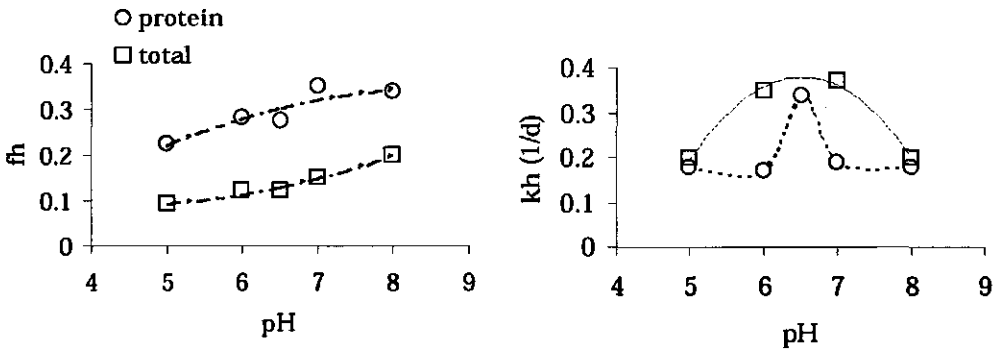

Figure 5.3: *The assessed relationship between the pH and the biodegradability (left) and the first order hydrolysis constant (right) of the proteins and total COD in primary sludge during a batch digestion at 35°C. Calculated from Boon (1994).*

Concerning the pH effect on the biodegradability of protein during the digestion of fish processing wastewater at methanogenic and acidogenic conditions Palenzuela-Rollon (1999) assumed an equal biodegradability at all pH's investigated of 0.8±0.01. From the results of the batch experiment this degradability only could be established at pH 8 because those at pH 4 to 7 were terminated before the conversion of protein had reached its asymptote. The main protein in the wastewater was myosin which structure almost remains unaffected by pH and temperature (Engbersen and de Groot, 1995). This supports the assumption that also at pH 4-7 the biodegradability was 0.8. Palenzuela-Rollon concluded that there was no significant effect of the pH on the value of the hydrolysis constant between acid and methanogenic conditions, except at pH 8 (Fig 5.4).

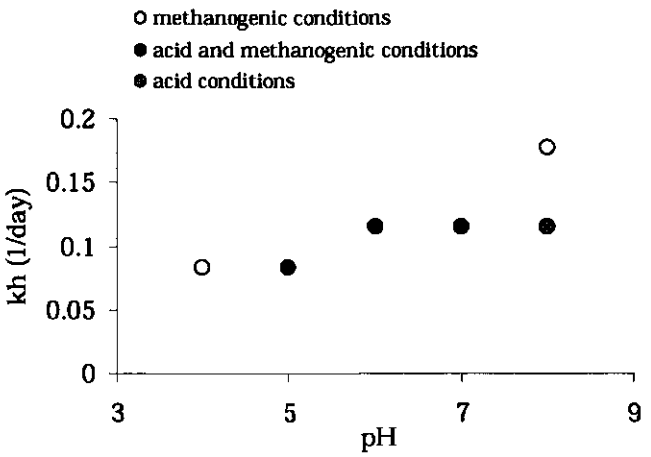

Figure 5.4: *The assessed biodegradability and first order hydrolysis constant of the proteins in fish processing wastewater during a batch digestion experiments at 30°C conducted at methanogenic and acidogenic conditions and different pH values (Palenzuela-Rollon (1999).*

Eastman and Ferguson (1981) conducted digestion experiments with primary sludge in CSTR's at 35°C, at very short retention times (9-72 h) and pH 5.17±0.04. Although they only calculated the kh and biodegradability on the basis of total COD, the hydrolysis of the separate components in the sludge were also followed. At pH 5.17 nitrogenous components were degraded very fast and followed first order kinetics (Fig 5.5). For carbohydrates only first order kinetics can be used for HRT 1.5 and 3 days. At retention times below 24h, a lower enzyme activity could have caused the hydrolysis kinetics to change from a surface limited to an enzyme-limited situation. Greases remained completely undegraded.

Calculation of the biodegradability and hydrolysis by using equation 5.5 results in a value for kh of 6.2 and 7.0 1/d and for the biodegradability of 0.30 and 0.31 for the protein and carbohydrates, respectively. In addition to the reactors operated at pH 5.17 Eastman and Ferguson (1981) also conducted two experiments at pH 5.85 and pH 6.67 and a retention time of 1.5 days. Although one HRT obviously does not allow calculation of the kh and biodegradability, the results clearly reveal that the biodegradability of both nitrogenous and carbohydrate compounds is positively effected by the increase of the pH from 5 to 7.

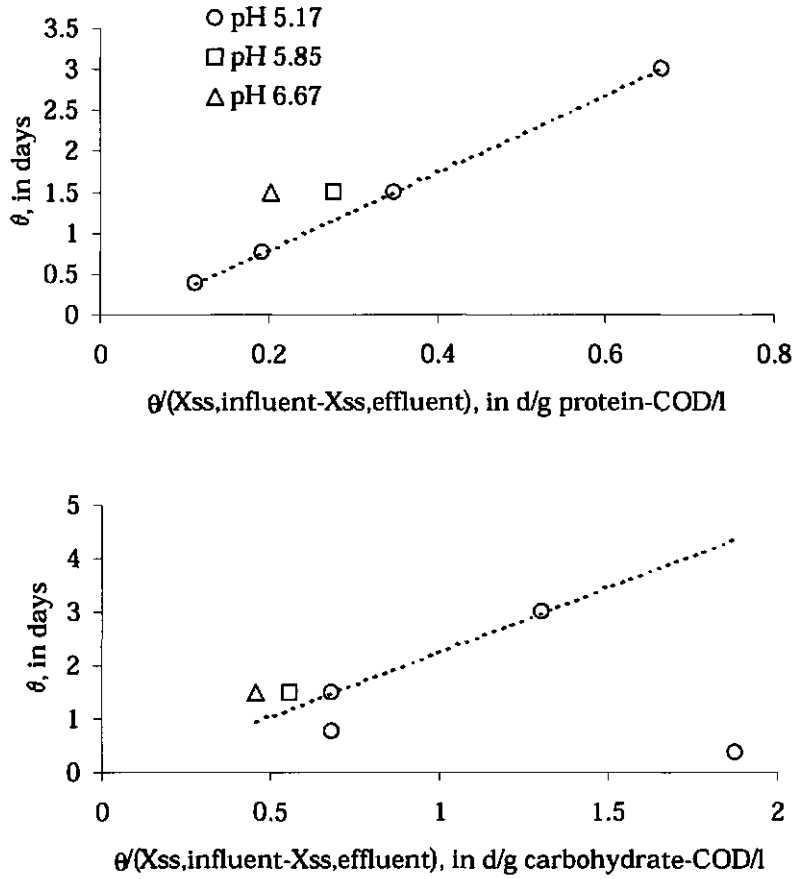

Figure 5.5: *Graphical presentation of the linearisation equation (eq 5.5) concerning the hydrolysis of protein (upper graph) and carbohydrates (bottom graph) in primary sludge at 35° and under acid conditions as calculated from the results obtained by Eastman and Ferguson (1981) (slope=$f_h \cdot X_{ss,influent}$, y-intercept=$-k_h^{-1}$)*

Miron et al. (2000) conducted digestion experiments with primary sludge in CSTR's at HRT's 3, 5, 8, 10 and 15 days at 25°C. Three reactors acidified and the pH reduced to 4.8, viz. those operated at an HRT of 3, 5 and 8 days. In two reactors, methanogenic conditions and pH=6.5 established, viz. the reactors at a HRT of 10 and 15 days. From the results Miron et al. (2000) it was concluded

that the hydrolysis process of none of the single components present in the sewage sludge proceeded according to first order kinetics.

5.5 Accumulation of hydrolysis intermediates

As discussed in section 5.2.1, both a batch set up and completely stirred tank reactor set up can be used to determine the hydrolysis constant for a certain type of waste(water). The main difference between these two methods is that in the batch set up never a steady state situation is reached, whereas this is a pre-condition in the continuous set up. In the batch set up intermediates, such as VFA, LCFA and H_2, can accumulate in time, which implies changing process conditions during the assessment of the k_h value.
In a CSTR in steady state, possibly accumulated intermediates remain at a constant level. However, as the assessment of the hydrolysis constant in a continuous set up requires at least three different HRT's, also here different levels of accumulated intermediates will prevail for the different HRT's. Eastman and Ferguson (1981) investigated the hydrolysis of lipids, proteins and carbohydrates during the acidification of primary sludge at different HRT's but at a constant pH (see also 5.4). Although likely different levels of accumulated intermediates prevailed at the different HRT's, a first order relation was established for the hydrolysis of protein and carbohydrate hydrolysis. This indicates that for proteins and carbohydrates the accumulation of degradation intermediates can be neglected for the hydrolysis of these components. Moreover, it also means that the hydrolysis constant of proteins and carbohydrates under acid conditions can also be determined in a batch set up, provided the pH is controlled (Palenzuella-Rollon, 1999).

With respect to the assessment of the hydrolysis constant for neutral lipids it can be concluded from the results in Chapter 4 that accumulated intermediates such as H_2 and LCFA do not seem to effect the hydrolysis constant.

5.6 The effect of the particle size distribution on the first order hydrolysis constant

For particulate substrates several researchers showed that the hydrolysis mechanism is surface related (Hills and Nakano 1984, Sanders et al. 2000). This means that in presence of an excess amount of enzymes the available surface to the hydrolysis is the rate-limiting factor (Hobson 1987, Sanders et al. 2000). The hydrolysis constant in the first order kinetics is however based on the degradation rate of the total mass. Obviously, the mass to surface ratio is an important controlling parameter for the value of the hydrolysis constant and is strongly related to the particle size distribution of the substrate (Chapter 2). According to the results of experiments of Hills and Nakano (1984) with tomato solid waste and of Sanders et al.(2000) (Chapter 2) with particulate starch with different initial size distributions, the average particle diameter of the substrate (D_p) multiplied by the sphericity of the particles (Φ_s) (Hills and Nakano, 1984) is linearly related to the hydrolysis constant (Fig 5.6).

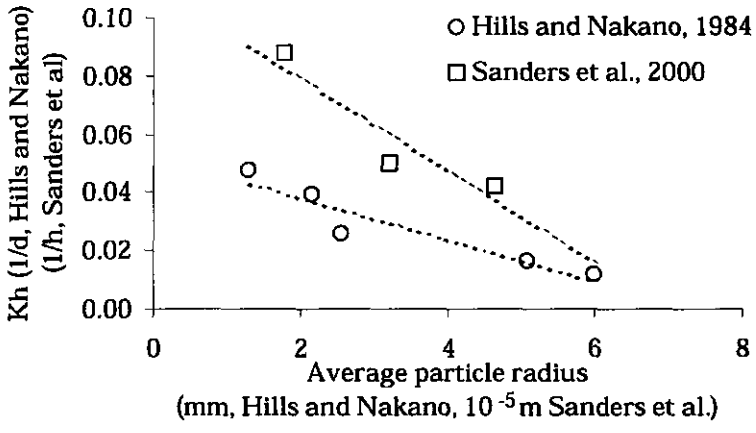

Figure 5.6: *The relation between the average particle radius of a substrate and the first order hydrolysis constant as assessed from Hills and Nakano (1984) and Sanders et al. (2000)*

The results of Perot et al. (1988) show a positive relation between mixing intensity and the hydrolysis rate. Based on the results presented in Chapter 4 the absence of methanogensis negatively effects the hydrolysis of neutral lipids, which likely can be attributed to changes in the particle size distribution of the lipids due to the lack of gas mixing.

Lipids can also have an effect on the bioavailability of other components. Palenzuella-Rollon (1999) digested fish processing wastewater with different lipid concentrations in batch experiments. The results showed that increasing the lipid concentration to ≥ 4300 mg COD.l^{-1}, the hydrolysis constant for proteins significantly decreased. Palenzuella-Rollon (1999) suggested that this may be due to physical hindrance by non-hydrolysed lipids and probably also LCFA, i.e. covering the surface of the proteins which results in a decrease of the amount of protein surface available to the hydrolysis.

5.7 Discussion

Although Eastman and Ferguson (1981) proposed first order kinetics for particulate complex heterogeneous substrates, these can only be applied when the rate limiting factor, in general the surface of the substrate, bioavailability or biodegradability does not alter during the conversion of the substrate. Especially for lipids the latter is not always the case. Reduction of the lipid water interface due to coagulation might be the reason that lipid hydrolysis does not follow first order kinetics under acidogenic conditions. First order hydrolysis kinetics of lipids were only assessed in a multiple flask batch experiment at methanogenic conditions (Chapter 4). The deviation from first order kinetics as found for lipid hydrolysis in CSTR experiments of O'Rourke (1968) and Miron et al. (2000) is probably related to problems encountered with sampling, viz. scum layers and adsorption of lipids to the reactor wall. The results also indicate that gas mixing effects the particle size distribution, and with that the hydrolysis rate of lipids. This implies that a value assessed for the hydrolysis constant of neutral lipids in a multiple flask batch experiment might not be applicable to a full-scale application in a CSTR.

When designing a reactor it is very tempting to use one of the many values for the first order constant reported in literature. However from above results and discussions it is clear that the use of these literature values is only legitimate with a detailed knowledge on the substrate, process conditions and calculation procedure from which the hydrolysis constant was obtained. As it is often difficult to find a hydrolysis constant obtained for the correct temperature and pH, corrections for both effects likely have to be made.

Corrections for temperature seem allowed as the hydrolysis increases with temperature according to the Arrhenius equation. For hydrolysis of total particulate COD and protein a standard free energy of activation of ~46 kJ/mol can be used. For the hydrolysis of carbohydrate components such as cellulose and starch the apparent standard free energy of activation is much higher i.e. ~181 kJ/mol. Temperature corrections to the hydrolysis constant of neutral lipids can

not be made, because the changes in temperature affect the solubility and coagulation behaviour of the lipids. These changes in bioavailablity are not accounted for in the Arrhenius relation.
Obviously corrections to account for differences in pH are quit difficult, as the effect of pH depends on the characteristics of the substrate and of the different enzymes involved in the process. From the results obtained in this chapter no general relation between the first order hydrolysis constant and the pH can be established. If the hydrolysis constant is assessed under methanogenic conditions the pH should be in the neutral range, viz. pH 7±0.5. For acid conditions it is recommended that the hydrolysis constant be assessed at several pH's.

Even when digester conditions and the substrates are similar, the biodegradability and kh can be different. Table 5.4 allows a comparison of the calculated hydrolysis of protein in primary sludge from data of O'Rourke (1968), Eastman and Ferguson (1981) and Boon (1994). It appears that the biodegradability of the proteins in raw domestic sewage remains rather constant, even at different pH's, but the hydrolysis constant is affected significantly even up to a factor of 30. A reason that could explain the differences in the hydrolysis constants in Table 5.4 is the difference in particle size distribution between the three primary sludge's. This would mean a 30 times smaller average particle size in the research of Eastman and Ferguson (1981) as compared to the particle size of the sludge used by Boon (1994). However, as in neither two of the researches particle size distribution was measured a conclusion on this matter can not be made.

Table 5.4: *Calculated first order hydrolysis constant and biodegradability of the protein in raw domestic sewage sludge at pH 5 and 7, from data of O'Rourke(1968), Eastman and Ferguson(1981) and Boon (1994)*

	kh (d^{-1})	fh	pH	T (°C)
Boon, 1994	0.2	0.32	7.0±0.2	35
O'Rourke, 1968	0.67	0.40	7.1±0.3	35
Boon, 1994	0.2	0.23	5.0±0.2	35
Eastman and Ferguson, 1981	6.2	0.31	5.17±0.04	35

5.8 Conclusions

- The first order hydrolysis constant of total COD, carbohydrates and proteins is related to digestion temperature via an Arrhenius relation. For total COD, protein and carbohydrates a standard free energy of activation of ~46 kJ/mol, ~46 kJ/mol and ~181 kJ/mol was observed, respectively.
- No general relation between the pH and the first order hydrolysis constant could be assessed.

- The first hydrolysis constant is affected by the particle size distribution of the substrate.
- In this chapter evidence is obtained that for waste(water) containing mainly protein and carbohydrates, the value for the first order hydrolysis constant can be assessed under acidic and methanogenic conditions in batch or CSTR system.
- For waste(water) that contains high concentrations of lipids the assessment of the hydrolysis constant for neutral lipids under acid conditions is impossible due to coagulation of the lipids. Under methanogenic conditions the hydrolysis constant can be assessed in a multiple flask batch system (section 5.2.1).

SUMMARY AND DISCUSSION

1 Introduction

During the anaerobic digestion of complex waste(water) the hydrolysis is the first and often the rate-limiting step. In the hydrolysis stage, polymeric compounds are converted by extra-cellular enzymes to soluble mono- or dimers that are readily available to the acidogenic bacteria.
The polymeric components in waste(water) that need to be hydrolysed can be found in different physical states, in particles, dissolved or emulsified. Particles are the most commonly found, for example 60-90% of the total COD in domestic sewage consists of particles (Elmitwalli, 2000). Significant amounts of dissolved polymers can be found in slaughterhouse wastewater (gelatine) or potato processing wastewater (dissolved starch). Emulsified polymers are usually lipids, for instance those prevailing in olive mill effluents.

The hydrolysis rate is most commonly described by a first order relation, in which the hydrolysis rate is linearly related to the amount of available biodegradable substrate (Eastman and Ferguson, 1981)

$$dX_{degr}/dt = -k_h.X_{degr}, \quad (1)$$

With:
X_{degr} = concentration biodegradable substrate (kg/m3),
t = time (days),
k_h = first order hydrolysis constant (1/day).

However, this relation is merely empirical and all physical and enzymatic aspects of the hydrolysis process are lumped together in the first order hydrolysis constant. Although this approach keeps the mathematical description of the hydrolysis rate simple, it does not clarify the complexity of the actual hydrolysis process. The latter makes the optimisation of the hydrolysis process difficult. In this thesis the hydrolysis kinetics during the anaerobic digestion was investigated to allocate the key factors in the hydrolysis process. Chapters 2 to 4 deal with the mechanisms of hydrolysis of particulate substrates, dissolved polymeric substrates and neutral lipids, respectively. Results of lab experiments and mechanistic hydrolysis models were used to elucidate the hydrolysis mechanisms. Chapter 5 evaluates the usefulness of the first order model based on the results presented in previous chapters. Moreover, in this chapter also guidelines are presented for the experimental determination of the first order constant.

2 Hydrolysis of particulate substrates

For particulate substrates, several authors have already provided evidence that the hydrolysis kinetics is surface related (Hobson 1987, Hills and Nakano 1984). In their approaches, it is presummed that the substrate particles are completely covered with bacteria that secrete the hydrolytic extra-cellular enzymes during digestion. As it is assumed that enzymes are excessively present, the hydrolysis rate is constant per unit area available for hydrolysis. The advantage of this approach is that the hydrolysis constant in this model is independent on the particle size of the substrate. This model will further be referred to as the Surface Based Kinetics (SBK) model:

$$\frac{dM}{dt} = -K_{sbk} * A \qquad (2)$$

With:
M = mass of substrate (kg),
t = time (days)
K_{sbk} = surface based hydrolysis constant (kg/m^2 day),
A = surface available for hydrolysis (m^2).

In Chapter 2 the surface based kinetics (eq 2) were elaborated, to describe the surface related hydrolysis kinetics for spherical particles in a batch digestion. In this model, it was assumed that the total number of substrate particles remains equal during the digestion. The model was verified in several batch experiments with particulate starch as a substrate. In these experiments three different substrates containing starch with different particle size distributions (PSD) were used. Two were obtained from fresh potatoes by wet sieving and for the third substrate, a commercially available starch was used. The substrates were batch digested at 30°C using granular sludge as inoculum and the hydrolysis efficiency was measured and fitted with the model. The results revealed that the surface based hydrolysis constants for the three substrates were equal, viz. 0.4±0.1 g starch/m^2/hour whereas the first order hydrolysis constant varied with the PSD. Moreover, for the commercial starch not only the hydrolysis efficiency but also the changes within the particle size distribution (PSD) of the starch was determined several times during the digestion with the use of light microscopy and image analysis. The obtained experimental PSD showed good similarity with the theoretical PSD from model calculations. The results of the experiments revealed that at constant pH and temperature, the amount of surface available for the hydrolysis is the most important parameter in the hydrolysis rate and all other parameters are of minor importance.
Because the composition of most (waste)water is more complex than an emulsion of particulate starch and the PSD is usually unknown, at this stage the SBK model cannot replace the first order model for the design of anaerobic digesters.

However, the results in this chapter reveal that the hydrolysis constants (k_h) obtained from literature cannot be extrapolated to similar substrates if the particle size distributions differ, as the first order model does not take the substrate surface into account.

3 Hydrolysis of dissolved polymer substrates

The results presented in Chapter 2 reveal that the rate of hydrolysis of particles is limited by the amount of surface available for the hydrolytic enzymes. When dealing with dissolved substrates the available amount of surface corresponds to the total amount of substrate. Therefore it is very likely that in case of dissolved polymeric substrates the amount of active enzymes is rate limiting. As investigations dealing with the kinetics of hydrolysis of dissolved compounds have indicated that the hydrolytic enzymes are located on the sludge (Goel et al. 1998, Confer and Logan 1998a) the sludge concentration could play a role in the hydrolysis of dissolved polymeric components.

Chapter 3 deals with research on the relation between the hydrolysis rate and the sludge concentration, by conducting laboratory experiments and by computer simulations. In the simulations, the hydrolysis of dissolved polymeric components was regarded as a general depolymerisation process. In this depolymerisation process it was assumed that the chemical bonds between the monomer units of the macromolecules break randomly until only monomers and dimers remain. The statistical relation for such a depolymerisation process, as proposed by Montroll and Shima (1940), was slightly modified to not only describe the evolution of the size distribution of the macromolecules but also to account for the enzyme activity during the degradation process.

From the results of the laboratory experiments presented in Chapter 3 and results presented in literature, it was established that for the hydrolysis of dissolved polymeric components (dissolved starch, gelatine) in batch experiments:

- the initial hydrolysis rate and sludge concentration are linearly related,
- the inverse initial hydrolysis rate and inverse initial substrate concentration are linearly related.

The results of the model simulations reveal that the mechanism of the enzymatic hydrolysis of dissolved polymers can be described as a random polymerisation process (Montroll and Shima, 1940).
Nevertheless, because the depolymerisation model requires a detailed knowledge on the composition of the substrate, the applicability of this model is limited to simple substrates.

4 Hydrolysis of lipids

A literature survey concerning the hydrolysis of lipids during anaerobic digestion indicated that methane production plays an important role in the hydrolysis of neutral lipids. The mechanism for this phenomenon remained however unclear. Therefore, in Chapter 4, the effect of the absence of methane production with respect to the rate of hydrolysis of neutral lipids was studied. The results show that under acidogenic conditions the hydrolysis of neutral lipids was slower as compared to methanogenic conditions. This lower volumetric hydrolysis rate under acidogenic conditions could not be attributed to a low pH, accumulation of long chain fatty acids or accumulation of di- or mono glyceride. The results indicated a non-causal relation between the hydrogen concentration and the volumetric hydrolysis rate. Calculations using a constant surface based hydrolysis rate of 0.19 mg COD/m^2/day showed that the decrease of the available lipid-water interface was less under methanogenic conditions. Apparently the production of biogas could have a positive effect on maintaining the lipid-water interface and subsequent higher volumetric hydrolysis rate.

5 Evaluation of the first order kinetics and the hydrolysis constant

The investigations presented in Chapters 2 to 4 reveal the most important factors for the hydrolysis mechanisms, but still a more suitable mathematical equation for the design of digesters treating complex waste(water) than the first order relation (eq. 6.1) could not be found. The identification of the essential factors for the hydrolysis rate did make it possible to designate the limitations of the first order relation. In Chapter 5 the first order kinetics were evaluated and the effects of digestion conditions were investigated through a literature survey. It was concluded that the hydrolysis only proceeds according to first order kinetics if no changes in the rate limiting step or the biodegradability occur during the degradation of a substrate. The first order hydrolysis constant seems system and substrate specific and the use of literature values for the hydrolysis constant is not advised as the correction for differences in pH often is difficult. Temperature corrections for the hydrolysis constant of protein and carbohydrates through the Arrhenius relation seem legitimate.

For waste(water) containing mainly protein and carbohydrates, first order kinetics can be established under acidic and methanogenic conditions in batch or CSTR systems. For waste(water) that contains high concentrations of lipids the assessment of the hydrolysis constant for neutral lipids under acid conditions is impossible due to coagulation of the lipid. Under methanogenic conditions the hydrolysis constant can be assessed in a multiple flask batch system. In a CSTR system no first order kinetics could be established for lipids, which was probably

caused by problems encountered with correct sampling, viz. scum layer formation and adsorption of lipid to the reactor wall. As (gas) mixing can differ between a laboratory batch and a full-scale CSTR-system, the subsequent effect on the lipid-water interface, as hypothesised in Chapter 4, might cause a difference in the prevailing k_h value of the two systems.

6 Discussion

In this thesis the hydrolysis mechanisms of particles, dissolved polymeric substrates and lipids were approached separately. However, in complex waste(water) these fractions usually occur side by side. When wastewater is treated in an up-flow reactor the particles become part of the sludge fraction whereas the dissolved components remain part of the water fraction. Therefore to achieve a good hydrolysis of the particles the SRT is the most important design parameter. For the hydrolysis of the dissolved polymers the hydraulic retention time is the most important design parameter.
As high concentrations of suspended solids make high volumetric loading rates in one-stage anaerobic reactors virtually impossible, a two-stage reactor system for complex wastewater is recommended, especially when low temperatures are applied (Zeeman and Lettinga, 1999). The first stage of such a two-stage system could be a high loaded UASB (Zeeman and Lettinga, 1999) or Anaerobic Filter reactor (Elmitwalli, 2000). In this reactor the particles in the wastewater are removed from the wastewater applying an HRT of 3-4h. In general, the hydrolysis of dissolved polymers is much faster than that of particles. Hydraulic retention times as low as 0.5h (at 30°C, pH 7) under acidogenic conditions appear long enough to establish a complete hydrolysis of dissolved components e.g. gelatine (Breure et al. 1985). For the design of complex wastewater with both particles and dissolved polymers, the hydrolysis rate of the particles rather than the dissolved polymers will be the major design parameter.
For the design of anaerobic reactors treating complex wastewater with a high level of neutral lipids several considerations have to be made. Lipids can cause severe problems during the digestion. Namely, accumulation of free LCFA and possible LCFA toxicity, due to the high lipid-water interface of the lipid emulsions (Batstone, 2000). Secondly formation of scum layers and wash out of sludge, due to the low specific density of the lipids can occur. Palenzuella-Rollon (1999) advises to remove lipids from the wastewater prior to anaerobic treatment to achieve a better process stability. This removal could be done in the first stage of a two-stage system as proposed by Zeeman and Lettinga (1999) or by a dissolved air flotation unit. The sludge produced in the first stage of a two-stage system as proposed by Zeeman and Lettinga (1999), Elmitwalli (2000) and Palenzuella-Rollon (1999) for the treatment of domestic sewage and fish processing wastewater is hardly stabilised. The prevailing short SRT in the first

step only provides some protein and carbohydrate hydrolysis while the absence of methanogenic activity limits the lipid hydrolysis. Application of sludge digestion at elevated temperatures can achieve stabilisation of the produced sludge and recovery of CH_4 gas.

While lipid containing wastewater is advised to be treated in a two stage system, concentrated slurries and waste with high lipid concentration should preferably treated in a one-stage system for two reasons. (1) The presented results clearly show that lipids will not be hydrolysed in absence of methanogenic activity. (2) The possible decrease of the lipid-water interface in the first stage of a two-stage sludge digester can even result in a longer required SRT in the second stage.

SAMENVATTING EN DISCUSSIE

1 inleiding

Complex afval(water), zoals rioolwater, slachthuisafvalwater, mest of afvalwater van de aardappelverwerkende industrie bevatten organische polymeren, zoals eiwitten, koolhydraten en vetten. Deze polymeren moeten tijdens de anaërobe vergisting eerst worden gehydrolyseerd alvorens ze door de micro-organismen kunnen worden opgenomen. Tijdens de hydrolyse worden de polymeren door extracellulair enzymen omgezet naar hun mono- en dimeren, die vervolgens weer opgenomen kunnen worden door micro-organismen. Polymeren in afvalwater kunnen in verschillende fysische toestanden voorkomen, in de vorm van deeltjes, opgelost of geëmulgeerd. De meeste polymeren komen voor in de vorm van deeltjes, zo bestaat het chemisch zuurstof verbruik (CZV) van bijvoorbeeld huishoudelijk afvalwater voor 60-90% uit deeltjes (Elmitwalli, 2000). Daarnaast zijn er ook afvalwaters die een aanzienlijke hoeveelheid opgeloste polymeren bevatten, bijvoorbeeld slachthuisafvalwater (gelatine) en afvalwater van de aardappelverwerkende industrie (opgelost zetmeel). Vetten in bijvoorbeeld afvalwater dat ontstaat tijdens de productie van olijfolie zijn over het algemeen geëmulgeerd.

De hydrolysesnelheid wordt vaak beschreven door middel van een eerste orde relatie, waarbij de hydrolysesnelheid lineair is met de hoeveelheid biodegradeerbaar substraat dat aanwezig is (Eastman and Ferguson, 1986).

$$dX_{degr}/dt = -k_h.X_{degr}, \qquad (1)$$

Waarin:
X_{degr} = concentratie biodegradeerbaar substraat (kg/m3),
t = tijd (dagen),
k_h = eerste orde hydrolyse constante (1/dag).

Deze eerste orde relatie tussen de hoeveelheid biodegradeerbaar substraat en de hydrolysesnelheid is echter slechts een empirische relatie en alle biologische en fysische aspecten van de hydrolyse kinetiek zijn samengenomen in de eerste orde hydrolyse constante. Hoewel door deze benadering de kinetische beschrijving van de hydrolyse eenvoudig blijft geeft de eerste orde relatie geen inzicht in de onderliggende biologische en fysische processen. Dit gebrek aan inzicht maakt het moeilijk om de hydrolyse tijdens de anaërobe vergisting van complexe substraten te optimaliseren.
In dit proefschrift is met behulp van laboratoriumexperimenten en mechanistische modellen de kinetiek van de hydrolyse onderzocht met als doel de hydrolyse kinetiek te verhelderen. In hoofdstuk 2 tot en met 4 worden achtereenvolgens de hydrolyse van deeltjes, opgeloste polymeren en vetemulsies

besproken. In hoofdstuk 5 wordt de eerste orde kinetiek geëvalueerd op basis van de conclusies uit de voorafgaande hoofdstukken. Bovendien worden in dit hoofdstuk richtlijnen gepresenteerd voor het bepalen van de eerste orde hydrolyse constante in een laboratoriumexperiment.

2 De hydrolyse van gesuspendeerde deeltjes

De resultaten van een aantal onderzoeken (Hobson 1987, Hills en Nakano 1984) geven indicaties dat de hydrolysesnelheid van deeltjes in complex afval(water) is gerelateerd aan het oppervlak wat beschikbaar is voor de extracellulaire enzymen. Tijdens deze onderzoeken wordt aangenomen dat deze enzymen in overmaat aanwezig zijn tijdens de vergisting en dat het oppervlak van de substraatdeeltjes volledig bezet is door de enzymen. Hierdoor kan worden gesteld dat de hydrolysesnelheid constant is per eenheid van oppervlakte dat beschikbaar is voor de hydrolyse (vergelijking 2). Een groot voordeel van een oppervlakte gerelateerde hydrolysesnelheid is dat de hydrolyse constante (K_{skb}) onafhankelijk is van de deeltjesgrootteverdeling van het substraat.

$$\frac{dM}{dt} = -K_{sbk} * A \qquad (2)$$

Waarin:
M = hoeveelheid substraat (kg),
t = tijd (dagen)
K_{sbk} = oppervlakte gerelateerde hydrolyse constante ($kg/m^2/day$),
A = oppervlakte beschikbaar voor de hydrolyse (m^2).

In hoofstuk 2 is met behulp van vergelijking 2 de hydrolyse van homogene bolvormige substraatdeeltjes tijdens een batchvergisting beschreven. Hierbij werd aangenomen dat de totale hoeveelheid deeltjes tijdens de vergisting gelijk blijft, dwz de deeltjes worden wel kleiner, maar vallen niet uiteen. Het model werd geverifieerd met behulp van een aantal batchexperimenten. In deze batchexperimenten (bij 30°C met anaëroob korrelslib als entmateriaal) werd de hydrolyse van substraten bestaande uit zetmeelkorrels met drie verschillende deeltjesgrootteverdelingen gevolgd. Van de substraten die werden gebruikt werden er twee verkregen door het zeven van gepureerde verse aardappelen. Het derde substraat was een commercieel verkrijgbaar zetmeel (Merck, pa). De oppervlakte gerelateerde hydrolyseconstante (K_{skb}) werd bepaald door het model te fitten op de resultaten van de batchproeven. De resultaten van de fit met het model laten zien dat de K_{skb} voor alle drie de substraten gelijk is, namelijk 0.4±0.1 g zetmeel/m^2/uur. K_{skb} is dus onafhankelijk van de deeltjesgrootteverdeling van

het substraat, dit in tegenstelling tot de eerste orde hydrolyse constante. Een tweede bewijs voor de geldigheid van het oppervlaktegerelateerde model werd verkregen door tijdens de vergisting van het commercieel verkrijgbare substraat de veranderingen in de deeltjesgrootteverdeling te bepalen door middel van microscopisch onderzoek en image analysis. De verschuivingen in de deeltjesgrootteverdeling tijdens het experiment en de door het model voorspelde verschuivingen bleken goed vergelijkbaar. Helaas zijn de deeltjes in afval(water) veel complexer dan de homogene bolvormige zetmeelkorrels. Bovendien is de deeltjesgrootteverdeling van de deeltjes meestal niet bekend. Daarom kan bij het ontwerp van reactoren voor de vergisting van complex afval(water) het oppervlaktegerelateerde model nog niet worden toegepast. De resultaten in hoofdstuk 2 laten echter wel zien dat bij een constante pH en temperatuur het oppervlak van het substraat de hydrolysesnelheid bepaald. Dit betekent dat aangezien bij de eerste orde kinetiek geen rekening wordt gehouden met het beschikbare oppervlak, eerste orde hydrolyse constanten (k_h) uit de literatuur niet gebruikt kunnen worden als de deeltjesgrootteverdeling van de betreffende substraten niet overeenkomen.

3 Hydrolyse van opgeloste polymeren

In hoofdstuk 2 werd geconcludeerd dat de extracellulair enzymen in overmaat aanwezig zijn in vergelijking met het beschikbare substraatoppervlak waardoor de grootte van dit oppervlak bepalend wordt voor de hydrolysesnelheid van deeltjes. Echter in het geval van opgeloste polymeren is het beschikbare oppervlak zeer groot zodat ook de activiteit van de enzymen een rol zou kunnen spelen bij de hydrolysesnelheid. Bovendien heeft onderzoek naar de kinetiek van de hydrolyse van opgeloste polymeren aangetoond dat de extracellulaire enzymen geassocieerd zijn met het slib (Goel et al 1998, Confer en Logan 1998a), zodat ook de slibconcentratie tijdens de hydrolyse een rol kan spelen. In hoofdstuk 3 is daarom de relatie tussen de slibconcentratie en de hydrolysesnelheid tijdens de vergisting van opgeloste polymeren door middel van batchproeven en modelsimulaties onderzocht. Tijdens de modelsimulaties is de hydrolyse beschouwd als een algemeen depolymerisatieproces. Hierbij is aangenomen dat de molecuulbindingen in de polymeren willekeurig breken totdat uitsluitend mono- en dimeren overblijven. De statistische relatie van een dergelijke depolymerisatie (Montroll en Shima, 1940) werd voor dit doel aangepast zodat niet alleen de veranderingen in de polymeergrootteverdeling konden worden beschreven, maar ook het effect van de enzymactiviteit.

De resultaten van de batchproeven en een literatuuronderzoek lieten zien dat tijdens de hydrolyse van opgeloste polymeren, zoals gelatine en opgelost zetmeel in een batchvergisting geldt dat:

- De initiële hydrolysesnelheid lineair is met de slibconcentratie

- De inverse initiële hydrolysesnelheid lineair is met de initiële substraat concentratie

Daarnaast laat de vergelijking van de modelsimulaties met de resultaten van laboratoriumexperimenten zien dat de hydrolyse van opgeloste polymeren beschreven kan worden als een algemeen depolymerisatieproces. Echter, voor toepassing van het depolymerisatiemodel is een zeer gedetailleerde kennis nodig van het betreffende substraat, zodat het model alleen geschikt is voor toepassing bij eenvoudige enkelvoudige substraten.

4 Hydrolyse van vetten

Literatuuronderzoek toonde aan dat plaatsvinden van methaanproductie invloed heeft op de hydrolyse van neutrale vetten tijdens de anaërobe vergisting. Het mechanisme achter deze relatie was echter onbekend. Daarom is in het onderzoek beschreven in hoofdstuk 4 het effect van de aanwezigheid van methaanproductie op de hydrolysesnelheid van neutrale vetten onderzocht. De resultaten van het onderzoek laten zien dat onder verzurende omstandigheden de hydrolyse van neutrale vetten langzamer verloopt dan onder methanogene omstandigheden. Dit kon echter niet worden toegeschreven aan een lagere pH, accumulatie van vrije hoge vetzuren of di- of monoglyceriden. Wel werd een non-causale relatie gevonden tussen de partiële waterstofspanning en de hydrolysesnelheid. Berekeningen waarbij werd aangenomen dat de oppervlakte gerelateerde hydrolyse constante tijdens de vergisting onder zowel methanogene als verzurende omstandigheden 0.19 mg CZV/m^2/dag was toonden aan dat de gasproductie een positief effect zou kunnen hebben op het in stand houden van de vetemulsie en daarmee ook een hogere hydrolysesnelheid.

5 Evaluatie van de eerste orde hydrolyse kinetiek en de hydrolyse constante

De resultaten van het onderzoek beschreven in hoofdstuk 2 tot en met 4 toonde de belangrijkste factoren in de hydrolysemechanismen aan, maar een beter toepasbare mathematische beschrijving van de hydrolyse processen dan de eerste orde relatie (Vergelijking 1) werd niet gevonden. De conclusies van hoofdstuk 2 tot en met 4 maakten het echter wel mogelijk de eerste orde kinetiek te evalueren en de grenzen waarbinnen de eerste orde kinetiek toegepast kan worden aan te wijzen. De resultaten van de evaluaties wijzen uit dat de hydrolyse alleen met behulp van een eerste orde relatie kan worden beschreven als er tijdens de vergisting van het substraat geen veranderingen in de snelheidsbepalende stap of de biodegradeerbaarheid van het substraat optreden. Daarnaast blijkt de hydrolyse constante zeer substraatspecifiek en het gebruik van constanten uit de literatuur moet worden afgeraden aangezien correcties voor verschillen in pH moeilijk zijn. Correcties voor verschillende vergistingstemperaturen voor hydrolyse constanten

van eiwitten en koolhydraten zijn wel mogelijk met behulp van de Arrhenius relatie. De eerste orde hydrolyse constant van afval(water) dat voornamelijk eiwitten en koolhydraten bevat kan onder methanogene als verzurende omstandigheden worden bepaald. Hiervoor kan zowel een batch als volledig gemengd doorstroom (VGD) systeem worden gebruikt. Als afvalwater hoge concentraties vetten bevat is het niet mogelijk om de hydrolyse constante te bepalen onder verzurende omstandigheden aangezien er tijdens het experiment waarschijnlijk coagulatie van het vet zal plaatsvinden. Onder methanogene omstandigheden kan de hydrolyse constante van vet wel worden bepaald. Hiervoor moet dan een 'multiple flask' batchproef worden uitgevoerd om problemen tijdens de bemonstering, door de aanwezigheid drijflagen en adsorptie van vet aan de wand van de reactor, te voorkomen. Deze problemen bij de bemonstering zijn waarschijnlijk ook de oorzaak van het ontbreken van een eerste orde relatie bij experimenten met VGD systemen uit onderzoeken gerapporteerd in de literatuur. Omdat, zoals voorgesteld in hoofdstuk 4, (gas)menging waarschijnlijk invloed heeft op de hydrolysesnelheid van vetten is de extrapolatie van de resultaten van batchexperimenten naar VGD systemen voor de praktijk waarschijnlijk niet mogelijk.

6 Discussie

In dit proefschrift zijn de mechanismen voor de hydrolyse van deeltjes, opgeloste polymeren en vetten apart benaderd. In de praktijk zullen deze fracties echter naast elkaar in het afval(water) voorkomen. Tijdens de behandeling van afvalwater in een opstroomreactor zullen de deeltjes uit het afvalwater voor een groot deel in het slibbed achterblijven. Daarentegen zullen de opgeloste polymeren deel uitblijven maken van de waterfractie. Voor een optimale hydrolyse van de deeltjes is de slibverblijftijd dus de belangrijkste ontwerpparameter terwijl voor de opgeloste polymeren de hydraulische verblijftijd de belangrijkste parameter is.
Een hoge concentratie gesuspendeerde deeltjes in afvalwater maakt de toepassing van hoge volume belastingen in één-fase anaërobe reactoren vrijwel onmogelijk. Daarom wordt voor dit type afvalwater, zeker bij toepassing van lage vergistingstemperaturen, een twee fasen systeem aanbevolen (Zeeman en Lettinga, 1999). De eerste fase van zo'n systeem zou een hoog belaste UASB reactor (Zeeman en Lettinga, 1999) of een anaeroob filter kunnen zijn (Elmitwalli, 2000). In de eerste fase worden de gesuspendeerde deeltjes uit het afvalwater verwijderd bij een hydraulische verblijftijd van 3-4 uur. Over het algemeen is de hydrolyse van opgeloste polymeren veel sneller dan die van deeltjes. Voor opgelost polymeren zoals, gelatine, is een hydraulische verblijftijd van 0,5 uur (30°C, pH 7) zelfs genoeg (Breure et al. 1985) zodat ook bij het ontwerp van reactoren voor afvalwater met zowel deeltjes als opgeloste polymeren, de hydrolysesnelheid van de deeltjes de belangrijkste parameter is.

Bij het ontwerp van anaërobe reactoren voor de behandeling van afvalwater met een hoog vetgehalte gelden andere overwegingen. Vetten kunnen zijn de oorzaak van verschillende problemen tijdens de vergisting, namelijk toxiciteit van vrije hoge vetzuren en vormingen van drijflagen en uitspoeling van biomassa. Palenzuella-Rollon (1999) adviseert daarom om het vet voor de vergisting te verwijderen. Dit kan in de eerste fase van een twee fasen systeem zoals voorgesteld door Zeeman en Lettinga (1999) of in een dissolved air flotatie (DAF) unit.
Het slib wat in de eerste fase van een twee fasen systeem voor de behandelingen van rioolwater en visverwerkingsafvalwater wordt geproduceerd is nog niet gestabiliseerd. De korte slibverblijftijd in de eerste fase reactor biedt alleen mogelijkheid tot het hydrolyseren van een deel van de eiwitten en koolhydraten. Bovendien wordt de hydrolyse van vet door de afwezigheid van methaanproductie geremd. Door het slib uit de eerste fase te vergisten bij hogere temperaturen kan een goed gestabiliseerd slib en methaan worden geproduceerd.

Bovenstaand wordt geadviseerd om vethoudend afvalwater in een twee fasen systeem te behandelen. Echter wanneer het gaat om de anaërobe behandeling van vethoudende slurries en vast afval is juist een één fase systeem het meest geschikt. Hiervoor zijn twee redenen aan te voeren: (1) De resultaten beschreven in dit proefschrift wijzen uit dat in afwezigheid van methaanproductie de hydrolyse van vetten niet of zeer langzaam verloopt. (2) Doordat onder de verzurende omstandigheden in de eerste fase van een twee fasen systeem coagulatie van vetten kan optreden zou de verblijftijd van de slurrie in de tweede fase langer moeten worden om een volledige stabilisatie te bereiken.

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Curriculum Vitea

Wendeline (Wendy) Theodora Maria Sanders werd op 13 april 1969 geboren te Eindhoven. Nadat op het Lorentz Lyceum te Eindhoven het Atheneum B diploma was behaald begon zij in september 1988 met een scheikunde studie aan de Universiteit Utrecht. Na het behalen van het propedeusediploma en het basisdoctoraal scheikunde besloot zij haar studie af te ronden bij de interfacultaire vakgroep milieukunde van de Universiteit Utrecht. Via deze vakgroep werd een 6-maands keuzevak milieuchemie gevolgd waarin onderzoek werd gedaan naar het adsorptiegedrag van organotinverbindingen aan de onderwaterbodem. Daarnaast werd een 9- maands afstudeervak gevolgd bij de vakgroep milieutechnologie van de Wageningen Universiteit met als onderwerp: 'de vorming van licht slib tijdens de biologische nutrientenverwijdering uit rioolwater'. Na het behalen van het doctoraaldiploma in september 1994 begon zij in diezelfde maand als assistent in opleiding bij de vakgroep milieutechnologie van de Wageningen Universiteit. Vanaf maart 2000 is zij bij dezelfde vakgroep werkzaam als tijdelijk projectmedewerker op het gebied van de behandeling van rioolwater in het Midden-Oosten.