Enzyme augmentation of a submerged anaerobic membrane bioreactor : effects on solids hydrolysis and membrane fouling

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**Enzyme augmentation of a submerged anaerobic membrane
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**SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING
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SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING

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ABSTRACT

Anaerobic hydrolysis of particulates presents both a challenge and an opportunity for raw sewage treatment using membrane bioreactors. A significant portion of the total chemical oxygen demand of raw sewage is in the form of organic particulates. Prior to assimilation by the anaerobic consortia, these particulates have to be hydrolyzed, which tends to be the rate limiting step. Enzyme augmentation, in the form of hydrolases, may be a potential solution. By enhancing hydrolysis, the overall biotransformation rate can be increased, chemical energy in the organic solids can be efficiently tapped, and solids accumulation can be better managed. Furthermore, it can potentially lead to a higher biogas production rate and a lower hydraulic residence time requirement.

Due to complete solids retention, anaerobic membrane bioreactors (AnMBRs) can be used to treat screened raw sewage directly, obviating the need for primary sedimentation and sludge digestion. However, slow hydrolysis rates under anaerobic condition may lead to significant solids accumulation in the bioreactors and consequently reduce their effective volumes. At high solids retention times typical for AnMBRs, solids accumulation tends to be further exacerbated. Therefore, it is proposed to supplement a mixture of crude hydrolases into AnMBRs, in an attempt to specifically enhance the hydrolysis of complex organics present in sewage. These complex substrates are typically constituted of proteins, carbohydrates and lipids in substantial proportions. Accordingly, crude hydrolases containing proteases, amylases and lipases were introduced in this study. An inherent benefit may be the potential retention of exogenous enzymes by the gel layers formed on the membranes, hence minimizing enzyme washout. It is also postulated that membrane fouling may be mitigated due to enhanced enzymatic hydrolysis of macromolecular foulants.

This study is divided into several phases, which attempts to elucidate the effects of enzyme augmentation on the performance of an AnMBR treating raw sewage. Firstly, the optimal pH and temperature for the anaerobic hydrolysis of organic particulates in

sewage were determined by statistical analysis. The ranges of pH, temperature and enzyme dosage studied were pH 5-9, 25-35 °C and 0-0.7% w/v respectively. Using full factorial design, the optimum conditions were determined as pH 7, 35 °C and 0.7% w/v of enzyme dosage. Higher temperatures and enzyme concentrations were found to be beneficial for enhancing anaerobic hydrolysis.

Secondly, a series of batch experiments were conducted to elucidate the effects of varying enzyme dosages on sludge characteristics. Zeta potential, particle sizes, floc compactness, settleability and hydrophobicity are the physicochemical properties evaluated in this study. Additionally, the impacts on the concentrations of loosely and tightly bound extracellular polymeric substances (EPS), and soluble microbial products (SMP) were assessed. Pearson's correlation test was adopted to statistically assess the linear correlations between these parameters. SMP protein, EPS protein, SMP carbohydrate, sludge volume index (SVI), fractal dimension (D_f) and contact angle are the factors identified as having strong and linear correlations with enzyme dosages. Positive relationships of exogenous enzymes with EPS protein, SMP carbohydrate, and SMP protein may be attributed to the localization of exogenous enzymes within the EPS matrix and the enzymatic hydrolysis of bound EPS into SMP. During the SVI analysis, sludge stratification was observed at enzyme dosages of 0.33 g/g TSS or higher, indicating the reduction in settleability. However, D_f and particle size distributions revealed insignificant changes in floc structures and sizes respectively, suggesting that deflocculation was not exacerbated. Increasing enzyme dosages resulted in higher sludge hydrophobicity, which could be contributed by the immobilized enzymes within the EPS matrix.

Thirdly, the effects of enzyme augmentation on the biological performance of a laboratory scale AnMBR treating synthetic raw sewage were ascertained. By supplementing a hydrolase mixture at dosages ranging from 0.9 to 18 mL/g influent COD, average total and volatile suspended solids declined by approximately 19% and 22% respectively, compared to the control without enzyme augmentation. Contrary to

initial expectation, insignificant improvement in COD removal from $94.5 \pm 0.5\%$ to $95.3 \pm 0.6\%$ was observed. Nevertheless, it is noteworthy that permeate quality was not compromised, suggesting the retention of exogenous enzymes within the AnMBR, which was corroborated by size exclusion chromatography. In general, average biogas production increased from 0.27 to 0.34 L/g influent COD, in spite of larger fluctuation attributed to perturbation of steady-state condition. Additionally, bound EPS concentrations declined and gave rise to higher SMP concentrations, suggesting the enzymatic hydrolysis of bound EPS into SMP, the latter possibly biomass associated. Low enzymatic activities were detected throughout the entire study, probably due to the instability of exogenous enzymes in the bioreactor environment.

Fourthly, batch and continuous filtration experiments were performed to investigate the effect of enzyme augmentation on membrane fouling. The hydrolase mixture was either applied directly as free enzymes, or immobilized covalently onto virgin membranes. Fouling mitigation effect of the former was evident throughout the entire study, judging from the transmembrane pressure (TMP) profiles. However, the latter lost their effectiveness when operated for an extended period of 20 days, after which TMP reached a similar plateau value of ~ 40 kPa relative to the control. During the continuous filtration experiment, chemical and particle size analyses of the cake layers revealed the enzymatic degradation of the macromolecular foulants formed on the membranes and the agglomeration of bioparticles respectively. The former was further corroborated by confocal images of bio-cakes stained for proteins and carbohydrates, which illustrated low fluorescence intensities and surface coverages. The latter could increase the bio-cake porosity, therefore conferring lower cake resistance based on the Carmen-Kozeny equation.

Lastly, indigenously produced hydrolases were recovered from anaerobic and aerobic waste sludges via ultrasonication and supplemented daily into the AnMBR. Enzyme recovery was optimized statistically using full factorial design, by varying sonication intensity (2.0 – 4.6 W/cm²) and duration (2–30 mins), resulting in the optimal condition

of 3.3 W/cm^2 and 30 mins. Under the proposed system, COD removal efficiencies improved slightly from $93.0 \pm 0.9\%$ to $94.2 \pm 1.2\%$ following the supplementation of anaerobic sludge extract, but declined to $91.5 \pm 1.8\%$ when dosed with its aerobic counterpart. Excitation-emission matrix analysis of the aerobic sludge extract allows the identification of humic and fulvic acids-like substances as the culprit. Biogas production increased inappreciably from 2.0 ± 0.2 to $2.1 \pm 0.6 \text{ L/d}$, but rose more substantially to $2.3 \pm 0.3 \text{ L/d}$ when supplemented with anaerobic and aerobic sludge extracts respectively. In spite of significant biogas generation, a shortcoming of applying the aerobic sludge extract was the exacerbation of membrane fouling, possibly due to its high SMP content.

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LIST OF ABBREVIATIONS

AnMBR	anaerobic membrane bioreactor
ANOVA	analysis of variance
BMP	biochemical methane potential
BESA	2-bromoethanesulfonic acid
COD	chemical oxygen demand
CLSM	confocal laser scanning microscopy
EEM	excitation-emission matrix
EPS	extracellular polymeric substances
EGSB	expanded granular sludge bed
HRT	hydraulic retention time
LCFA	long chain fatty acids
MWCO	molecular weight cut-off
MF	microfiltration
OLR	organic loading rate
PBS	phosphate-buffered saline
PVDF	polyvinylidene fluoride
SMP	soluble microbial products
SEM	scanning electron microscope
SEC	size exclusion chromatography
SRT	solids retention time
SVI	sludge volume index
TSS	total suspended solids
TMP	transmembrane pressure
UASB	upflow anaerobic sludge blanket
UF	ultrafiltration
VFA	volatile fatty acids
VSS	volatile suspended solids

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Teo C.W., Wong P.C.Y. (2014). Enzyme augmentation of an anaerobic membrane bioreactor treating sewage containing organic particulates. *Water Research*, 48, 335-344.

Teo C.W., Lee J.Y., Wong P.C.Y. (2014). Effects of supplementing free and immobilized hydrolases on fouling mitigation in anaerobic membrane bioreactors for raw sewage treatment. *Journal of Membrane Science*, submitted.

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CHAPTER 1: GENERAL INTRODUCTION

1.1. Introduction

The predicaments confronting mankind in the 21st century are inevitably the water and energy shortage crises. Anaerobic biotechnology can be an effective remedy to address and overcome these compelling issues, by coupling the stabilization of biodegradable pollutants with the reclamation of renewable energy. Through the widespread adoption of anaerobic wastewater treatment, conventional water reclamation plants can be revolutionized into holistic treatment facilities with net energy generation and clean water production.

In the realm of sewage treatment, anaerobic biotechnology has mainly been applied to sludge stabilization in conventional digesters. Concentrated sludge streams ensure high chemical oxygen demand (COD) loads into the digesters with concomitantly high rates of methane production. If aerobic sludge stabilization is adopted instead, large aeration volume is required to ensure that the overall process will not be limited by oxygen mass transfer. On the other hand, domestic wastewater has been conventionally treated using aerobic biotechnologies, especially the activated sludge process. The rationale for this phenomenon is that domestic wastewater contains low COD which can be easily oxidized by aeration. However, aerobic treatment has some inherent shortcomings that compelled a reassessment of conventional sewage treatment. Aerobic treatment incurs high aeration cost, typically 15-25% of the overall operational cost (Mueller et al., 2002). It also engenders high sludge treatment and disposal costs, which can add up to more than 50% of the total construction and operational costs (Rowe et al., 1995). On the contrary, anaerobic treatment requires no oxygen and produces little sludge. Typical net yields of aerobic and anaerobic sludges are about 0.5 and 0.1 kg VSS/kg COD removed, respectively (Eckenfelder et al., 1988). Furthermore, biomethane can be recovered as a renewable energy. Although the methane yield from sewage treatment is low, typically in the range of 0.08-0.18 m³/kg COD removed (Noyola et al., 1988),

substantial amount of bioenergy can still be generated due to high sewage volumes, up to nine times higher than the electricity required for operation (Liao et al., 2006).

Application of anaerobic biotechnology to municipal wastewater has been scarce until recent developments in upflow anaerobic sludge blanket (UASB) technology, which established anaerobic sewage treatment in tropical regions with COD removal efficiencies of 65-80% at organic loading rates (OLRs) lower than 3 kg COD/m³/d and hydraulic residence times (HRTs) between 6-10 h (Foresti, 2002). Other high rate anaerobic processes like anaerobic fluidized bed reactor, anaerobic filter, and expanded granular sludge bed (EGSB) reactor have also been developed for the treatment of low strength wastewaters, of which UASB and EGSB have been more widely adopted in full scale sewage treatment (Jae et al., 2007). However, high solids concentration can negatively affect granular sludge bed formation due to blockage of liquid distribution channels and favorable bio-growth on particulate surfaces (Lettinga et al., 1991). This shortcoming can be circumvented by the use of anaerobic membrane bioreactor (AnMBR), which can ensure high concentration of floccular biomass due to membrane retention, therefore preventing the washout of slow growing methanogens. Furthermore, organic particulates in raw sewage can be effectively retained by microfiltration (MF) or ultrafiltration (UF) membranes, leading to improved solids degradation (Lew et al., 2009). Liao et al. (2006) evaluated the applicability of AnMBR for domestic wastewater treatment and concluded that there are expected opportunities for treating raw sewage and primary sewage effluent. A comparison of previous studies on the performances of AnMBR and UASB for sewage treatment revealed that AnMBR can achieve relatively lower effluent COD and suspended solid concentrations. Moreover, direct treatment of raw sewage using AnMBR obviates the need for primary sedimentation and separate sludge digestion, hence streamlining the entire treatment process.

Due to the aforementioned advantages, AnMBR can be a valuable expansion to the range of wastewater treatment technologies currently employed in the industry. It offers

excellent physical suspended solids removal, coupled with the biochemical digestion of dissolved organics. Nevertheless, there are also shortcomings and complications to be addressed. Firstly, membrane fouling is a prevalent problem in MBR operations, which engenders higher pressure drop and pumping requirements. Secondly, moderate control of pH is appropriate for methanogens to function favorably, typically within the pH range of 6.4-7.8 (Grady et al., 1999), even though several anaerobic studies demonstrated otherwise (Taconi et al., 2008; Leerdam et al. 2008). Thirdly, slow anaerobic hydrolysis, high solid retention times (SRT), and complete solids retention can lead to solids accumulation and low treatment efficiencies (Seghezzo et al., 2005).

Domestic wastewater contains approximately 500-800 mg/L of total solids, out of which 155-330 mg/L of the solids are suspended (US EPA, 2008). With a considerable amount of suspended solids present, OLR cannot be too high in order to achieve satisfactory treatment efficiency. Otherwise, excessive solids accumulation can reduce the performance and stability of AnMBR owing to exacerbation of cake fouling. Therefore, improving solids hydrolysis represents an important step in raw sewage treatment, considering that hydrolysis is rate limiting, especially under psychrophilic condition (Henze et al., 2008; Lettinga et al., 2001; Graaff et al., 2010). With the right concoction of enzyme blend, organic particulates are promptly converted to their soluble equivalents, which tend to be more amenable to biodegradation. The corollaries may be reduced solids accumulation and enhanced biogas production.

Membrane fouling is a critical phenomenon that can influence the economic and operational feasibility of AnMBR. Membranes represent a significant proportion of the capital costs, and operations can be adversely affected by fouled membranes requiring frequent cleaning and replacement. In order to maintain a constant flux operation, fouling necessitates a higher transmembrane pressure (TMP) to compensate the resulting flux reduction. MBR fouling is induced by a multitude of foulants like inorganic precipitates, colloids, extracellular polymeric substances (EPS), soluble microbial products (SMP) and cell agglomerates, of which EPS and SMP were

identified as the major culprits (Meng et al., 2009; Le-Clech et al., 2006). Specifically, high molecular weight EPS and SMP (>10 kDa) were to be blamed (Jang et al., 2005).

Hydrolases are a group of non-specific enzymes that can catalyze the hydrolysis of organic particulates and macromolecular foulants. Therefore, enzyme augmentation, in the form of hydrolases, may potentially reduce solids accumulation and mitigate membrane fouling in AnMBR. Furthermore, Aquino et al. (2006) noticed that fouled MF membranes could retain macromolecules, giving rise to the speculation that enzyme washout may be minimized. Currently, application of AnMBR for sewage treatment has been largely confined to preliminary trials at laboratory and pilot plant scales. The main reasons for the lack of full scale implementation are slow reaction kinetics and low biogas yields. The reduction in volumetric productivity due to membrane fouling further downplays its applicability. Enzyme augmentation is a potential solution that attempts to address and resolve these salient issues. If successfully applied, widespread implementation of AnMBR for raw sewage treatment may ultimately be the result.

Due to the complex nature of raw sewage, a mixture of hydrolases is required for effective degradation. As sewage typically comprises of 40-60% proteins, 25-50% carbohydrates, and 8-12% lipids (Asano, 2007), it is desirable for the enzyme pool to contain hydrolases targeting these specific macromolecules, like proteases, glucosidases and lipases. Nevertheless, cost remains a pertinent issue governing their application in wastewater treatment. If a combination of enzymes is required, it is more economically feasible to adopt crude enzymes, instead of purified ones, considering the polluted nature of wastewaters. Other than cost consideration, enzyme stability in the bioreactor environment must also be addressed. In order to exhibit reasonable amounts of catalytic activities, treatment conditions like pH, temperature and ionic strength should be within the acceptable ranges for the individual enzymes. These parameters are typically satisfied in biological sewage treatment, considering the low wastewater strength and moderate conditions.

1.2. Scope and objectives

This thesis studied the effects of enzyme augmentation, in the form of crude hydrolases, on the performance of an AnMBR treating synthetic raw sewage containing organic particulates. Emphasis was placed on the operation at mesophilic temperature (30-32 °C) and high SRT (50 d), under which solids accumulation was expected. Various doses of a hydrolase mixture were supplemented directly into the bioreactor. The technical and economical feasibilities of enzyme supplementation in AnMBR were investigated and discussed in details.

Two main research hypotheses were investigated and corroborated with the experimental results. Firstly, it is hypothesized that biogas production can be enhanced as a result of enzyme augmentation. This may be attributed to the enhanced enzymatic hydrolysis of organic particulates into monomers, which can be microbially assimilated for methane generation. Secondly, it is postulated that membrane fouling may be mitigated, since macromolecular foulants were also invariably the target of enzymatic hydrolysis. By reducing the concentrations of these foulants, lower operational TMP is achievable under constant flux. These hypotheses underpin the overall study, of which the main objective is to assess the effects of enzyme augmentation on solids hydrolysis and membrane fouling. The secondary objectives are to investigate any detrimental effects on sludge characteristics and to explore alternative enzyme sources.

1.3. Organization of thesis

This report comprises of eight chapters. Introduction and literature review constitute the first and second chapters respectively. In Chapter 3, a series of anaerobic batch experiments were performed in an attempt to determine the optimum conditions for the anaerobic hydrolysis of organic particulates in sewage. Temperature, pH and enzyme dosage were the variable factors selected and the results were analyzed statistically using full factorial design. The impacts of supplementing enzymes on the

physicochemical characteristics of anaerobic sludge were examined using Pearson's correlation test in the fourth chapter. Chapters 5 and 6 entail the thorough investigation of the effects of enzyme augmentation on the biological and membrane performances of AnMBR respectively. Chapter 7 looks into the possibility of recovering enzymes from waste sludge using ultrasonication as an alternative to commercial enzymes. Lastly, conclusions and recommendations for future studies were presented in Chapter 8. The first and second hypotheses in Section 1.2 are corroborated in Chapters 5 and 6 respectively.

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CHAPTER 2: LITERATURE REVIEW

2.1. AnMBRs for municipal wastewater treatment

Anaerobic membrane bioreactors (AnMBRs) couple excellent physical retention of solids by membranes and biological stabilization of organic substrates by anaerobic microorganisms to yield high quality permeate. Compared to conventional processes employing clarifiers, MBRs can achieve complete solids removal as well as retain pathogens and slow growing microbes. The latter include methanogens that produce biogas via anaerobic respiration, which can be combusted for heat or energy generation. Additionally, sludge production is low in anaerobic processes, in which only 4% of the initial carbon reserve is converted into sludge compared to 48% in aerobic ones (Dieter et al., 2008). Regardless of these advantages, anaerobic degradation is slow relative to its aerobic counterpart. The anaerobic microbial community consists of slow growing microbes such as aceticlastic methanogens with doubling times of several days (Henze et al., 2008), and undergoes acclimatization from few weeks to months.

Microfiltration (MF) or ultrafiltration (UF) membranes are typically installed in AnMBRs, possessing nominal pore sizes in the ranges of 0.1-10 μm and 1-100 nm respectively. MF membranes can effectively reject almost all suspended solids and bacterial cells, whereas UF membranes additionally retain colloids and viruses. These membranes function mainly via sieving mechanisms to retain compounds larger than their pore sizes. However, smaller compounds may also be retained due to inertial impaction and diffusion interception (Nath, 2008). It is generally inappropriate to select membranes with smaller pore sizes like nanofiltration or reverse osmosis membranes, as fouling will be exacerbated at the high operational TMPs required.

In general, there are three types of AnMBR configurations, namely side-stream, internally submerged and externally submerged, as illustrated in Fig. 2-1. Internally submerged AnMBRs have outer skin membrane modules installed within, whereas the

modules are submerged in an external chamber for externally submerged ones. Side-stream AnMBRs function under crossflow filtration at 1-5 m/s and 2-7 bars (Judd, 2006). On the other hand, submerged AnMBRs operate at low TMPs of less than 1 bar, typically applied through vacuum suction. Coupled with lower footprint requirement, internally submerged configuration may be the preferable choice for municipal wastewater treatment.

Fig. 2-1: Different AnMBR configurations: (a) side-stream, (b) internally submerged, (c) externally submerged (Liao et al., 2006).

Applicability of AnMBRs for low-strength wastewaters was assessed by Liao et al. (2006), who concluded that there is scope for the treatment of raw sewage and primary effluent using the technology. Jae (2007) experimented with a 4 L side-stream AnMBR equipped with polytetrafluoroethylene tubular membranes (nominal pore size of 1 μm) for synthetic sewage treatment at 25 °C. Under organic loading rates of 1-2 kg

COD/m³/d, the bioreactor could be operated continuously for 280 days with satisfactory permeate quality (< 40 mg COD/L), over 6-12 h HRTs. Huang et al. (2008) studied the application of two 6 L submerged AnMBRs equipped with plate and frame membrane modules (0.45 µm pore size) for the treatment of low-strength synthetic wastewater (550 mg COD/L). The two AnMBRs could be operated continuously for five months with COD removal efficiencies of around 99%, and biogas productions of 0.023 and 0.028 L CH₄/g VSS/d at SRTs of 30 and 60 days respectively. Hu et al. (2006) investigated three 3 L submerged AnMBRs for synthetic wastewater treatment (460±20 mg COD/L) and achieved more than 90% COD removals even at a low HRT of 3 h. All these studies allude to the possibility that AnMBRs can achieve similar treatment performances as their aerobic counterpart, with the additional benefit of energy generation.

Nevertheless, the abovementioned studies dealt with particulate-free wastewaters. Preconceived notion of primary sedimentation before biological treatment, as adopted from conventional sewage treatment processes, could influence the decision to pump pre-settled sewage directly into ANMBRs. In the current literatures, there are only few that studied the treatment of raw sewage containing organic particulates using AnMBRs. One reason for the lack of such studies may be solids accumulation at high SRTs. As a result, membrane fouling could be exacerbated as TMP was found to increase linearly with particulate concentration (Lew et al., 2009). Fouling aggravation may also be due to the formation of more acidogenic bacteria, which increased sludge viscosities and reduced floc sizes (Jeison et al., 2009). Using a 17 L submerged AnMBR, Wen et al. (1999) investigated the treatment of real domestic wastewater, screened to < 1 mm, with COD and TSS in the ranges of 100-2,600 mg/L and 100-1,000 mg/L respectively. High COD removals of over 97% were attained at 4-6 h HRTs, which corresponded to organic loadings of 0.5-12.5 g COD/L/d. On the other hand, Saddoud et al. (2007) employed a 50 L side-stream AnMBR for the treatment of real raw sewage, possessing COD and TSS concentrations of 685±46 mg/L and 380±9 mg/L respectively. Higher HRTs of 15-60 h, corresponding to lower organic loadings

of 0.23-2 g COD/L/d, were required to achieve a stable COD removal of 88%. Although different reactor configurations were adopted, it seems that more research is necessary to ascertain the potential of raw sewage treatment using AnMBRs.

2.2. Enhancing anaerobic hydrolysis of organic particulates in raw sewage

Anaerobic hydrolysis is a biochemical process whereby complex macromolecules are hydrolyzed by facultative and obligate anaerobes into soluble monomers, through a series of extracellular enzymatic reactions. Proteins are hydrolyzed by proteases into amino acids; lipids are hydrolyzed by lipases into long chain fatty acids (LCFA) and glycerol; carbohydrates are hydrolyzed by glucosidases into simple sugars. The hydrolysates can then pass through microbial cell walls to be consumed as substrates. The hydrolysis of lipids is much slower relative to carbohydrates and proteins, and requires a minimum SRT of 10 days for complete degradation at 35 °C, compared to 3 days for carbohydrates and proteins (Grady et al., 1999). More details on the functionalities of different hydrolases can be found in the reviews by Whiteley and Lee (2006) and Guo and Xu (2011).

Irimi et al. (2004) reviewed the anaerobic hydrolysis mechanisms of particulate substrates. One mechanism involves the secretion of exo-enzymes by hydrolytic bacteria into the supernatant, which hydrolyze the substrates upon contact. Another mechanism involves the attachment of hydrolytic bacteria onto the particulates directly, followed by the excretion of exo-enzymes. The hydrolytic bacteria can also utilize its attached ecto-enzymes for hydrolysis upon attachment. Isaacson (1990) stated that cell attachment onto particulates can achieve a greater extent of hydrolysis, compared to the secretion of exo-enzymes into the supernatant. The reason provided is that fresh enzymes are continually supplied by the attached cells, and deactivated enzymes are constantly removed by microbial catabolism, hence exposing new sites for hydrolysis.

A theoretical model was proposed by Vavillin et al. (1996), in which bacterial colonization and particulate hydrolysis produce monomers for bacterial growth and the resultant daughter cells further attach onto other particulates. Mohapatra (2006) also agreed that bacterial attachment and enzyme secretion at particle surfaces play a significant hydrolytic role. Using optical microscopy, Sanders et al. (2000) observed that starch particles were totally covered by bacterial cells after a few hours of anaerobic digestion. Hence, particulate hydrolysis can be described as a surface-based degradation phenomenon, whereby particulates with higher specific surface areas are hydrolyzed faster (Dimock and Morgenroth, 2006).

Organic particulates represent approximately 60% of the total organic carbon in municipal wastewater (Bitton, 2005), with an upper bound of 85% of the total COD (Tarek et al., 2001). Biomethanization of these organics consists of a series of catabolic reactions using carbon dioxide as the electron acceptor, as illustrated in Fig. 2-2. Among the four sequential stages of anaerobic degradation, solids hydrolysis tends to be rate limiting in sewage treatment (Seghezzo et al., 2005). Therefore, development of strategies to enhance solids degradation is imperative in anaerobic sewage treatment.

Hydrolysis can be primarily enhanced under optimal temperature, pH and retention time. In general, hydrolysis proceeds faster at higher temperatures according to Arrhenius equation. Although Rollon (1999) stated that the optimal pH for anaerobic hydrolysis is near neutral, Isaacson (1990) pointed out that this pH maximizes methanogenesis instead of hydrolysis. Specifically, pH 5.6-6 is optimum for hydrolysis and acidification, whereas pH 6.8-7.2 is optimum for methanogenesis (Riva, 1992). The optimal conditions are probably substrate-specific. Perot et al. (1988) found that the optimal pH and temperature for anaerobic sludge hydrolysis are pH 6.8 and 50°C respectively. For the anaerobic digestion of primary sludge and starch-rich wastewater, a HRT of 30 h was determined to be optimal (Maharaj et al., 2001).

Fig. 2-2: Anaerobic pathways for the degradation of organic particulates.

Enzymatic treatment had been investigated by several researchers as a potential technique to enhance anaerobic hydrolysis in wastewater treatment. Mendes et al. (2006) studied the anaerobic batch digestion of lipid-rich wastewater, augmented with porcine pancreas lipase (0.5% w/v). Higher COD removal (76.4%) and biogas production (354 ± 34 mL) were reported, compared to the control without enzyme augmentation (40% and 209 ± 47 mL). Slightly higher biogas production and COD removal (78.2% and 445 ± 29 mL) were observed when the wastewater was enzymatically pre-treated for 12 h. Nonetheless, pre-treatment may not be feasible for municipal wastewater treatment owing to large volumetric requirement for reaction tanks. Turkdogan-Aydinol et al. (2011) investigated the supplementation of crude proteases, amylases and lipases (0.2 mL/L of wastewater) to UASB reactors for the treatment of real raw sewage (993 ± 259 mg COD/L). Higher COD removals ($73 \pm 8\%$)

were observed, relative to the control without enzyme addition ($64\pm5\%$). Batch experiments alluded to the importance of dose optimization, because excess enzymes remained as residual COD. From these studies, it appears that enzyme augmentation may be beneficial for raw sewage treatment by improving effluent quality and biogas production. A comprehensive list of literatures on the application of hydrolytic enzymes for waste or wastewater treatment is presented in Table 2-1. All the studies reported marginal to significant improvements in treatment performance.

2.3.Fouling mitigation in AnMBRs

Membrane fouling in AnMBRs is influenced by several physicochemical and biological phenomena, which contribute to total hydraulic resistance (R_T) according to Equation 2-1,

$$R_T = R_{cp} + R_c + R_g + R_{pb} + R_m \quad (2-1)$$

where R_{cp} is the resistance attributed to concentration polarization, R_c and R_g are the resistances conferred by the formation of bio-cake and gel layers respectively, R_{pb} is the resistance contributed by inter-pore blocking, and R_m is the intrinsic resistance of membrane.

At the initial stage of filtration, primary gel layer forms even at sub-critical flux, due to the diffusion and adsorption of macromolecules and their aggregates onto membrane surface. Gel layer tends to be shear resistant due to its cross-linked structure, and confers high R_g due to a lack of pore connectivity (Wang and Waite, 2008). Ionic bridging of cations with the anionic groups of biopolymers reinforces the gel layer (Wang et al., 2008). The concurrent accumulation of solutes creates a concentration gradient that exerts R_{cp} . Foulant penetration into the membrane pores contribute to R_{pb} via pore narrowing or constriction.

Table 2-1: Enzyme augmentation studies for anaerobic waste/wastewater treatment (control results are highlighted in italic font)

Enzyme ¹	Waste/Wastewater ²	Reactor configuration ³	COD removal / solubilization ⁴ (%)	Biogas production (mL/d)	Reference
Crude lipase (0.5% w/v)	Dairy wastewater (21-52 g COD/L)	Batch (500 mL)	76.4 <i>40</i>	354±34 <i>209±47</i>	Mendes et al. (2006)
Crude protease, amylase and lipase (2 mL/L of wastewater)	Raw sewage (993±259 mg COD/L)	USAB (21 L)	73±8 <i>64 ± 5</i>	–	Turdogan-Aydinol et al. (2011)
Crude lipase (0.1% w/v)	Synthetic dairy wastewater (3,068±623 g COD/L)	UASB (1.8 L)	90±2.74 <i>82 ± 7.15</i>	–	Leal et al. (2006)
Crude lipase (0.1% w/v)	Synthetic dairy wastewater (3.5 g COD/L)	Batch (1.5 L)	90.5±3.4 <i>32.4 ± 9.7</i>	–	Rosa et al. (2009)
Crude cellulase (2 U/mL of wastewater)	Swine wastewater (8,528 mg COD/L)	Batch (50 mL)	76.3 <i>65.6</i>	30 25	Lee et al. (2008)
Crude multi-enzymes (0.25 g/L)	Spent grain (5 g/L)	Batch (250 mL)	45-60 <i>40-42</i>	600-800 <i>500-700</i>	Bochmann et al. (2007)
Crude carbohydrase, protease and lipase (0.02-4% v/v)	Raw food waste (146.1±7.4 g COD/L)	Batch (1.5 L)	30-60 <i>0</i>	–	Kim et al. (2005)

¹ Dosage in parenthesis

² Feed concentration in parenthesis

³ Reactor volume in parenthesis

⁴ COD removal and solubilization are reported for treatment of wastewater and solid waste respectively

Table 2-1: Enzyme augmentation studies for anaerobic waste/wastewater treatment (control results are highlighted in italic font)

Enzyme ¹	Waste/Wastewater ²	Reactor configuration ³	COD removal / solubilization ⁴ (%)	Biogas production (mL/d)	Reference
Pure trypsin (920 U/g)	Dewatered sewage sludge (167-176 mg COD/g)	Batch (20 L)	22.1 <i>0</i>	–	Nagle et al. (1992)
Pure protease and α -amylase (6% w/w)	Secondary sewage sludge (7,783 mg COD/L)	Batch (250 mL)	68.4 <i>10</i>	–	Yang et al. (2010)
Crude lipase (0.1% w/v)	Poultry slaughterhouse effluent (1181±371 mg COD/L)	Batch (100 mL)	85 <i>53</i>	43.75 9.25	Valladao et al. (2007)
Pure cellulase, protease and lipase (0.1% w/w)	Anaerobic digester sludge (31 g/L)	Batch (250 mL)	29 <i>6.1</i>	–	Parmar et al. (2001)
Pure maganese peroxidase (1 mg/L)	Municipal solid waste (0.9 kg/L)	Anaerobic landfill bioreactor (15.4 L)	73 <i>24</i>	15.25 <i>3.06</i>	Jayasinghe et al. (2012)

¹ Dosage in parenthesis

² Feed concentration in parenthesis

³ Reactor volume in parenthesis

⁴ COD removal and solubilization are reported for treatment of wastewater and solid waste respectively

The conditioning organic layer, being a nutrient source, encourages microbial attachment, microcolony formation and biofilm maturity in sequential steps (Watnick and Kolter, 2000). Convective drag induces the deposition of bulk sludge on the membrane, while the ionic bridging between metal ions and biopolymers enhances the compactness of the attached biomass (Meng et al., 2007). Altogether, bio-, organic and inorganic fouling resulted in the formation of bio-cake above the gel layer (Lee et al., 2008), exerting R_c . 95-98% of R_T was contributed by R_c , when filtering aerobically or anaerobically digested sludge under dead-end configuration, using mixed acetate cellulose filters with 0.4 μm pore size (Ramesh et al., 2007). Under constant flux operation, an increase in filtration resistance will lead to higher operational TMP, according to Darcy's equation,

$$J = \frac{\Delta P}{\mu R_T} \quad (2-2)$$

where ΔP represents the TMP, μ denotes the permeate viscosity and J symbolizes the instantaneous permeate flux. Higher TMP raises the energy demand for permeate suction, which imposes additional operational cost.

High cell concentrations are prevalent in biological sewage treatment. Therefore, a significant proportion of R_T in AnMBR may be attributed to biofouling. Active microorganisms, cell debris and EPS contribute to the biofilm architecture, of which EPS are responsible for its structural integrity. EPS are heterogeneous polymeric matrices consisting mainly of proteins, carbohydrates, glycoproteins, and other compounds like lipids, humic substances, nucleic acids and urionic acids (Wingender et al. 1999). EPS act primarily as glue in biofilms to facilitate gene and substrate transfers, and may also protect microbes against biocides and retain embedded enzymes (Flemming et al., 2007). Considering the stabilization effect of EPS, one strategy for biofilm dispersion is to target and degrade these compounds. In a MBR system, EPS modify the membrane surface property and alter the cake layer structure, potentially

contributing to R_T . Specific R_c was found to be positively correlated to bound EPS concentration using dead-end filtration (Cho et al., 2005). In particular, the protein and carbohydrate fractions of EPS were identified as the main foulants (Teychene et al., 2008).

Bound EPS, being insoluble in nature, can be classified into loosely and tightly bound fractions, of which loosely bound EPS demonstrated a good correlation with membrane fouling (Wang et al., 2009) and contributed significantly to R_T (Ramesh et al., 2006a). Loosely bound EPS are slime polymers that can move freely between the cells, whereas tightly bound EPS are capsular polymers tightly associated with the cell walls. On the other hand, soluble EPS are dissolved polymers released into the supernatant due to endogenous decay or substrate utilization, and used for cell communication, metal chelation and toxicity protection (Mesquita et al. 2010). Soluble EPS are the same as SMP according to the unified theory proposed by Laspidou and Rittmann (2002), although experimental results revealed that their physicochemical characteristics were different (Ramesh et al., 2006b).

Both EPS and SMP were identified as the main factors influencing fouling during recent reviews (Meng et al., 2009; Le-Clech et al., 2006). Other than reinforcing the bio-cake structure, bound EPS can affect sludge characteristics like hydrophobicity, surface change, flocculability and sludge viscosity, which may further aggravate fouling (Meng et al., 2009). Concentrations of EPS protein are typically higher than that of EPS carbohydrate in MBRs, with maximum values of 120 and 40 mg/g TSS for the former and the latter respectively (Le-Clech et al., 2006). SMP, being present in the supernatant, have a tendency to cause pore narrowing or constriction during their convective passage across the membrane. Adsorption of SMP onto fouling layers and sludge flocs are also possible fouling mechanisms, with the latter contributing to the pool of bound EPS. Recently, Wang et al. (2007) discovered that the affinity clustering of EPS and SMP can lead to the formation of biopolymer clusters, which contribute to higher R_c .

EPS were identified as the main foulant in some studies (Cho et al., 2005; Chang et al., 1998), whereas SMP were identified in others (Rosenberger et al., 2002; Geng and Hall, 2007). The contribution of SMP to R_T ranges widely from 17-81% (Le-Clech et al., 2006). The discrepancy may be attributed to differences in operational fluxes, membrane properties and feed characteristics. Firstly, passive adsorption of SMP onto membrane surface occurs below the critical flux determined for biomass, hence contributing significantly to R_T . Above critical flux, their significance declines due to convective flow of bulk sludge towards the membrane, inducing cake formation and EPS deposition. Secondly, the fouling propensities of EPS and SMP vary with membrane materials, due to differences in membrane characteristics such as surface charge, porosity and hydrophobicity. For example, polyethersulfone membrane experienced more irreversible fouling by EPS due to higher roughness and hydrophobicity, as compared to polyvinylidene fluoride and polyacrylonitrile membranes (Zhang et al., 2008). Thirdly, utilization of easily biodegradable substrates tends to yield less SMP, relative to complex ones, due to lower substrate residuals in the supernatant (Le-Clech et al., 2006). These residuals are commonly measured as part of SMP in the filtered supernatant. Strictly speaking, SMP should be quantified after removing the undegraded substrates (Aquino and Stuckey, 2004).

Supplementation of hydrolytic enzymes to catalyze the degradation of macromolecular foulants can potentially mitigate fouling. The enzymatic hydrolysability of EPS and SMP had been demonstrated in several studies involving sludge digestion (Pei et al., 2010), biofilm degradation (Molebela et al., 2010) and synthetic wastewater treatment (Jiang et al., 2008). Hodgson et al. (1993) performed the dead-end microfiltration of a carbon-limited marine bacterium (SW8), pretreated with 100 mg/mL of proteolytic enzyme at 30°C for 30 mins, in order to assess the effect of EPS modification on filtration performance. EPS hydrolysis, as evidenced from transmission electron microscopy, reduced filtration resistance by 2-3 times relative to the unmodified control. Howell and Velicangil (1982) studied the dead-end ultrafiltration of albumin and hemoglobin (0.5% w/v each) separately at 207-221 kPa for 22 h, using polysulfone

membranes (10 and 30 kDa) adsorbed or cross-linked with proteases (0.2-0.5% w/v). The series of batch experiments revealed 25-78% improvement in permeate yield compared to the untreated control, possibly owing to the presence of a primary enzyme layer that retarded gel formation. Although these studies demonstrated the effectiveness of free and immobilized hydrolases on fouling mitigation using a pure culture or defined medium, the same strategy may also conceivably alleviate MBR fouling.

2.4. References

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CHAPTER 3: OPTIMIZATION OF ANAEROBIC HYDROLYSIS IN MUNICIPAL WASTEWATER TREATMENT WITH EMPHASIS ON ENZYME AUGMENTATION

3.1. Introduction

An attractive feature of anaerobic digestion is the concomitant stabilization of organic matter and production of bioenergy. This technology can be potentially exploited to recover biogas directly from raw municipal wastewater, obviating the need for primary sedimentation. In anaerobic digestion, hydrolysis of suspended solids tends to be rate limiting, especially for substrates with high solids content. This is also the case for municipal wastewater treatment (Seghezzo et al., 2005). Hydrolysis is also a vital process for the degradation of dissolved macromolecules and soluble microbial products (SMPs) that are prevalent in biological wastewater treatment.

Municipal wastewater contains approximately 500-800 mg/L of total solids with a suspended solids concentration of 155-330 mg/L (US EPA, 2008), which represents up to 85% of the total chemical oxygen demand (COD) (Tarek et al., 2001). In terms of organic composition, domestic wastewater typically comprises of 40-60% of proteins, 25-50% of carbohydrates and 8-12% of oil and fats (Asano et al., 2007). These organic solids and soluble macromolecules have to be hydrolyzed to monomers (<1 kDa) before they can be assimilated into cells (Burgess and Pletschke, 2008). Proteins are hydrolyzed into amino acids, lipids are hydrolyzed into long chain fatty acids (LCFAs) and glycerol, and carbohydrates are hydrolyzed into simple sugars, of which carbohydrates are known to be easily hydrolyzable (Zeeman and Sanders, 2001). Due to their predominance in municipal wastewater, hydrolysis represents an important step in reducing the effluent COD and improving overall treatment efficiency.

Anaerobic hydrolysis kinetics can be enhanced by operating at the optimal pH and temperature. In general, hydrolysis rate is higher under thermophilic compared to

mesophilic condition. Within each range, hydrolysis proceeds faster at higher temperatures according to the Arrhenius equation. Conventionally, the optimum pH is at near neutral (Rollon, 1999), although Isaacson (1990) pointed out that neutral pH aims to maximize the rate of methanogenesis; hydrolysis may have a different optimum, specifically pH 5.6-6.0 (Riva, 1992). Furthermore, the optimum pH may vary according to the substrate composition and the hydrolytic species present. For example, the optimum pH of holocellulytic bacteria can range widely from 3.0 to 11.0 (Isaacson, 1990).

Other than pH and temperature optimization, enzyme augmentation in the form of hydrolases can further improve the treatment performance. Enzymatic hydrolysis pretreatment of lipids-rich wastewater using low cost lipase was investigated by Adriano et al. (2006). Higher COD removal (69-80%) and biogas production (10-12 mL/g COD) were observed in comparison with the control without enzymatic pretreatment (COD removal of 40% and biogas production of 6mL/g COD). Moreover, it was found that direct addition of enzymes into the bioreactor is viable and attractive, achieving high COD removal of 76.4%. The feasibility of enzymatic hydrolysis was also demonstrated by other authors (Leal et al., 2006; Rosa et al., 2009; Lee et al., 2008).

The main objective of this research is to determine statistically the optimum conditions for the anaerobic hydrolysis of synthetic raw sewage, including an assessment on the feasibility of enzyme augmentation. Anaerobic batch experiments were also conducted to ascertain the hydrolysis kinetics at neutral pH, with or without methanogenesis, in order to assess the significance of methanogenesis on anaerobic hydrolysis.

3.2. Materials and Methods

3.2.1. Anaerobic batch experiments

Anaerobic hydrolysis experiments were conducted in batches spanning 20 days each. 500 mL standard Erlenmeyer flasks sealed with rubber stoppers and customized flasks (Ying Kwang, Singapore) fitted with Miniert valves (614250, VICI International, Switzerland) were used as batch reactors. The latter consisted of an 8-10 mm diameter port with tap valve installed ~5 cm from the bottom for liquid sampling. The flask headspace was sparged with nitrogen gas for approximately 2 mins before closure to create anaerobic condition. Incubating shakers (Multitron Standard, Infors HT, Switzerland) provided the necessary temperature (25-35 °C) and agitation (100 rpm). pHs were adjusted daily using 6 N HCl and 6 N NaOH. 0.1 M of 2-Bromoethanesulfonic acid (BESA) was added to inhibit methanogenesis at pH 7 (Sparling and Daniels, 1987). Alternatively, heat treatment at 100 °C for 1 h was employed. Heating at 75-121 °C for 0.25-2 h is typically adopted to inhibit methanogenesis in seed sludge (Hu et al., 2008).

The synthetic wastewater used in the batch experiments was prepared to simulate raw municipal wastewater containing suspended solids. The soluble constituent of the wastewater was prepared according to the OECD guideline for synthetic sewage (OECD, 2001), and diluted twice to give a COD of 150 mg/L. The insoluble constituent of the wastewater was simulated by 250 mg/L of dog food with a measured COD of 400±50 mg/L (ALPO, Purina, USA). ALPO dog food was selected because it has similar organic composition as primary sludge (Kim et al., 2003). The dog food was crushed using mortar and pestle, followed by sieving through 2 mm mesh. This gave a total COD of 550±50 mg/L for the synthetic concoction. Seed sludge was obtained from the anaerobic digester at Ulu Pandan Water Reclamation Plant, Singapore. The seed sludge was washed twice with phosphate buffered saline (PBS) (Crystal PBS buffer, Bioline, UK) to remove residual sCOD by centrifugation at

2,000g for 5 mins per cycle. Approximately 5,000 mg TSS/L of washed sludge was added to simulate the high biomass concentration in MBR.

The exogenous hydrolases employed were in the form of a customized liquid blend (BioCat Microbials, USA) containing proteases, lipases and amylases. The measured activities of proteases and amylases were 28 and 23 U/mL respectively, whereas lipase activity was not detected using the titration assay by Freire et al. (1997). The crude blend was purified using a 100 mL stirred cell (Ying Kwang, Singapore) equipped with a 10 kDa membrane disc (Amicon PM 10, Millipore, USA) for 2 exchange cycles to remove the low molecular weight propylene glycol before each experiment. To preserve the enzyme activities, the stirred cell was submerged in an ice bath and a low nitrogen pressure of 2 bar was applied. After purification, the measured protease and amylase activities of triplicated samples were 10.4 ± 0.8 and 19.0 ± 3.0 U/mL respectively.

The degree of particulate hydrolysis was estimated using Equation 3-1 by analyzing the periods where hydrolysis dominates, as indicated by increasing sCOD:

$$\text{Degree of hydrolysis} = \frac{\Delta \text{sCOD} + \Delta \text{COD}_{\text{H}_2} + \Delta \text{COD}_{\text{CH}_4}}{\text{Particulate COD}_{\text{initial}}} \quad (3-1)$$

where $\Delta \text{COD}_{\text{H}_2}$ is the sCOD converted into biogas as H_2 (8g COD/g H_2) and $\Delta \text{COD}_{\text{CH}_4}$ is the sCOD converted into biogas as CH_4 (4g COD/g CH_4). Hence, the degree of hydrolysis was expressed in grams of COD hydrolyzed per gram of particulate COD. Microbial assimilation of COD for growth was not considered in the calculation due to insensitivity of the VSS test and low electron equivalents transferred for cell synthesis (5-15% of ultimate BOD) under anaerobic condition (Rittmann and McCarty, 2002). By adopting a typical yield of 0.08 g VSS/g COD (Tchobanoglous et al., 2003), 44 mg VSS/L is theoretically produced upon complete degradation, which is easily obscured by high VSS concentrations in the batch reactors.

The degree of acidogenesis was calculated from Equation 3-2:

$$\text{Degree of acidogenesis} = \frac{\Delta \text{sCOD}_{\text{VFA}}}{\text{sCOD}_{\text{initial}} + (\text{Particulate COD}_{\text{initial}})(\text{Degree of hydrolysis})} \quad (3-2)$$

where $\Delta \text{sCOD}_{\text{VFA}}$ is the change in COD equivalents of VFA (expressed in g COD/L). Concentrations of acetic acid, propionic acid and butyric acid were converted to COD equivalents using conversion factors of 1.066g COD/g $\text{C}_2\text{H}_4\text{O}_2$, 1.512g COD/g $\text{C}_3\text{H}_6\text{O}_2$ and 1.816g COD/g $\text{C}_4\text{H}_8\text{O}_2$ respectively. The degree of acidogenesis was expressed in grams of sCOD acidified per gram of sCOD present.

The first order hydrolysis constant (k_h ; expressed in d^{-1}) was estimated using non-linear regression based on the first order kinetic equations for substrate and product concentrations respectively (Vavilin et al., 2008):

$$S = S_0 e^{-k_h t} \quad (3-3)$$

$$P = P_0 + \alpha S_0 (1 - e^{-k_h t}) \quad (3-4)$$

where S_0 and P_0 are the initial substrate and product concentrations (expressed in mg/L) respectively, and α is the unitless conversion coefficient from substrate to product.

Statistical analysis was performed to determine the optimum conditions for anaerobic hydrolysis, using Design of Experiments software (Design-Ease 8.0, Stat-Ease, USA). Full factorial design at two levels with three central points was applied. The factors studied were pH (5.0-9.0), temperature (25-35 °C) and enzyme dosage (0-0.7% w/v). The ranges were selected with due consideration to economic feasibility.

3.2.2. Analytical methods

TSS, VSS and sCOD were quantified according to the Standard Methods (APHA, 1998). Dissolved organic carbon (DOC) was analyzed using the TOC ASI-V analyzer (Shimadzu, Japan). Samples for sCOD and DOC analyses were filtered using 0.45 μm Nylon syringe filters (F2602-1, Scientific Procurement, Singapore). Biogas production was measured by the syringe displacement method (Owen et al., 1979). Biogas composition was measured by a gas chromatograph-thermal conductivity detector (GC 7890A) equipped with GS-GasPro and HP-Plot Molesieve columns, and a gas chromatograph-flame ionization detector (GC 6890) equipped with a DB-FFAP column respectively, all of which were procured from Agilent cooperation, USA. Helium was the carrier gas. Protein concentration was determined using a micro BCA protein assay kit (23235, Thermo Scientific, USA) using bovine serum albumin as the calibration standard. Ammonia-nitrogen was measured with the colorimetric assay kit containing salicylate (10031, Hach, USA). Carbohydrate determination was carried out by adapting the modified phenol-sulfuric acid method with glucose as the calibration standard (Rao and Pattabiraman, 1989). Absorbance was measured at 490 nm after the acidified solution reacted with 50 μL of 80% phenol for 30 mins at 25°C.

3.3. Results and Discussion

3.3.1. Optimum conditions for anaerobic hydrolysis

Two distinct intervals can be discerned from the temporal profiles, which are episodes of rising and declining sCOD. The former indicates the solubilization of organic particulates and hydrolysis dominates, whereas the latter reveals the microbial assimilation of soluble substrates and their biomethanization. Table 3-1 presents the calculated degrees of hydrolysis from the periods of rising sCOD (shown inside parentheses in days) and the total degree of hydrolysis under the various conditions. The periods were categorized chronologically into Periods 1 to 3. Only sCOD

deviations of more than 3% with and 1% without the addition of BESA were considered as significant. The selected values correspond to the standard deviation of 1-3% expected for a variety of wastewater samples (Boyles, 1997). As the sCOD increments after Day 10 may be significantly contributed by the release of intracellular compounds arising from cell death and lysis due to starvation, these periods were omitted in the calculation of the total degree of hydrolysis.

To minimize the competing effect of methanogenesis at pH 7, which acts as a sink for sCOD, methanogens were selectively inhibited using either BESA or heat treatment. Both methods yielded a maximum total degree of hydrolysis of 1. It is possible for the calculated values to exceed 1, due to SMP generation derived from the enzymatic hydrolysis of bound EPS. In these cases, the results were reported as the maximum. EPS hydrolysis is inferred from control experiments without substrate addition at pH 7.0 and 30 °C, during which 11 mL and 4 mL of biogas were generated respectively, with and without 0.7% (w/v) of enzyme dose. The disparity in the periods where hydrolysis dominates is probably due to the presence of a variety of macromolecules with distinct rates of hydrolysis under different conditions. As a general guideline, anaerobic hydrolysis of carbohydrate is faster than that of protein, and lipid degradation proceeds with the slowest kinetics (Rollon, 1999).

Due to the low biogas generation and methane proportion, the COD equivalent attributed to methane production ($\Delta\text{COD}_{\text{CH}_4}$) can be negligible. A maximum of 13% CH_4 was detected in the biogas at pH 5, none was noted at pH 7 following BESA supplementation, and 3% CH_4 was detected at pH 9. Therefore, it is presumed that the methanogens were effectively inhibited by BESA, even those hidden within the wall biofilms of the batch reactors. No hydrogen gas was detected throughout the entire study, probably due to the occurrence of hydrogenotrophic methanogenesis which keeps the H_2 partial pressure low. The total biogas production for the entire duration of 20 days ranged from 13-57 mL at pH 5, 8-15 mL at pH 7, and 14-33 mL at pH 9.

Table 3-1: Degree of hydrolysis under the investigated conditions according to full factorial design.

Conditions	Period 1	Period 2	Period 3	Total[@]
pH 9, 35 °C, 0% (w/v)	0.870 (Day 0-10)	0.705 (Day 14-17)	-	0.870
pH 9, 35 °C, 0.7% (w/v)	0.622 (Day 3-9)	0.258 (Day 15-20)	-	0.622
pH 9, 25 °C, 0.7% (w/v)	0.415 (Day 2-6)	0.048 (Day 8-10)	0.217 (Day 16-20)	0.463
pH 9, 25 °C, 0% (w/v)	0.258 (Day 0-1)	0.120 (Day 6-9)	0.210 (Day 10-13)	0.378
pH 5, 25 °C, 0.7% (w/v)	0.303 (Day 0-1)	0.157 (Day 7-9)	0.525 (Day 10-13)	0.460
pH 5, 35 °C, 0.7% (w/v)	0.892 (Day 0-2)	0.217 (Day 8-9)	0.378 (Day 14-17)	1
pH 5, 35 °C, 0% (w/v)	0.280 (Day 0-1)	0.438 (Day 8-14)	0.187 (Day 15-20)	0.430
pH 5, 25 °C, 0% (w/v)	0.375 (Day 0-2)	0.120 (Day 8-9)	0.205 (Day 10-15)	0.495
⁺ pH 7, 30 °C, 0.35% (w/v)	1.967 (Day1-9)	0.235 (Day 9-14)	0.905 (Day 15-16)	1
⁺ pH 7, 30 °C, 0.35% (w/v)	1.875 (Day 0-2)	0.362 (Day 7-9)	1.400 (Day 13-15)	1
⁺ pH 7, 30 °C, 0.35% (w/v)	1.062 (Day 0-2)	0.950 (Day 3-9)	1.275 (Day 13-20)	1
[#] pH 7, 30 °C, 0.35% (w/v)	1.120 (Day 0-1)	0.575 (Day 2-8)	0.323 (Day 13-16)	1
[#] pH 7, 30 °C, 0.35% (w/v)	1.248 (Day 0-7)	0.067 (Day 8-10)	0.090 (Day 14-15)	1
[#] pH 7, 30 °C, 0.35% (w/v)	0.984 (Day 0-1)	0.477 (Day 2-8)	0.560 (Day 13-15)	1

[@] Calculated from first 10 days of each batch experiment

⁺ Repetition of center condition (BESA); [#] Repetition of center condition (Heat treatment)

From the statistical analysis, the coded equation for the degree of hydrolysis expressed in unitless terms was determined ($p < 0.0001$):

$$\frac{1}{\sqrt{\text{Degree of hydrolysis}}} = 1.26 - 0.14(B) - 0.054(C) - 0.048(A)(B) + 0.064(A)(C) - 0.028(B)(C) + 0.12(A)(B)(C) \quad (3-5)$$

where A and B are the coded pH and temperature of the sludge suspension respectively, and C is the coded concentration of augmented enzymes. The low, center and high levels were assigned the coded values of -1, 0 and 1 respectively. An inverse square root transformation was fitted into the equation as determined from Box Cox plot analysis.

As deduced from Equation 3-5, positive correlations exist between the degree of hydrolysis with temperature and enzyme concentration, suggesting that hydrolysis can be enhanced by increasing the operating temperature and/or augmenting hydrolytic enzymes. pH exists as part of the interaction terms in the equation, implying that pH acts synergistically with other parameters to affect hydrolysis. Therefore, the influence of pH could not be determined from the equation directly. The 3D surface plots at various pH values are illustrated in Fig. 3-1. The interactions between the various factors are better visualized from the respective plots constructed using the equation. On close inspection, there is an outlier at pH 9, 35 °C and 0.7% (w/v), possibly due to enzyme inhibition under this condition.

Fig. 3-1: 3D surface plots under the respective pH: (a) pH 5.0, (b) pH 7.0, and (c) pH 9.0. Degree of hydrolysis, temperature and enzyme concentration are expressed in gram of COD hydrolyzed per gram of particulate COD, degree Celsius and percent (weight/volume) respectively.

As illustrated in the 3D surface plots (Fig. 3-1), temperature affects the degree of hydrolysis to a greater extent compared to enzyme dosage due to steeper contour. This can also be inferred from Equation 3-5 where the coefficient of temperature is approximately 3-fold higher than that of enzyme concentration. However, it is not economically feasible to heat municipal wastewater owing to the large volumes to be treated regularly. Enzyme augmentation may be a better option if the enzymes can remain catalytically active for a sustained period. Furthermore, the inactive enzymes are recoverable as bioenergy, as inferred from Equation 3-6.

The predicted optimum conditions are pH 7.0, 35 °C and 0.7% (w/v) of enzyme dosage. Other researchers had also observed high rates of hydrolysis at neutral pH during the treatment of primary and activated sludge (Gomec and Speece, 2003; Perot et al., 1988), kitchen waste (Zhang et al., 2005) and urban solid wastes (Dinamarca et al., 2003). For municipal wastewater treatment, this is an important finding as it is not economically feasible to optimize the overall process by employing two separate stages for hydrolysis and methanogenesis.

From the statistical analysis, the equation for biogas production (in actual factor) was also determined ($p < 0.001$):

$$\text{Biogas production} = 10.386 + 26.428(C) \quad (3-6)$$

A positive correlation exists between biogas production (expressed in mL) and enzyme concentration (expressed in % w/v), suggesting that biogas production can be enhanced by augmenting the hydrolytic activities in the bioreactor.

3.3.2. Kinetic analysis of enzymatic hydrolysis

The kinetics of enzymatic hydrolysis with and without methanogenesis (inhibited using BESA) was investigated under the center condition (Table 3-1). Ammonium is released as one of the final products during protein degradation and its production rates indirectly represent the kinetics of protein hydrolysis, as illustrated in Fig. 3-2.

Fig. 3-2: Temporal profiles of ammonium production (a) with and (b) without methanogenesis. Non-linear regression lines (dotted) are obtained by least square fitting of the experimental data.

The first order hydrolysis constants for protein degradation were estimated from ammonium production using Equation 3-3. With methanogenesis, the predicted values for k_h and α are 0.780 d^{-1} and $0.815 \text{ g NH}_4^+\text{-N /g protein}$ respectively. Without methanogenesis, the predicted values for k_h and α are 0.515 d^{-1} and $0.666 \text{ g NH}_4^+\text{-N /g protein}$ respectively. Lower hydrolysis rate observed under non-methanogenic condition can be attributed to the accumulation of intermediates like VFA and LCFA, resulting in thermodynamically unfavorable reactions. The graphs approached plateaus by Day 6, indicating that a significant proportion of protein was hydrolyzed before then. The prediction of k_h from ammonium production is kinetically possible if hydrolysis proceeds at a slower rate compared to acidogenesis, which can be ascertained by comparing the degrees of hydrolysis and acidogenesis, as calculated from Equations 3-3 and 3-4 respectively. Lower degree of hydrolysis suggests that hydrolysis is more rate-limiting, vice versa. By examining the degrees of hydrolysis and acidogenesis from Day 1 to 9, it was found that hydrolysis was rate-limiting on Day 2, whereas acidogenesis was rate-limiting on Day 3 and 9. On the remaining days, both the degrees of hydrolysis and acidogenesis were similar, implying that methanogenesis may be the rate-limiting step. Since acidogenesis was seldom rate-limiting, the rates of ammonium production could adequately represent the rates of protein hydrolysis.

The first order hydrolysis constants for the degradation of soluble protein and carbohydrate were also determined from non-linear regression using Equation 3-3. However, protein degradation did not follow the first order degradation kinetics as the coefficients of determination (R^2) were both zero (results not shown). This is probably due to the detection of exogenous enzymes by the protein assay that are not easily hydrolyzable. Approximately 21.4 ± 0.6 and $23.1 \pm 0.7 \text{ mg/L}$ of recalcitrant proteins were detected with and without methanogenesis respectively, which likely include exogenous enzymes and SMP. The time profiles for carbohydrate degradation are shown in Fig. 3-3.

Fig. 3-3: Temporal profiles of carbohydrate degradation (a) with and (b) without methanogenesis. Non-linear regression lines (dotted) are obtained by least square fitting of the experimental data.

Good fits to the experimental results based on first order kinetics were not observed for carbohydrate degradation, whereby the R^2 values with and without methanogenesis were 0.805 and 0.544 respectively, as similarly observed by Miron et al. (2000) for primary sludge hydrolysis. Regardless of the poor fits, the predicted values for k_h were 0.121 and 0.080 d^{-1} with and without methanogenesis respectively. Although the statistical significance of the difference was not assessed due to a lack of repetition, carbohydrate hydrolysis was seemingly slower under non-methanogenic condition. Combined with the results obtained from ammonium production (Fig. 3-2), it could be

generally deduced that anaerobic hydrolysis proceeds slower under non-methanogenic condition. This provides a strong justification for inhibiting methanogenesis in order to accurately determine the degree of hydrolysis shown in Table 3-1.

3.4. Conclusions

From the full factorial experiments, anaerobic hydrolysis of organic particulates was found to be optimum at pH 7.0, 35°C and 0.7% (w/v) of enzyme dosage within the respective ranges studied. The degree of particulate hydrolysis was determined primarily from the increase in COD solubilization. Higher operating temperature and/or enzyme concentration generally led to higher rate of hydrolysis, whereby hydrolysis was enhanced to a greater extent by the former. However, as it is not economically feasible to heat municipal wastewater, low-cost crude enzymes coupled with suitable immobilization strategies may be the preferred option. Furthermore, enzyme augmentation was found to enhance biogas production. Combined with the optimal pH of 7.0, a single-stage anaerobic bioreactor treating raw sewage could be supplemented with crude hydrolases to improve its hydrolysis kinetics.

Kinetic analysis of anaerobic hydrolysis was also performed at pH 7, 30°C and 0.35% (w/v) of enzyme dosage. Protein hydrolysis was slower under non-methanogenic condition, as deduced from the first order kinetics of ammonium production. Kinetic analysis of carbohydrate degradation also revealed the same trend, therefore providing a strong justification for inhibiting hydrolysis in order to accurately determine its kinetics.

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CHAPTER 4: EFFECTS OF VARYING ENZYME DOSAGES ON THE PHYSICOCHEMICAL PROPERTIES OF ANAEROBIC SLUDGE

4.1. Introduction

Enzymes can catalytically and selectively enhance the degradation of a target compound, where biotransformation is inherently rate limiting. In the context of wastewater treatment, low-cost crude enzymes may be utilized as high degree of purity is not essential. For example, crude hydrolases produced by *Pencillium restrictum* using agro-industrial waste as the substrate may be adopted (Cammarota and Freire, 2006). Enzyme mixtures remain active and functional under typical operating conditions, although the overall enzymatic activity is suboptimal due to disparate pH optima of different enzymes (Burgess and Pletschke, 2008). Unless chemical inhibitors are present at significant concentrations, enzyme augmentation may be feasible in wastewater treatment. Supplementation of crude hydrolases could enhance the hydrolysis of organic particulates and soluble macromolecules, making the hydrolysates more amenable to subsequent biodegradation.

However, enzyme augmentation may also affect sludge characteristics, such as rheology, surface potential, hydrophobicity, dewaterability and fractal property. Wawrzynczyk et al. (2008) found that exogenous hydrolases were partially attached onto waste activated sludge via cationic interaction, which could modify its surface potential and hydrophobicity. Some of these effects may be undesirable. For example, excessive hydrolysis of bound extracellular polymeric substances (EPS) can lead to deflocculation (Sheng et al., 2010). Watson et al. (2004) had observed deflocculation in a methanogenic bioreactor after the addition of proteases, cellulases and β -glucosidases (10% w/v each), which was explained by the enzymatic hydrolysis of peptide and glycosidic bonds within the flocs. On the other hand, improved filterability and dewaterability of activated sludge were noticed after supplementing α -amylase and β -

glucanase (Ayol et al. 2008). Since these modifications can have direct influence on treatment performance, it is of both scientific and practical relevance to ascertain the effects of enzyme augmentation on sludge characteristics before implementing in anaerobic membrane bioreactors (AnMBRs).

The main objective of this study is to investigate the effects of increasing enzyme dosages on anaerobic sludge characteristics, and to propose a suitable dosage to minimize any detrimental eventualities. Loosely and tightly bound EPS concentrations, soluble microbial products (SMP) concentration, zeta potential, hydrophobicity, floc compactness, settleability and particle sizes are some of the physicochemical parameters statistically analyzed by Pearson's correlation coefficient. It is postulated that low enzyme dosages may aid in sludge flocculation due to electrostatic and hydrophobic interactions with multivalent cations, whereas higher dosages may induce deflocculation due to excessive enzymatic hydrolysis of bound EPS. This study will hopefully provide new and valuable insights on the complex interactions between floc constituents and exogenous enzymes. After identifying the salient factors, an appropriate dosage will be recommended to downplay these undesirable consequences and to strike a balance between enzymatic hydrolysis and intermolecular attraction.

4.2. Materials and Methods

4.2.1. Biotic batch experiments

50 mL centrifuge tubes were utilized as anaerobic batch reactors to evaluate the effects of increasing enzyme dosages on sludge properties. Experiments were performed in triplicates and varying enzyme concentrations were added: 0 g/g TSS, 4.20 g/g TSS, 1.68 g/g TSS, 0.34 g/g TSS and 0.08 g/g TSS. There was less focus on higher enzyme dosages due to concern over economic feasibility. The reactors were sparged with nitrogen gas for 5 mins before closure and wrapped with parafilm to minimize oxygen intrusion. 2.5 g TSS/L of acclimatized anaerobic biomass was added to each reactor.

The seed sludge was obtained from an anaerobic digester treating municipal sludge (Ulu Pandan Water Reclamation Plant, Singapore) and acclimatized to a soluble synthetic sewage (OECD 303A) at a sludge loading rate of 0.28 g COD/g VSS/d for 2 weeks prior to experimentation. The reactors were incubated for 1 day in an incubating shaker (Multitron Standard, Infors HT, Switzerland) at 30 °C and 150 rpm. Information about the crude enzymes utilized and the purification technique involved can be found in Chapter 3.

4.2.2. Analytical methods

Particle size distribution (PSD) of the sludge suspension was measured by a particle size analyzer (Mastersizer 2000, Malvern, England) with a detection range of 0.02-2,000 μm . Each sample was homogenized at 1,350 rpm and analyzed thrice with a scattered light detector that converts the signals into a volume-based size distribution. From the raw PSD results, volume-weighted mean and fractal dimension (D_f) were determined. D_f was estimated from the gradient derived from plotting the log of light scattering intensity versus the log of light scattering vector, based on their power law relationship (Li et al., 2008). Observation of power law behavior, as indicated by a linear plot, suggests the development of fractal structure (Guan et al., 1998). High D_f value implies dense and compact floc structure, and vice versa.

Bound EPS were separated into the loosely and tightly bound fractions by adapting the methodology described by Li and Fang (2007). Sludge suspension was centrifuged at 4000g for 5 mins and SMP were recovered from the supernatant by filtering through 0.45 μm syringe filters (Supor membrane, Pall, USA). Cell pellets were resuspended using PBS solution and vortexed for 1 min. This was followed by centrifugation at 4000g for 10 mins and loosely bound EPS were recovered as the supernatant. Cell pellets were then homogenized with PBS and tightly bound EPS were extracted using cation exchange resin (Lin et al., 2009). The washed suspension and exchange resin (80g/g TSS) were added to a tightly sealed glass bottle (50 mL) and reacted in an ice

bath for 1.5 h under magnetic stirring of 600 rpm. Tightly bound EPS were recovered as the supernatant after centrifugation at 17,400g for 20 mins. EPS and SMP were reported as the sum of protein and carbohydrate concentrations. Analytical methods for protein and carbohydrate concentrations are described in Chapter 3.

Zeta potentials of macromolecular solutions and sludge suspensions were analyzed using Zetasizer (Nano-ZS, Malvern, England). 10-100 measurements were executed automatically at 25 °C using disposable capillary cells (DTS1061, Malvern, England), until stable and consistent results were obtained. Sludge suspensions were vortexed for 1 min prior to analyses in an attempt to homogenize the flocs. Sludge hydrophobicity was ascertained by contact angle measurements using a goniometer (OCA 15, Dataphysics, Germany) at 25 °C. Sludge suspension was filtered through a 1.5 µm filter paper (934-AH, Whatman, USA) and dried at 103 °C for 1 day before characterization. 10 µL of distilled water droplet was deposited onto the sludge layer using a microliter syringe. Static contact angle was measured after the droplet stabilized on the surface.

Sludge settleability was analyzed using sludge volume index (SVI) by adapting the Standard Methods (APHA, 1998). Sludge suspension of known solids concentration was transferred to a 50 mL centrifuge tube, tightly sealed with parafilm, and allowed to settle for 30 mins. SVI (mL/g) was expressed as the volume occupied by 1 g of sludge and calculated using the modified formula,

$$\text{Modified SVI} = \frac{V \times 1000}{\text{TSS} \times 0.05} \quad (4-1)$$

where V denotes the volume of settled sludge after 30 mins (mL/L), and TSS represents the total suspended solids concentration (mg/L).

Most of the factors are normally distributed as determined by the Shapiro-Wilk test (Shapiro and Wilk, 1965), thereby justifying the use of parametric tests even though they are fairly robust to violations of normality assumption (Sara et al., 2010).

Pearson's correlation coefficients, ranging from -1 to +1, were determined using the SPSS statistical package (SPSS version 21.0, IBM, USA). -1 or +1 signifies perfect correlation between the two parameters, coefficient between -0.4 to +0.4 indicates weak correlation, and 0 represents absence of relationship (Li et al., 2008). Weak correlations were not considered in this study. Correlations were statistically significant at a 95% confidence interval ($p < 0.05$). Bonferroni correction was also applied to conservatively reduce the probability of obtaining false-positive results (type I errors) when performing multiple comparison tests (Sokal and Rohlf, 1995).

4.3. Results and Discussion

4.3.1. Identification of major correlations between enzyme dosages and sludge properties

Table 4-1: Pearson's correlation coefficients and p-values for assessing linear correlations between enzyme dosages and sludge characteristics.

	Enzyme dosages		
	r_p	p-value	Number of observations
Zeta potential of sludge	0.578	0.080	10
Zeta potential of tightly bound EPS	-0.024	0.948	10
Zeta potential of loosely bound EPS	0.199	0.581	10
SVI	0.772	0.001 ^{#,+}	15
Protein in SMP	0.986	0.000 ^{#,+}	10
Protein in loosely bound EPS	0.980	0.000 ^{#,+}	10
Protein in tightly bound EPS	0.770	0.009 [#]	10
Carbohydrate in SMP	0.961	0.001 ^{#,+}	10
Carbohydrate in loosely bound EPS	0.552	0.098	10
Carbohydrate in tightly bound EPS	-0.363	0.302	10
Contact angle	0.677	0.006 [#]	15
Volume weighted mean	-0.286	0.302	15
D_f	-0.627	0.012 [#]	15

[#] Statistically significant at two tailed level ($p < 0.05$)

⁺ Statistically significant after Bonferroni correction

According to Table 4-1, seven out of thirteen factors were identified as having strong and linear correlations with enzyme dosages ($r_p > 0.4$, $p < 0.05$). These include protein concentrations in SMP and bound EPS, carbohydrate concentrations in SMP, SVI, D_f and contact angles. After Bonferroni adjustment, only four factors displayed strong statistical associations with enzyme dosages at a very conservative critical value of 0.003. While these factors deserve special attention, the remaining ones should not be neglected to avoid false-negative results (type II errors).

4.3.2. Effects of exogenous enzymes on bound EPS and SMP concentrations

Fig. 4-1: Effects of exogenous enzymes on (a) protein in SMP ($r_p = 0.986$, $p < 0.003$), (b) carbohydrate in SMP ($r_p = 0.961$, $p < 0.003$), (c) protein in loosely bound EPS ($r_p = 0.980$, $p < 0.003$) and (d) protein in tightly bound EPS ($r_p = 0.770$, $p < 0.05$).

It is evident that increasing enzyme dosages induced the accumulation of proteins (Figs. 4-1(a), (c), and (d)), which were preferentially segregated and localized into SMP, loosely and tightly bound EPS. Enzyme localization into cell-free and -bound fractions

was also observed in other studies (Frolund et al., 1995; Guellil et al., 2001; Parawira et al., 2005; Goel et al., 1998). Other than the abovementioned distributions, some of the exogenous enzymes may be autolyzed or biodegraded, especially under low substrate loadings. In the instance of adding 1.68 g/g TSS of enzymes, there was an increase in protein concentrations of 99.4 ± 23.3 , 10.7 ± 3.5 and 18.5 ± 5.6 mg/L in SMP, loosely and tightly bound EPS respectively, relative to the control. Therefore, the majority of the exogenous enzymes seemingly existed as cell-free ones, which could also be inferred from the trendline gradients. Protein mass balance indicated that ~38.7% of these enzymes was biodegraded, autolyzed or cell bounded. Proteins adsorbed onto cell walls were not quantified in this study.

Higher hydrolytic activities of free enzymes compared to cell-bound ones were noticed during the anaerobic digestion of vegetable and flower wastes (Zhang et al., 2007), and of solid potato waste (Parawira et al., 2005), suggesting the fractionation of larger amounts of enzymes in the supernatant. On the other hand, other authors observed that the majority of hydrolytic enzymes existed within the bound EPS fraction in activated sludge (Frolund et al., 1995; Goel et al., 1998) and the pellet fraction in anaerobic sludge (Whiteley et al., 2002), reflecting a lack of consensus on their localization. Varying degrees of electrostatic and hydrophobic interactions between the enzymes and the EPS constituents under different experimental conditions may account for the discrepancies.

In many instances, researchers believed that hydrolytic enzymes are predominantly cell associated. However, it is noteworthy that they studied indigenously produced enzymes, whereas exogenous enzymes were supplemented in this study. As hydrolytic microbes tend to be entrapped within flocs, the secreted enzymes may be significantly retained during their diffusion across the EPS matrices. In the context of enzyme augmentation, exogenous enzymes were dosed directly into the bulk solution. Therefore, higher amounts of proteins could be detected in the supernatant fraction. Other than exogenous enzymes, these proteins might also include undegraded substrates and SMP.

Linear correlation between enzyme dosages and carbohydrates in SMP was also observed (Fig. 4-1(b)), possibly owing to the enhanced enzymatic hydrolysis of sludge constituents. Insoluble carbohydrates within the bound EPS or the organic particulates can be enzymatically solubilized into oligosaccharides or simple sugars, which are detectable by the phenol-sulfuric acid assay (Dubois et al., 1956). Confer and Logan (1997) found that partially hydrolyzed intermediates (<10 kDa) accumulated in the supernatant during aerobic hydrolysis of dextrin and dextran using wastewater cultures. Similar occurrence may be expected under anaerobic condition. The increase in SMP carbohydrate seemingly corresponded to a general decline in tightly bound EPS carbohydrate from 8.04 ± 1.28 mg/L to 7.63 ± 0.71 , 7.44 ± 0.41 and 7.07 ± 2.81 mg/L following the supplementation of 0.34, 1.68 and 4.20 g/g TSS of enzymes respectively. However, the negative linear correlation was not strong ($r_p = -0.363$, $p = 0.302$) using Pearson's correlation and the differences in means were not statistically significant ($F = 0.259$, $p = 0.892$) using analysis of variance (ANOVA). Therefore, discretion has to be exercised when analyzing these results. Nevertheless, since EPS hydrolysis by exogenous enzymes was noticed during sludge digestion (Pei et al., 2010; Ayol et al., 2007) and biofilm degradation (Molebela et al., 2010), the observed decline could be ascribed to the catalytic actions of the exogenous enzymes.

4.3.3. Effects of exogenous enzymes on SVI

Without too much emphasis on the outlier at 0.34 g/g TSS of enzyme dosage, higher enzyme dosages supposedly led to increments in SVI (Fig. 4-2). This observation may be attributed to poorer sludge settleability caused by deflocculation, which arises from the enhanced enzymatic hydrolysis of bound EPS. Deflocculated sludge could exacerbate membrane fouling due to pore blockage by colloidal bio-particles (Meng and Yang, 2007). Fortunately, deflocculation was not severe in this study, as evidenced by similar volume-weighted means for floc diameters according to ANOVA ($F = 0.736$, $p = 0.587$). Alternatively, the preferential localization of exogenous enzymes within bound EPS (Figs. 4-1(c) and (d)) might cause poor settleability. In a critical review by

Liu and Fang (2003), many past studies revealed a linear positive correlation between SVI and EPS, suggesting better settleability at lower EPS concentrations.

Fig. 4-2: Linear correlation between SVI and enzyme concentrations ($r_p = 0.772$, $p < 0.003$).

Stratifications were noticed in the enzyme-treated biomass after 30 mins of settling, which might lead to overestimations of SVI values. The dark brownish solids were stratified into top and bottom regions, with the middle region being relatively clear. Partial enzymatic hydrolysis of sludge probably released lighter flocs into the top region. The volumes occupied by the top and bottom regions were illustrated in Table 4-2. SVI of deflocculated sludge was calculated using the highest measurable volume of 50 mL, which possibly overestimated the actual settleability. For example, 5 mL of stratified sludge in both the top and bottom regions corresponded to an SVI value of 40 mL/g if only the settleable portion was considered. Overestimation of SVI value can possibly explicate the wide deviation between the outlier at 0.34 g/g TSS of enzyme and the other two points. Therefore, basic SVI computation may not be suitable when the sludge was segregated into dual layers.

Table 4-2: Volumes occupied by the stratified sludge layers in SVI analysis.

Enzyme dosage (g/g TSS)	Volume of top region (mL)	Volume of bottom region (mL)
0	-	10
	-	10
	-	10
0.08	-	10
	-	10
	-	10
0.34	-	15
	-	15
	7.5	5
1.68	7.5	<5 [#]
	7.5	<5 [#]
	5	5
4.20	7.5	<5 [#]
	5	5
	2.5	7.5

[#] Minimum measurable volume using the calibrated scale of the centrifuge tubes is 5 mL.

To overcome the limitation of SVI analysis in this study, the volumes occupied by the top and bottom regions were linearly regressed and analyzed separately (Fig. 4-3). The samples with settled volumes of less than 5 mL were not considered. The increasing and decreasing trends of the volumes of top and bottom regions respectively with higher dosages substantiated the hypothesis that sludge flocs were enzymatically hydrolyzed into smaller fractions. The total volume remained largely unchanged, except for the outlier at 0.34 g/g TSS of enzyme, which resulted in a low coefficient of determination ($R^2 = 0.084$). Since the top region likely comprised of loose flocs with low solids concentrations, it is conceivable that sludge solubilization occurred at dosages higher than 0.34 g/g TSS, if the total volume did not increase concomitantly after deflocculation.

Fig. 4-3: Volumes occupied by stratified sludge at different enzyme dosages.

4.3.4. Effects of exogenous enzymes on D_f

Fig. 4-4: Impact of increasing enzyme dosages on D_f ($r_p = -0.627$, $p < 0.05$).

D_f of biological aggregates typically varied from 1.7 to 2.5 (Lin et al., 2010). Specifically, D_f of upflow anaerobic sludge blanket sludge and activated sludge fell within the ranges of 2.76-2.82 and 2.3-2.5 respectively (Mu and Yu, 2006). Pearson's correlation test revealed negative linear relationship between enzyme concentrations and D_f (Fig. 4-4), implying looser and less compact flocs with increasing enzyme dosages. Nevertheless, it should be noted that ANOVA test indicated statistically insignificant differences ($F = 2.32$, $p = 0.126$). Closer inspection of the results showed

that D_f fluctuated within a narrow range of 2.422 to 2.448, suggesting minor structural changes in the flocs. By comparing with the literature values, D_f of the anaerobic sludge adopted in this study fell within the typical range for biological aggregates and could be classified as relatively compact. Nevertheless, these are composite values and the poorly settled flocs in the upper region might possess lower D_f , which were not quantified separately. The minor alterations of floc structures corresponded to insignificant changes in the PSDs of the sludge suspensions, as illustrated in Fig. 4-5.

Fig. 4-5: PSDs of sludge suspensions supplemented with varying enzyme dosages from 0 to 4.2 g/g TSS.

Although poorly settleable flocs were visually observed during the SVI test, deflocculation was not evident from PSD and D_f analyses (Fig. 4-4 and 4-5). It was postulated that the enzyme-treated biomass was predominated by well settleable flocs, compared to their poorly settleable counterpart. Therefore, the latter might be obscured by the former and undetected by the particle size analyzer. Deflocculation may be more pronounced over a longer time horizon, therefore warranting further investigation on the long-term performances of AnMBRs under the influence of these enzymes.

4.3.5. Effects of exogenous enzymes on contact angle

Fig. 4-6: Effects of enzyme augmentation on the contact angles of anaerobic sludge ($r_p = 0.677$, $p < 0.05$).

The anaerobic sludge was hydrophobic in nature, as evidenced by contact angles larger than 45° (Liu et al., 2003). Higher contact angles, signifying increments in hydrophobicity, were generally noted with increasing enzyme dosages (Fig. 4-6). This observation coincides with the accumulation of proteinaceous compounds within the flocs (Figs. 4-1(c) and (d)), alluding to the hydrophobic nature of these enzymes. Larger contact angles were also observed when varying concentrations of bovine serum albumin and bovine glycoprotein (0.1-100 $\mu\text{g/mL}$) were pretreated with pronase P (20 $\mu\text{g/mL}$) and analyzed on petri dishes using the bubble contact angle method (Fletcher and Marshall, 1982). However, the differences in this study were not statistically significant according to ANOVA ($F = 2.29$, $p = 0.131$), probably due to insufficient enzyme doses applied. Repeated dosages in the long run may induce more significant response. In a number of past studies, higher sludge hydrophobicity evidently improved bioflocculation (Liu and Fang, 2003). This inherent benefit can partially counteract the deflocculation phenomenon induced by the enzymatic hydrolysis of bound EPS.

4.4. Conclusions

This study investigated the short-term effects of varying enzyme dosages on various sludge characteristics. By adopting Pearson's correlation test, SMP protein, EPS protein, SMP carbohydrate, SVI, D_f and contact angle were identified as factors having strong and linear correlations with enzyme dosages. Pertinent findings from this study were summarized in the following points:

- Increasing enzyme dosages induced the accumulation of proteins, which were preferentially segregated and localized into the SMP and the bound EPS fractions. Majority of the exogenous enzymes existed in the former compared to the latter. Other than the abovementioned distributions, some of the exogenous proteins were unaccounted by mass balance, possibly autolyzed, biodegraded or cell bounded.
- Higher carbohydrate concentrations were also observed in the SMP fraction with higher dosages, which could be ascribed to the enhanced enzymatic hydrolysis of insoluble carbohydrates within the bound EPS.
- Positive and linear correlation between SVI and enzyme concentrations was observed, implying reductions in sludge settleability at higher enzyme dosages (>0.34 g/g TSS). Sludge stratification was noticed in the enzyme-treated biomass during SVI analysis, possibly owing to the release of lighter flocs into the top region after partial hydrolysis and deflocculation. An upper bound dose of 1.68 g/g TSS was recommended based on the results from this study.
- On the other hand, D_f and PSDs of sludge suspensions revealed insignificant changes in the floc structures and sizes respectively, suggesting that deflocculation was not exacerbated.
- Increasing enzyme dosages resulted in higher sludge hydrophobicity, which may be explained by the accumulation of hydrophobic enzymes in the bound EPS.

Nonetheless, these results should be validated by those from long-term continuous experiments in AnMBR. Microbial acclimatization to the exogenous enzymes presumably moderates the accumulation of proteins. Additionally, complication arises from prolonged exposure of flocs to shear forces in AnMBR.

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CHAPTER 5: ENZYME AUGMENTATION OF AN ANAEROBIC MEMBRANE BIOREACTOR TREATING SEWAGE CONTAINING ORGANIC PARTICULATES

5.1. Introduction

Anaerobic sewage treatment possesses desirable attributes such as energy generation, low sludge production, low nutrient requirement, and elimination of expenses associated with the provision of oxygen. However, a major disadvantage compared to its aerobic counterpart is a poorer effluent quality requiring additional downstream polishing to meet discharge standards. This shortcoming can be partially circumvented by employing anaerobic membrane bioreactors (AnMBRs) in which suspended solids are completely retained by micro- or ultra-filtration membranes, thereby producing solids-free effluents even under high hydraulic throughputs. Apart from active biomass, these solids include particulate materials originating from the influent and from cell decay. The former is dependent on the influent solids concentration, while the latter is considerable at large solids retention times (SRTs).

Solids typically exert 30-70% of the total chemical oxygen demand (COD) in raw sewage (Prashanth et al., 2006), and may even be as high as 85% (Tarek et al., 2001). In an anaerobic environment, the hydrolysis rate of suspended solids is low ($0.3-0.7 \text{ d}^{-1}$), about half that of aerobic hydrolysis (Henze et al., 1997), leading to the observation that anaerobic hydrolysis tends to be rate limiting in sewage treatment (Seghezzo et al., 2005). Prashanth et al. (2006) investigated the biochemical methane potential of synthetic wastewater (500 mg COD/L) containing varying proportions of particulate and soluble COD. A declining methane production rate with increasing particulate concentration was observed. Therefore, potential inhibition of methanogenesis at higher particulate concentrations may impose a practical limit on the maximum allowable solids load.

By treating screened raw sewage directly using AnMBRs, primary sedimentation and separate sludge digestion are obviated, allowing conventional sewage treatment processes to be streamlined. Although AnMBR appears feasible for raw sewage treatment (Liao et al., 2006), solids accumulation, especially at high SRTs, may impede its widespread implementation. Solids accumulation in AnMBRs is contributed by complete sludge retention and slow hydrolysis kinetics, which may reduce the proportion of active biomass. As a result, COD removal efficiencies could be detrimentally affected due to lower specific methanogenic activities (Prashanth et al., 2006). Declines in both methanogenic and non-methanogenic activities can be expected in AnMBRs paralleling similar observation in aerobic MBRs (Hasar et al., 2002).

Supplementing hydrolytic enzymes to AnMBRs may reduce solids accumulation and enhance biomethane generation. A mixture of these enzymes can conceivably enhance the hydrolysis of raw sewage, which typically comprises of 40-60% proteins, 25-50% carbohydrates, and 8-12% lipids (Asano, 2007). Furthermore, enzyme augmentation and membrane filtration potentially complement each other as the fouling layers formed on membrane surfaces could prevent enzyme washout (Aquino et al., 2006; Fan and Huang, 2002) and the impairment of effluent quality. Previous enzyme augmentation studies on lipid-rich wastewaters indicated that higher COD removal efficiencies and biogas production rates could be achieved by supplementing lipases (Leal et al., 2006; Mendes et al., 2006). In municipal sludge digestion, the addition of proteases and amylases enhanced the rate of sludge solubilization (Yang et al., 2010). Recently, Turkdogan-Aydinol et al. (2011) applied crude enzymes containing proteases, amylases and lipases to mesophilic upflow anaerobic sludge blanket (UASB) reactors treating real raw sewage containing 993 ± 259 mg COD/L. Higher COD removal efficiencies ($73 \pm 8\%$) were reported with an enzyme dosage of 2 mL/g of influent COD compared to that without augmentation ($64 \pm 5\%$). Higher process stability was also exhibited in the former.

Soluble macromolecules, which constitute part of municipal wastewater and membrane foulants, are also invariably the target of hydrolytic enzymes. Hydrolysis of these large molecules to low molecular weight monomers (< 1 kDa) is essential prior to microbial assimilation (Burgess et al., 2008), which can be promoted by enzyme catalysis. Confer and Logan (1997) observed the formation of intermediates (2-10 kDa) in the supernatant during the hydrolysis of bovine serum albumin using wastewater cultures. These oligomers may also be enzymatically hydrolyzed into monomers. Consequently, there may be less accumulation of intermediates and higher substrate bioavailability to acidogens.

The objective of this study is to investigate the long-term effects of enzyme augmentation on the biological performance of a laboratory scale AnMBR fed with synthetic sewage containing organic particulates. COD removal efficiency, volatile fatty acids (VFA), solids concentration and biogas production were monitored as performance indicators. Potential retention of exogenous enzymes by fouling layers was also monitored using size exclusion chromatography (SEC) and enzyme activity assays. Coupled with other physicochemical parameters including bound extracellular polymeric substances (EPS), soluble microbial products (SMP), and particle sizes, substrate-enzyme interactions within the AnMBR were identified and elucidated. As the biodegradation of exogenous enzymes was expected, their anaerobic biodegradability was assessed by biochemical methane potential (BMP) tests.

5.2. Materials and Methods

5.2.1. AnMBR setup and operation

Fig. 5-1: Schematic diagram of AnMBR setup. Solid and dotted lines indicate flow directions and data transfers respectively. Open arrows indicate feedback controls.

The study was performed in a 8.6 L laboratory-scale submerged AnMBR (Fig. 5-1) with a working volume of 5 L. Two vertical baffles were installed to create upcomer and downcomer regions. Two triangular blocks were also fixated at the corners of the reactor bottom to minimize dead zones. Flat sheet polyvinylidene fluoride membrane (HVLP, Millipore, USA) with a 0.45 μm nominal pore size and 0.05 m^2 effective membrane area was housed in a customized module (Ying Kwang, Singapore) and placed vertically between the baffles. Biogas was recirculated at a rate of approximately 1.5 L/min using a gas pump (EW-07520-47, Cole Parmer, USA) and coarse bubbles were supplied via two tube diffusers at the bottom. Synthetic raw sewage (550 ± 50 mg COD/L) was introduced semi-continuously at a 1.17 h interval using a peristaltic pump (EW-07520-57, Cole Parmer, USA) to maintain a volumetric loading rate of 1.12 kg COD/ m^3/d . The soluble fraction of the wastewater was prepared

according to the OECD guideline for synthetic sewage (OECD 303A) and diluted twice to give a COD of 150 mg/L. The particulate portion was constituted by 250 mg/L of dry dog food (400 ± 50 mg COD/L) (ALPO, Purina, USA) that was crashed and sieved to sizes of less than 2 mm. ALPO dog food was selected because of its similar organic composition compared to primary sludge (Kim et al., 2003). The influent solids were homogenized by a large capacity magnetic stirrer (SB 301, Stuart, UK) in the feed tank. Permeate was withdrawn using a suction pump (EW-77521-57, Cole Parmer, USA) at a flowrate of ~ 0.43 L/h to achieve a flux and hydraulic retention time (HRT) of 8.5 LMH and 11.7 h respectively. 100 mL of sludge was wasted daily to maintain a 50 d SRT. Seed sludge obtained from an anaerobic digester treating municipal sludge (Ulu Pandan Water Reclamation Plant, Singapore) was acclimatized to the synthetic feed for 6 months prior to experimentation.

Membrane flux, pH, and liquid level were maintained by a programmable logic controller. HCl and NaOH (3 N each) were automatically dosed once the pH exceeded the desired range of 7.0 ± 0.5 . Biogas production rate, permeate flowrate and transmembrane pressure (TMP) were measured by a thermal mass flowmeter (EW-32707-00, Cole Parmer, USA), a turbine flowmeter (S111, McMillan, USA) and a high accuracy pressure transducer (EW-68075-02, Cole Parmer, USA), respectively. Temperature was maintained between 30 and 32 °C by a heating tape and controller (TTM-004, TOHO Electronics, Japan). The AnMBR was operated with a 8 mins-on and 2 mins-off permeate suction-relaxation cycle.

Customized liquid blend (BioCat Microbials, USA) containing proteases, lipases and amylases was purified using a dead-end stirred cell equipped with a 10 kDa membrane disc (Amicon PM 10, Millipore, USA) to remove the low molecular weight propylene glycol before use. The stirred cell was pressurized with nitrogen gas at 2 bars while submerged in an ice bath. The retained enzymes were reconstituted in phosphate buffered saline (PBS) (Crystal PBS buffer, Bioline, UK) to the original volume, yielding a measured COD of 10.9 mg/mL. The purified enzymes were added into the bioreactor daily after sludge and permeate sampling. Following 42 days of baseline

study, enzyme augmentation commenced on Day 43 at a dosage of 0.9 mL/g of influent COD. The dosage was systematically increased to 3.6 mL/g on Day 64, and 18 mL/g on Day 85. These dosages were equivalent to 0.5, 2 and 10 mL/L of the feed volume, respectively. Incremental doses were supplemented to minimize the effect of organic shock loads. All results were expressed as mean $\pm$ standard deviation, unless otherwise stated.

5.2.2. Analytical methods

Molecular weight distributions of soluble samples were obtained using an integrated SEC system (PL-GPC 50 Plus, Agilent, USA) equipped with two PL aquagel-OH mixed columns in series and a refractive index detector. Deionized water was delivered as the eluent at a flowrate of 0.7 mL/min. The injector port, oven and detector were maintained at 30 °C. Calibration over the range 1-600 kDa was performed using dextran.

Protease activity was determined by the protease activity assay kit (Sigma Aldrich, USA) with casein as the substrate. One unit of protease activity is defined as the amount of enzyme that hydrolyses casein to produce 1 μ mol/min of tyrosine at pH 7.5 and 35 °C. Amylase activity was determined using the dinitrosalicylic acid method (Bernfeld, 1955) with soluble starch as the substrate. One unit of amylase activity is defined as the amount of enzyme that hydrolyses starch to liberate 1 mg of maltose in 3 mins at pH 7 and 25 °C. Protease and amylase activities were normalized by VSS and expressed as a standardized unit (μ mol/min/g VSS) to facilitate the comparison of results. Sludge samples were withdrawn weekly for the quantification of enzymatic activities of bound EPS, SMP and washed sludge. Enzymatic activity of cell pellets was calculated as the difference between the enzymatic activities of washed sludge and bound EPS. The sludge samples were diluted 10 times and dispersed under mild ultrasonication of 20 W for 1 min prior to analyses, in order to minimize the mass transfer resistances of enzyme substrates and products across the EPS matrix.

BMP of exogenous enzymes was assessed using anaerobic digester sludge, as adapted from Owen et al. (1979). BMP assays, performed in duplicates, were conducted in 150 mL serum bottles sealed with 20 mm rubber serum caps. 2,500 mg TSS/L of anaerobic biomass and 0.52 g COD/g VSS of synthetic feed were introduced to both the control and the test assays, with the addition of purified enzymes (18 mL/g of feed COD) to the latter. The anaerobic biomass was washed twice at 4,250g for 30 mins at 4 °C, and resuspended with the defined media. The headspace was sparged with nitrogen gas for 10 mins prior to closure. Incubation was performed for 28 days in an incubating shaker (Multitron Standard, Infors HT, Switzerland) at 35 °C and 150 rpm.

Bioreactor sludge and permeate were sampled 1-2 times per week and analyzed immediately unless otherwise stated. The soluble portion of the bioreactor sludge was obtained by centrifugation at 3,350g for 20 mins at 4 °C, followed by filtration using 0.45 µm syringe filters (Supor membrane, Pall, USA). Details of other analytical procedures and methods are described in Chapters 3 and 4.

5.3. Results

5.3.1. Effects of enzyme augmentation on AnMBR performance

During the first 42 days, the suspended solids concentration ($2,441 \pm 58$ mg TSS/L, $2,208 \pm 49$ mg VSS/L) and COD removal efficiency ($94.5 \pm 0.5\%$) were steady, indicating that the bioreactor was functionally stable. Fig. 5-2 shows a slight reduction in solids concentration from the baseline (Day 0-42) to $1,970 \pm 395$ mg TSS/L ($1,720 \pm 348$ mg VSS/L) following enzyme augmentation (Day 43-109), corresponding to a marginal decline in average VSS/TSS ratio from 0.90 to 0.87. Specifically, higher enzyme dosages led to larger declines, wherein VSS concentrations were $2,033 \pm 76$, $1,812 \pm 94$ and $1,283 \pm 275$ mg/L for 0.5, 2 and 10 mL/L of enzyme dosages respectively. Segmented regression lines for VSS were added to aid in trend visualization. Other

than Day 64 to 84, the regression lines illustrated decreasing trends following repeated doses, suggesting that enzymatic solubilization outcompeted microbial growth. Declining trends in VSS/TSS ratios were also evident between Day 50 and 84, followed by a slight increase towards the final phase of the study (Day 84-109).

Fig. 5-2: Temporal profile of solids concentration in AnMBR. Dotted lines indicate segmented regression lines for VSS.

In spite of the observed reduction in VSS concentrations, treatment performance in terms of COD removal was not compromised (Fig. 5-3). COD removal efficiencies improved slightly from the initial $94.5 \pm 0.5\%$ (Day 0-42) to $95.3 \pm 0.6\%$ following enzyme augmentation (Day 43-109). Specifically, the removal efficacies increased marginally with higher enzyme dosages, yielding COD removals of 95.0 ± 0.6 , 95.5 ± 0.9 and $95.5 \pm 0.5\%$ for 0.5, 2 and 10 mL/L of enzyme dosages respectively. Bioreactor and permeate soluble COD (sCOD) vacillated in the ranges of 45-85 mg/L and 16-33 mg/L respectively. The observed fluctuation in bioreactor sCOD during the control period may be induced by the semi-continuous feeding regime adopted. With reference to the mean lines, supernatant quality appeared to improve initially by virtue of enzyme

catalysis, but deteriorated subsequently due to COD accumulation. Total COD declined from 3358 ± 318 to 2688 ± 608 mg/L following enzyme supplementation, which corresponded to the VSS reduction trend (Fig. 5-2).

Fig. 5-3: Temporal profiles of sCOD in AnMBR and in permeate, and total COD. Dotted lines indicate segmented mean lines for sCOD.

5.3.2. Biogas and VFA production

Biogas production represents the energy recovery potential of an anaerobic system and determines its overall economic feasibility. The relative percentage of CH_4 and CO_2 ranged between 74-93% and 7-26% respectively for 12 samples quantified throughout the study. Temporal variation in biogas production (expressed as L biogas/g influent COD) is shown in Fig. 5-4. On average, higher biogas production was observed following enzyme augmentation, increasing from 0.27 ± 0.08 to 0.34 ± 0.27 L/g. Methane yield (expressed as L CH_4 /g influent COD removed) improved correspondingly from 0.24 ± 0.06 to 0.32 ± 0.25 L/g, as calculated using a COD removal of 95%. Deviations from the theoretical yield of 0.38 L/g at 30 °C could be accounted for by cell synthesis

(5-10%), methane dissolution (~10%), and biomass adsorption (Angelidaki and Sanders, 2004). Closer inspection revealed higher biogas production with increasing enzyme dosages, except when enzyme dose was adjusted from 2 to 10 mL/L, after which the biogas production declined from 0.37 ± 0.3 to 0.29 ± 0.13 L/g.

Fig. 5-4: Temporal profiles of VFA in AnMBR and in permeate, and biogas production.

VFA are important intermediates in anaerobic processes, where excessive accumulation indicates process instability and overload. Fig. 5-4 illustrates the VFA concentrations (expressed as COD equivalents) in the bioreactor and in the permeate, which were constituted predominantly by propionic and acetic acids. Prior to enzyme augmentation, low and stable VFA concentrations of 15 ± 4 mg COD/L were observed in the bioreactor. VFA-COD represented approximately 22.7% of the supernatant sCOD, indicating that VFA did not accumulate excessively. The remaining sCOD may comprise of partially degraded substrates and SMP. Significant fluctuations in VFA concentrations following

enzyme augmentation (Day 43-109) were noticed. The extent and the upper bound of those fluctuations appear to increase at higher enzyme doses. The spike in VFA concentrations may be due to enhanced enzymatic hydrolysis and subsequent acidification of the feed, and/or microbial fermentation of exogenous enzymes into VFA. In some instances, VFA dropped to below detection, which was similarly noted by others treating low-strength wastewaters (Huang et al., 2008; Saddoud et al., 2007). The decline may be attributed to the adaptation of aceticlastic methanogens to higher organic loadings, hence improving the bioconversion of VFA into methane (Fuchs et al., 2003; Hu and Stuckey, 2006). A general trend was evident where VFA accumulated after a stepwise dose increment and decreased subsequently.

5.3.3. EPS and SMP concentrations

Macromolecular EPS and SMP are major membrane foulants in MBRs (Le-Clech et al., 2006), which may be degraded into smaller oligomers and monomers by hydrolytic enzymes, subjected to reactivity and mass transfer limitations. Difference in bound EPS and SMP concentrations with and without enzyme augmentation can be discerned from Fig. 5-5. Following enzyme augmentation, the EPS protein concentration declined from 23 ± 3 to 11 ± 3 mg/g VSS, and the EPS carbohydrate concentration declined from 4.0 ± 0.4 to 3.2 ± 0.9 mg/g VSS. The decrease may be due to the enzymatic hydrolysis of bound EPS into SMP, specifically biomass associated products (BAP) according to the unified theory for EPS and SMP (Laspidou and Rittman, 2002). This hypothesis was corroborated by an observed increase in SMP protein concentration from 10 ± 2 to 21 ± 7 mg/L and SMP carbohydrate concentration from 1.5 ± 0.7 to 5.7 ± 2.7 mg/L. The same trend was evident after expressing as COD equivalents. Pei et al. (2010) and Ayol et al. (2007) similarly observed that the addition of hydrolases led to the degradation of bound EPS via a reduction of its protein and carbohydrate contents.

Fig. 5-5: Comparison of EPS and SMP concentrations with and without enzyme augmentation. Error bars represent standard deviations from 6 samples without, and 10 samples with enzyme augmentation. Samples were taken weekly.

5.3.4. Particle size distributions

Fig. 5-6: Particle size distributions of sludge suspensions on Day 40, 61, 82 and 107, representing periods with 0, 0.9, 3.6 and 18 mL/g of enzyme dosages respectively.

The abiotic influence of the exogenous enzymes (6.3 mL/g) on the particulate feed (5 g/L) was preliminarily ascertained in duplicated batch studies, with the addition of sodium azide (1% w/v) to inhibit microbial activities (Batt et al., 2007). The volume-weighted average diameters declined from 553 ± 9 to 292 ± 5 μm after 15 mins of incubation at 30 °C. Demonstration that the supplemented enzymes are reactive toward the organic particulates suggests that floc sizes will decline following enzyme augmentation, unless there are other biotic interactions. Particle size distributions of the sludge suspensions measured on Days 40, 61, 82 and 107, representing the periods with 0, 0.9, 3.6 and 18 mL/g of enzyme dosages respectively, are presented in Fig. 5-6. On Day 40, particle sizes in the bioreactor ranged from 2 to 1,200 μm , with a volume-weighted average diameter of 71 μm . Contrary to the initial conjecture, the volume-weighted average diameters (enzyme dosages expressed in parentheses) increased gradually to 86 μm (0.9 mL/g), 87 μm (3.6 mL/g) and 115 μm (18 mL/g) following higher dosages.

5.3.5. Enzymatic activities of EPS, SMP and cell pellets

Quantifying the enzymatic activities of EPS, SMP and cell pellets would elucidate the fate and localization of the exogenous enzymes inside the AnMBR. Measured protease and amylase activities in the EPS, SMP and pellet fractions from the bioreactor are shown in Table 5-1. Amylase activities were generally higher than protease activities, which could be ascribed to the differences in the methodologies adopted. For example, the longer incubation time in the protease assay (10 mins) compared to that in the amylase assay (3 mins) could give rise to a lower time-averaged protease activity, due to decreasing reaction velocity with time as substrate depletes and product accumulates (Purich and Allison, 2000). The effect is apparently greater than the presumably significant expression of microbial proteases stimulated by the proteinaceous feed. The departure from expectation notwithstanding, the reported values were in the same order of magnitude as that of sludges from sewage treatment plants (Yu et al., 2008). Larger

spread of experimental values observed here probably reflected the temporal variations in enzymatic activities.

Table 5-1: Enzymatic activities ($\mu\text{mol min}^{-1} \text{g}^{-1}\text{VSS}$) of bound EPS, SMP and cell pellets expressed as mean $\pm$ standard error of 3 to 6 samples at various enzyme dosages.

Daily enzyme dose (mL/L)		Supernatant	Bound EPS	Cell pellets
0	Protease activities	2.3 $\pm$ 0.7	7.3 $\pm$ 1.1	11 $\pm$ 1
	Amylase activities	20 $\pm$ 11	82 $\pm$ 9	60 $\pm$ 13
0.2	Protease activities	0.6 $\pm$ 0.6	4.7 $\pm$ 1.2	9.9 $\pm$ 1.2
	Amylase activities	29 $\pm$ 20	63 $\pm$ 9	54 $\pm$ 39
2.0	Protease activities	ND	9.9 $\pm$ 2.0	10 $\pm$ 2
	Amylase activities	ND	110 $\pm$ 23	40 $\pm$ 14
10	Protease activities	1.2 $\pm$ 1.3	8.7 $\pm$ 1.7	9.9 $\pm$ 1.7
	Amylase activities	38 $\pm$ 43	156 $\pm$ 18	26 $\pm$ 11

ND = not detected.

Throughout this study, the majority of the hydrolytic activities were detected in bound EPS and cell pellets, without any discernible trends in preferential localization within the former or the latter. The low enzymatic activities detected in the supernatant, as similarly observed by others in activated sludge process (Guellil et al., 2001; Frolund et al., 1995), were suggestive that it may not be energetically feasible for microbial secretion of enzymes into the bulk solution due to dilution effect. No significant increase in enzymatic activities was detected following enzyme augmentation, except for a rise in amylase activity of 90% in both the supernatant and the bound EPS, when

10 mL/L of enzyme dose was applied. The undiluted enzyme blend contained approximately 19 U/mL of amylase, considerably higher than the protease activity of 10.4 U/mL, which could explicate the accumulation of amylase activity detected.

5.3.6. Size exclusion chromatography

SEC chromatogram represents the molecular weight distribution of soluble compounds, where larger compounds elute faster and display shorter retention times. As the molecular weights of the proteases and amylases utilized are 30 and 55 kDa respectively, a benchmark cut-off of 30 kDa was selected to assess the retention of these enzymes within the AnMBR. The SEC chromatograms indicate that the fouled membranes can partially (Figs. 5-7a and c) or fully (Fig. 5-7b) retain colloids, enzymes and other soluble macromolecules within the bioreactor. High output intensity of ~54 units for the filtered supernatant on Day 59 (Fig. 5-7b) suggested the retention and detection of significant amounts of colloids and/or biopolymer clusters. These compounds, which elute at around 12-17 mins, cannot possibly be single enzyme molecules as the peak is outside the resolving range of the column (> 600 kDa). The right shift and intensity reduction of permeate chromatograms compared to that of supernatant in Fig. 5-7 (a) and (c) suggest the partial degradation of organics during their passage across the membrane biofilm. Negative peaks were observed in some instances, which occur when the refractive indices of the solutes are lower compared to those of the mobile phase (Figueruelo et al., 2006).

Fig. 5-7: SEC chromatograms of permeate and bioreactor supernatant on (a) Day 31, (b) Day 59, and (c) Day 94.

5.3.7. BMP assays

BMP assays were adapted to assess the anaerobic biodegradability of the exogenous enzymes by monitoring the daily biogas production and performing a supernatant sCOD balance, as illustrated in Fig. 5-8. The cumulative biogas volumes produced in 28 days, with and without enzyme supplementation, were 39.0 ± 1.4 mL and 12.5 ± 0.7 mL respectively. In the former case, supernatant sCOD declined to a pseudo-steady-state value of ~ 65 mg/L after 12 days, corresponding to a decline in protein concentrations from 111 ± 5 to 20 ± 2 mg/L. Higher biogas production indicated the biomethanization of the enzyme blend, which amounts to an addition of ~ 350 mg COD/L. On the other hand, a stable sCOD value of ~ 61 mg/L was attained after 5 days in the latter, suggesting that the exogenous enzymes were slowly biodegradable. Nevertheless, complete degradation was observed with an average biogas yield of 0.36 L/g COD removed. Negligible VFA accumulation was noted, being less than 10% of supernatant sCOD for all experiments. Protein and carbohydrate SMP made up a

significant proportion of supernatant sCOD (29-85%), and the remaining may include lipids, monomers, humic and nucleic acids, etc.

Fig. 5-8: BMP assays (a) without, and (b) with enzyme augmentation. Error bars represent ranges of duplicates.

5.4. Discussion

Fig. 5-9: Predicted ratios of S_p to X_v at various k_h . Scattered points are experimentally determined values, expressed as mean $\pm$ standard deviation for 3-6 points.

Although it seems desirable to treat raw sewage containing organic particulates directly using AnMBRs, solids accumulation is expected due to complete solids retention, slow hydrolysis kinetics, and high operating SRTs. Using acridine orange cell staining technique, Munch and Pollard (1997) found that particulate COD constituted a significant proportion of insoluble COD when dealing with particulate containing feed. This occurrence can be illustrated by performing steady-state mass balances on organic particulates (S_p) and volatile suspended solids (X_v) (Wong, 2012):

$$S_p = \frac{\theta_x}{\theta} \left(\frac{S_p^o}{1 + k_h \theta_x} \right) \quad (5-1)$$

$$X_v = \frac{\theta_x}{\theta} \left\{ \frac{(S_p^o/c_p)}{1+k_h\theta_x} + \frac{Y[1+(1-f_d)b\theta_x]}{1+b\theta_x} \left[S^o + \frac{S_p^o k_h \theta_x}{1+k_h\theta_x} - S \right] \right\} \quad (5-2)$$

where S_p^o , X_i^o and S^o represent the concentrations of influent organic particulates, inert organics and soluble organics respectively. The COD to mass ratio of organic particulates is denoted by c_p , Q and Q_w denote the influent and waste flow rates respectively, V stands for the reactor volume, k_h symbolizes the first order hydrolysis rate constant, θ is the HRT ($=V/Q$), and θ_x is the SRT ($=V/Q_w$). Other microbial kinetic constants include Y , the true yield, b , the endogenous decay coefficient, and f_d , the fraction of active biomass that is biodegradable. Therefore, the ratio of S_p to X_v can be determined:

$$\frac{S_p/c_p}{X_v} = \frac{S_p^o/c_p}{\left(S_p^o/c_p \right) + \frac{Y[1+(1-f_d)b\theta_x](1+k_h\theta_x)}{1+b\theta_x} \left[S^o + \frac{S_p^o k_h \theta_x}{1+k_h\theta_x} - S \right]} \quad (5-3)$$

Using the parameter values employed in this study ($S_p^o/c_p = 228$ mg/L, $Q = 10.2$ L/d, $Q_w = 0.1$ L/d, $V = 5$ L) and a typical k_h of 0.5 d⁻¹, S_p was estimated to be 893 mg/L, resulting in an accumulation factor (S_p/S_p^o) of 3.9. Assuming that the VSS reductions (Fig. 5-2) were totally contributed by the hydrolysis of organic particulates, k_h was back-calculated from the reduced S_p values using Equation (5-1), which increased to 0.63 and 0.92 d⁻¹ when 0.2 and 2 mL/L of enzyme doses were applied respectively. Complete hydrolysis of organic particulates occurred when 10 mL/L of enzyme was supplemented. Fig. 5-9 illustrates the unitless ratios of S_p to X_v at various k_h , as predicted using Equation (5-3) by adopting typical Y , f_d and b of 0.08, 0.85 and 0.03 d⁻¹ respectively (Rittmann and McCarty, 2001). Particulate accumulation ranged from 0.28 to 0.46 for k_h of 0.3-0.7 d⁻¹ associated with anaerobic systems, which may justify the proposed augmentation strategy. Experimentally determined VSS values (Fig. 5-2) corresponding to the respective k_h of 0.5, 0.63 and 0.92 d⁻¹ were slightly lower than that predicted using Equation (5-2), probably due to actual kinetic constants deviating slightly from the adopted values. Furthermore, it should be noted that these time-

independent equations do not take into account the steady-state perturbations induced by the non-continuous and stepwise dosing regimen. As such, the estimated hydrolysis rate constants should be construed as average values in a dynamic steady-state environment.

The results from this study suggest that the supplementation of hydrolytic enzymes can improve the process performance of AnMBR treating raw sewage by reducing solids accumulation (Fig. 5-2) and increasing biogas production (Fig. 5-4). Enzyme-enhanced solubilization of organic particulates facilitates their subsequent biodegradation, as dissolved macromolecules are approximately 6-28 times easier to hydrolyze anaerobically compared to suspended solids by inferring from their respective hydrolysis rate constants (Henze et al., 1997).

The decline in solids concentration (Fig. 5-2) could be ascribed entirely to the enhanced hydrolysis of organic particulates and bound EPS by the exogenous hydrolases, which are unlikely to act on active bacterial cells due to the complex glycopeptides structure of cell walls requiring specific bacteriolytic enzymes to break down (Stominger et al., 1967). This hypothesis was experimentally verified by Sesay et al. (2006), who demonstrated that the enzymatic hydrolysis of activated sludge using protease and amylase did not induce cell lysis. Enhanced enzymatic hydrolysis of bound EPS and organic particulates were demonstrated in Sections 5.3.3 and 5.3.4 respectively. The former is hydrolyzed and solubilized into BAP, which tends to be poorly biodegradable and accumulates in the supernatant (Noguera et al., 1994). Enzymatic hydrolysis of macromolecular BAP probably occurred, albeit at a lower degradation rate than bound EPS, as inferred from the lower model-fitted constants for BAP utilization relative to EPS hydrolysis under anaerobic condition (Aquino and Stuckey, 2008). Slight decline in average VSS/TSS ratio suggested the occurrence of the latter and the accumulation of inorganic materials.

COD removal efficiencies were not compromised following enzyme augmentation (Fig. 5-3), which could further substantiate the hypothesis by Stominger et al. (1967). If the active cells were enzymatically degraded, microbial activities would be adversely affected. On the contrary, minor improvements in COD removal were noticed, which may be due to the enzymatic catalysis of inert organics. However, the improvements were insignificant compared to the average increase of 14% reported by Turkdogan-Aydinol et al. (2011). Better effluent qualities can be achieved using AnMBR compared to USAB (Liao et al., 2006), as evidenced by the higher COD removal by the former. Therefore, there may be less room for improvement in this study. Additionally, the observed discrepancy may be due to the higher organic loading ($12.9 \text{ kg COD/m}^3/\text{d}$) adopted in their study. Biotransformation of intermediates is thermodynamically favored at low organic loading rates, thus reducing the effectiveness of enzyme augmentation. Nevertheless, it is noteworthy that supplementing enzymes with a measured COD of 10.9 g/L did not deteriorate the permeate quality, possibly owing to the retention of macromolecules within the AnMBR as demonstrated using SEC (Fig. 5-7). By considering the size demarcation above which refractive indices in the permeate are relatively lower, Aquino et al. (2006) estimated the nominal pore size of fouled microfiltration membranes to be 30 kDa . In this study, the cut-off was estimated to be around $3\text{-}20 \text{ kDa}$.

Although the exogenous enzymes were contained within the AnMBR, enzymatic activities generally did not register any appreciable increase (Table 5-1), possibly due to the competing effect of microbial degradation. Binner et al. (2011) observed the degradation of exogenous enzymes within an hour after supplementing to anaerobic digesters. Biodegradation is probably slower in this study as the active biomass concentrations were much lower compared to typical anaerobic digestion processes. Judging from the delayed response of biogas production in the BMP tests (Fig. 5-8), it can be inferred that the exogenous enzymes were slowly but completely biodegradable. Since the enzyme activity values represent the catalytic efficiencies of residual enzymes one day after the supply of fresh enzymes, it can be concluded that the

stability of exogenous enzymes could not be maintained for long periods, thus necessitating regular supplementation.

From an operational perspective, it is desirable to utilize the catalytic abilities of the active enzymes, while recovering bioenergy from the inactive ones. The catalytic effects of the exogenous enzymes were evident in this study (Fig. 5-2 to 5-6), suggesting that the enzymes were stable for a short duration (<1 d). Modest increases in supernatant sCOD (Fig. 5-3) and SMP protein (Fig. 5-5) concentrations following enzyme augmentation suggest low accumulation of exogenous enzymes and their bioconversion into methane. The accumulated enzymes were likely inactivated, as deduced from the enzyme activity profile (Table 5-1). Higher supernatant sCOD may also be contributed by VFA accumulation (Fig. 5-4). Enzyme accumulation in the AnMBR was consistent with the BMP results (Fig. 5-8) showing that the exogenous enzymes were slowly biodegradable. Nevertheless, the anaerobic consortia could be acclimatized to the degradation of these enzymes after a period of time, thus encouraging their methanization. Therefore, improved biogas production may be derived from the methanization of accumulated particulates and exogenous enzymes, with the latter occurring via proteolysis, autolysis and/or biological catabolism. Higher biogas yields were observed with increasing enzyme dosages, except for a marginal decline during the transition from 2 to 10 mL/L (Fig. 5-4). This may be due to slight perturbation caused by enhanced hydrolysis (Hearns, 2006), as suggested by the accumulation of VFA from Day 81 to 95 (Fig. 5-4). Since the supernatant sCOD and the SMP protein did not accumulate excessively, it may be presumed that the majority of the inactivated enzymes were biodegraded and recovered as methane.

A noteworthy observation is that the particle sizes of the sludge suspensions increased modestly with higher enzyme dosages (Fig. 5-6), even though some EPS compounds were enzymatically degraded (Fig. 5-5). This may be because of the stabilization effect of enzymes on sludge flocculation. Proteins promote flocculation by virtue of the hydrophobic interactions between amino groups (Raszka et al., 2006), and can bind

with divalent cations (Ca^{2+} and Mg^{2+}) for floc stabilization (Wuertz et al., 2003). In this study, these cations were abundantly present in the synthetic feed and tap water. Surprisingly, the reduction in bound EPS concentration (Fig. 5-5) did not lead to deflocculation, which was supported by the observation by Liao et al. (2001) that bioflocculation was governed more by the physicochemical properties than the amounts of EPS. Nevertheless, it is expected that higher enzyme dosages would lead to deflocculation as hydrolytic activity takes precedence over the floc stabilization effect. Watanabe et al. (1998) similarly observed that the addition of proteases and amylases (0.1% w/v) did not contribute to the deflocculation of a marine bacterium (*Rhodovulum* sp.). Although slightly higher enzyme dosages were applied here (0.02-0.42% w/v), it was apparently still insufficient to constitute deflocculation.

Notwithstanding the desirable effects of enzyme augmentation, its practicality hinges on the costs of crude enzymes. Approximate costs of crude protease and amylase are US\$5/kg (Zhang et al., 2011) and US\$10/kg (Zhang, 2011) respectively, which may be further moderated by adopting solid state fermentation (SSF) using reusable substrates such as agro-industrial wastes (Nigam and Pandey, 2009). For instance, SSF can reduce the production cost of cellulose, widely prepared commercially by submerged fermentation, from US\$20/kg to US\$0.20/kg. Wastewater sludge also presents an interesting alternative as a highly nutritional substrate for enzyme production (Tyagi et al., 2009). Other alternative strategies may also be explored, which include enzyme immobilization and recovery, provision of optimal conditions, and dose optimization. Indigenous hydrolases may be recovered from excess sludge by physical disruption (Jung et al., 2002) and immobilized onto suitable carriers to improve enzyme stability (Aitken, 1993). Coupled with optimization study, it is envisaged that crude hydrolases derived from reusable sources can be economically feasible for the treatment of particulate-containing wastewaters. Economic considerations aside, the wider implication of this study is that other types of enzymes may be beneficially supplemented into AnMBR and eventually recovered as bioenergy, without impairing the permeate quality. Other enzymes worth considering for wastewater applications are

peroxidase and cyanides for phenol and cyanide degradation respectively (Case et al., 1999; Basher et al., 1993), amylase for bio fouling mitigation (Yen et al., 2009), and cellulase for sugar recovery from lignocelluloses (Champagne and Li, 2009).

5.5. Conclusions

An attempt was made to streamline sewage treatment processes by treating screened raw sewage directly using AnMBR, hence obviating the need for primary sedimentation and separate sludge digestion. However, steady-state models indicated the occurrence of particulate accumulation under typical operating conditions. Therefore, crude hydrolytic enzymes were introduced to enhance the solubilization of these particulates. The results indicated that enzyme augmentation could reduce solids accumulation without compromising the effluent quality. Additionally, lower EPS and higher SMP concentrations were observed, suggesting the enzymatic hydrolysis of the former to the latter. Despite the occurrence of enzymatic degradation, the proportion of larger sized floc particles increased, implying the presence of a competing floc stabilization effect of the exogenous enzymes. Both lower EPS concentrations and larger floc sizes are likely to mitigate membrane fouling. Therefore, enzyme augmentation of AnMBRs should be further investigated in terms of filtration performance. Another inherent feature of supplementing enzymes to AnMBRs is their partial or complete retention by fouling layers, hence minimizing enzyme washout. This occurrence was demonstrated by comparing the SEC chromatograms of the supernatant and the permeate. Despite of their retention, enzymatic activities were not concomitantly elevated, suggesting that the exogenous enzymes were inactivated within one day.

5.6. References

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CHAPTER 6: EFFECTS OF SUPPLEMENTING FREE AND IMMOBILIZED HYDROLASES ON FOULING MITIGATION IN ANAEROBIC MEMBRANE BIOREACTORS FOR RAW SEWAGE TREATMENT

6.1. Introduction

The recent focus on reducing energy consumption in sewage treatment (Kim et al., 2010) has directed a trend towards anaerobic processes, including the employment of anaerobic membrane bioreactors (AnMBRs). Although AnMBRs possess inherent advantages such as the elimination of outlay associated with oxygen provision, biogas generation and low sludge production, membrane fouling control and membrane cleaning still incur sizable expenses. For instance, as much as 50% of the operating cost of MBR is associated with gas scouring (Verrecht et al., 2010). Several complementary or adjunctive strategies include supplementing with additives such as powdered and granular activated carbons (Kim et al., 2010; Hu and Stuckey, 2007), coagulants (Mishima and Nakajima, 2009), and quorum quenchers (Yeon et al., 2008). The main mechanistic functionalities of these additives are different. For example, quorum quenchers target autoinducers involved in intercellular communication, thereby mitigating biofouling. On the other hand, activated carbons scour membrane surfaces and coagulants aggregate foulants into floc particles, both belonging to physicochemical removal mechanisms. However, as microbial macromolecules are significant contributors to membrane fouling in MBRs (Le-Clech et al., 2006; Meng et al., 2009), a more selective control strategy may be warranted.

A different approach involves utilizing enzymes that have an affinity towards microbial macromolecules, thereby promoting their catabolism. This is the basis behind enzyme cleaning agents such as protease, amylase, cellulase and peroxidase (Puspitasari et al., 2010; Maartens et al., 2002), and underpins the supplementation of crude hydrolases into bioreactors to enhance solids hydrolysis (Turkdogan-Aydinol et al., 2011). The latter may be imperative for AnMBRs treating particulate containing wastewaters,

owing to solids accumulation at high sludge retention times (Münch and Pollard, 1997). Therefore, the dual benefits of mitigating membrane fouling and alleviating solids accumulation should render the proposed strategy attractive for AnMBR application.

Microbial macromolecules include both extracellular polymeric substances (EPS) and soluble microbial products (SMP). EPS are heterogeneous polymeric matrices in which microbial cells are embedded, whereas SMP are by-products excreted during cell growth and EPS hydrolysis (Laspidou and Rittmann, 2002). Both EPS and SMP are composed largely of proteins, carbohydrates, glycoproteins, and small quantities of lipids, humic substances, nucleic acids and urionic acids (Wingender et al., 1999). As such, supplementation of hydrolases could mitigate fouling by reducing the amounts, or through structural simplifications of EPS and SMP. The premise was partially supported by demonstrations of the reactivity of these enzymes towards macromolecular EPS in sludge digestion (Ayol et al., 2007; Pei et al., 2010) and biofilm degradation (Molobela et al., 2010).

The benefits of supplementing free or immobilized hydrolases on membrane filtration have been demonstrated previously. Hodgson et al. (1993) observed that the addition of 100 mg/mL proteases to a carbon-limited bacterial suspension containing 0.1% bovine serum albumin resulted in 2-3 times reduction in filtration resistance, which was ascribed to the modification of EPS matrix. Howell and Velicangil (1982) filtered 0.5% albumin and hemoglobin separately using ultrafiltration membranes immobilized with 0.2-0.5% proteases and noted 25-78% higher fluxes over 22 h periods, compared to the control without enzymes. Therefore, hydrolytic enzymes can conceivably alleviate MBR fouling unless their biotic interactions with bioreactor contents lead to inactivation.

Presently, the costs of crude protease and amylase produced via submerged fermentation are approximately US\$5/kg (Zhang et al., 2011) and US\$10/kg (Zhang, 2011) respectively, which do not make the process overtly favorable. However, such

costs are likely to be substantially reduced when solid state fermentation of reusable substrates such as agro-industrial wastes (Satyanarayana et al., 2012; Nigam and Pandey, 2009) and wastewater sludge (Tyagi et al., 2009) becomes more widespread. For example, the manufacturing cost of cellulase can be reduced from US\$20/kg to US\$0.20/kg by switching from submerged to solid state fermentation (Nigam and Pandey, 2009). Assuming a similar extent of reduction, the projected costs of protease and amylase would be US\$0.05/kg and US\$0.10/kg, respectively, which could render the process feasible. Advancements in protein engineering and molecular farming techniques will further enhance the applicability of enzymes (Tarr, 2003).

The objective of this paper is to investigate the effects of enzyme augmentation on the filtration performances of two laboratory scale AnMBRs treating synthetic sewage. It is hypothesized that a mixture of hydrolases can mitigate fouling by enhancing the hydrolysis of EPS within the cake layers, therefore reducing their cohesiveness and promoting their back transport with the aid of shear induced by gas sparging. Other than assessing filterability using transmembrane pressure (TMP), the physicochemical characteristics and compositions of membrane foulants were analyzed to elucidate the fouling mitigation mechanisms. The undesirable effect of excessive enzyme dosages on the flocculability of bulk sludge, if any, was also investigated.

6.2. Materials and Methods

6.2.1. AnMBR setup and operation

Batch and continuous filtration experiments were performed in two similar submerged AnMBRs with working volumes of 0.7 L and 5 L respectively, except for the absence of computer control in the former. The latter underwent re-acclimatization to a synthetic sewage for approximately 3 months prior to experimentation, as it had been used previously for an enzyme augmentation study (Chapter 5). After the acclimatization phase, total suspended solids concentration was constant at ~2.3 g/L,

suggesting the attainment of steady state. The same AnMBR setup and synthetic feed illustrated in Chapter 5 were adopted here.

Each batch filtration experiment was carried out using seed sludge that underwent prior batch cultivation with the synthetic feed at 1.12 kg COD/m³/d for at least 2 weeks, in order to degrade any residual organic particulates. The concentrated sludge was diluted to 2.5 g TSS/L, and the pH adjusted to 7.0±0.2 prior to experimentation. Each constant flux filtration run lasted 1-2 h, which was terminated when TMP exceeded 0.5 bar. Flowrate and TMP were measured by a variable area rotameter (Key Instruments, USA) and a digital pressure gauge (Cecomp Electronics, USA) respectively. Tap water was added manually via an influent port to maintain a constant liquid level. Each experimental set comprising of a test run and a control run was performed on the same day. 20X and 50X diluted enzyme blends were applied as free hydrolases for the first 2 filtration sets, corresponding to 45 and 18 mL/g influent COD respectively. Operational fluxes were 40 LMH for the former, and 20 LMH for the latter. The third set using membranes with immobilized hydrolases was performed at 40 LMH. These values were selected above critical fluxes to observe accelerated fouling behaviors under short filtration times. Headspace biogas was recirculated at a rate of approximately 1.5 L/min to provide adequate mixing and to reduce solids deposition. Variable speed peristaltic pumps (Masterflex console drive, Cole Parmer, USA) were used for liquid pumping and gas recirculation. Flat sheet polyvinylidene fluoride membranes (HVLP, Millipore, USA), with a nominal pore size of 0.45 µm, were installed in a customized membrane module (Ying Kwang, Singapore). Details of the operating conditions of the long-term AnMBR are described in Chapter 5. All results were expressed as mean ± standard deviation, unless otherwise stated. Data smoothing was performed using a curve fitting software (TableCurve 2D, SPSS, USA).

Methodology for immobilizing enzymes onto membranes was adapted from Lozano et al. (2002). The virgin membrane was coated with an ultra-thin gel layer obtained by filtering gelatin (1% w/v) for 1 h at 25 °C. Excess gelatin was gently washed off the

membrane using deionized water, followed by cross-linkage with glutaraldehyde (2% v/v) for 6 h at 30 °C under 150 rpm of shaking. After washing off excess glutaraldehyde, the treated membrane was soaked in the enzyme blend for 20 h at 6 °C. 100 mg/L of acylases was also added to minimize the biodegradation of immobilized enzymes during the continuous filtration experiment (Yeon et al., 2008). Excess enzymes were washed off using PBS.

6.2.2. Analytical methods

Molecular size distribution (MSD) of the supernatant was determined with Zetasizer (Nano-ZS, Malvern, England), which has a detection range of 0.3 nm to 10 µm. Measurements were repeated 15 times, and the averaged scattered light signal was converted into a volume-based distribution.

Micrographs of fouled membranes were captured using a scanning electron microscope (SEM) (EVO 50, Carl Zeiss International, Germany). Membrane samples were fixated using glutaraldehyde (2% v/v) for 1.5 h, followed by washing thrice with cacodylate buffer (0.1 M). After fixation, the samples were dehydrated in succession using 50%, 70%, 85% and 95% ethanol for 10 mins each. Before analysis, the membranes were mounted onto carbon tapes and sputter-coated with platinum for 20-30 s at 20 mV.

The major functional groups of the membrane foulants were characterized by Fourier transform infrared spectroscopy (FTIR) (IR Prestige 21, Shimadzu, Japan). Samples were dried and preserved in a freeze dryer (ALPHA 1-4 LD, Martin Christ, Germany) prior to analysis. . A horizontal attenuated total reflectance accessory (ATR-8200HA, Shimadzu, Japan) was employed, in which the membrane was fixed onto a ZnSe prism. The interferometer was desiccated using silica gel. 45 scans were performed in the range of 650-4000 cm⁻¹ at a resolution of 4 cm⁻¹.

Spatial imaging of EPS on fouled membranes was performed using a confocal laser scanning microscope (CLSM) (A1R, Nikon Instruments, USA), after staining with Concanavalin A and SYPRO orange (Invitrogen, USA) to target carbohydrates and proteins respectively, according to the methodology described by Lin et al. (2009). Green and red channels signify the presence of proteins and carbohydrates respectively. Detector gains and magnifications were adjusted to the same level for all the images to ensure comparability. A z-projection of the cake surface was represented by a 2D image, whereas all the stained regions with a 0.9 µm step progression along the z-axis were reconstructed into a 3D image. The former was quantitatively analyzed using Image J software (NIH, USA).

Soluble samples were obtained by centrifuging the sludge suspensions at 3,350 g for 20 mins at 4 °C, followed by filtering the supernatants through 0.45 µm syringe filters (Supor membrane, Pall, USA). Other analytical procedures and methods are elaborated in Chapters 3, 4 and 5.

6.3. Results and Discussion

6.3.1. Laboratory scale AnMBRs

Scaled TMP profiles for the batch filtration experiments are shown in Fig. 6-1. Scaling was done by subtracting TMPs from the initial TMP to reconcile the slight difference of the latter between the control and test runs. From the juxtaposed graphs, it was evident that both free and immobilized enzymes were effective in alleviating fouling by retarding TMP developments. To quantitatively assess the extent of fouling mitigation, a new terminology known as the relative percentage of TMP reduction (RPTR) was introduced and calculated according to the following equation:

$$RPTR = \left(\frac{TMP_{control} - TMP_{test}}{TMP_{control}} \right) \times 100\% \quad (6-1)$$

where $TMP_{control}$ and TMP_{test} represent the TMP values obtained from the control and test bioreactors at a specific time, respectively. After approximately 25 mins of filtration, RPTR values for the experiments utilizing 20X and 50X diluted enzymes plateaued at $54\pm4\%$ and $45\pm2\%$ respectively (Figs. 6-1(a) and (b)). Therefore, it seems that increasing enzyme concentrations could improve the filterability of the sludge suspensions, probably due to faster catalysis of macromolecular foulants (Xiong and Liu, 2010). On the other hand, RPTR values for the study involving immobilized enzymes (Fig. 6-1(c)) increased to 55% after 12 mins, followed by a gradual decline to 26% at the end of the filtration run. The observed trend could be explained by the targeted actions of immobilized enzymes on the initially attached foulants in close proximity (Howell and Velicangil, 1982), which were less effective as the growth of fouling layers progressed. This limitation may be circumvented by the use of free enzymes, which possess considerable flexibility to target more foulants via advective drag or diffusion through preferential transport channels inside the fouling layers (Nagata et al., 1989; Drioli and Giorno, 2009).

Fig. 6-1: TMP profiles of (a) 20X and (b) 50X diluted free enzymes, and (c) immobilized enzymes during the batch filtration experiments, with their respective controls.

Under continuous filtration, the scaled TMP profiles (Fig. 6-2) were characterized by rapid increases in TMP within the first 5 days, followed by slightly fluctuating plateaus that suggested pseudo steady state between advective drag and back transport (Le-Clech et al., 2006). A biogas recirculation rate of 1.5 L/min, equivalent to a superficial velocity of 4.6 m/h, was probably insufficient to control fouling. Similar TMP profiles were observed in other AnMBR studies that applied low recirculation rates of 0.25-1.5 L/min (Lin et al., 2009; Gao et al., 2011). From Day 0 to 2, TMPs increased rapidly to 19 kPa and 31 kPa for the control and the experiment involving immobilized enzymes respectively, indicating lower membrane permeability for the latter, which could be due to the compaction of the immobilized protein layers (Scott, 1995). Signs of fouling mitigation appeared on Day 3-20, during which the TMPs recorded for the study utilizing immobilized enzymes were relatively lower than that of the control, possibly owing to the catalytic effects of enzymes and the hydrophobic repulsion between the immobilized layers and foulants (Chen et al., 1992). After Day 20, the TMP profiles for both studies were similar, in which a plateau of ~40 kPa was reached. This suggests that the overall filterability was predominantly influenced by the formation of consolidated cake layers, as deduced from the high TMP. On the other hand, with the supplementation of free enzymes, the effect of fouling alleviation was evident throughout the 28 days, as deduced from lower TMPs relative to the control. This phenomenon can be attributed to the enhanced enzymatic hydrolysis of macromolecular EPS (see Section 6.3.2).

Fig. 6-2: TMP profiles of control, enzyme immobilized membranes, and free enzymes during the continuous experiment. Loess-smoothed lines were included to illustrate underlying trends. Smoothing was performed by least-squares fitting to the data points using a moving window of 6 days.

6.3.2. EPS and SMP concentrations

Comparisons of the concentrations of EPS and SMP obtained during the continuous filtration experiment are illustrated in Fig. 6-3. Following the use of immobilized enzymes, EPS protein reduced slightly from 18 ± 2 to 17 ± 1 mg/g TSS whereas SMP protein increased from 15 ± 3 to 18 ± 2 mg/L, possibly due to shear stress induced by gas bubbling, which partially erodes clusters of immobilized hydrolases into the supernatant and indirectly causes EPS hydrolysis. On the other hand, after supplementing with free enzymes, EPS protein decreased to 12 ± 3 mg/g TSS and EPS carbohydrate seemingly fell to 3.2 ± 1.0 mg/g TSS, which could be ascribed to the enzymatic hydrolysis of EPS into SMP, specifically biomass associated products (BAP) according to the unified theory for EPS and SMP (Laspidou and Rittmann, 2002). BAP tend to accumulate in the supernatant due to their poor biodegradability (Noguera et al., 1994). This assertion was corroborated by higher SMP concentrations, whereby SMP protein increased to 19 ± 2 mg/L and SMP carbohydrate rose to 5.2 ± 2.4 mg/L. Hence, the fouling mitigation effect (Fig. 6-2) could be attributed to the reductions in bound

EPS concentrations, as suggested by others (Gao et al., 2010; Wang et al., 2009). Bound EPS can induce membrane fouling through two transport mechanisms, which involve their release into the supernatant via shear-induced erosion or microbial hydrolysis (Wang et al., 2009; Meng et al., 2010), or their direct contact with the membranes via advective drag on the bulk sludge. Nevertheless, it is noteworthy that the slight increase in SMP did not exacerbate fouling in both instances, as evidenced in Fig. 6-2.

Fig. 6-3: Comparisons of EPS and SMP concentrations in the continuous filtration experiment. Error bars represent standard deviations from 4 samples measured weekly.

Comparisons of EPS and SMP concentrations within the fouling layers, normalized by the membrane areas, are shown in Fig. 6-4. In terms of COD equivalents, EPS concentrations in the cake layers were significantly lower after applying immobilized enzymes (1.0 ± 0.1 mg COD/g TSS/cm²) and free enzymes (0.8 ± 0.3 mg COD/g TSS/cm²), compared to the control (1.5 ± 0.2 mg COD/g TSS/cm²). Similarly, SMP concentrations in the cake layers were relatively lower, whereby the normalized values are 6.3 ± 0.9 , 4.2 ± 0.7 and 3.6 ± 0.7 mg COD/L/cm² for the cases of the control,

immobilized and free enzymes respectively. The results corresponded to average reductions of 30.6% and 46.0% in EPS, and 34.2% and 42.9% in SMP, following the supplementation of immobilized and free enzymes respectively.

Fig. 6-4: Comparisons of EPS and SMP concentrations within the fouling layers. Error bars represent standard deviations from three different sections on fouled membranes.

Effective fouling mitigation by free hydrolases (Fig. 6-2) could be a combined outcome of the reductions in bound EPS concentrations (Fig. 6-3), EPS and SMP concentrations within the fouling layers (Fig. 6-4), and sludge cake concentrations. Deposited sludge cakes on three membrane samples were quantified to be 86 ± 18 mg TSS/L/cm², much lower than 155 ± 24 mg TSS/L/cm² for the control. Based on Carmen-Kozeny equation, higher porosity due to EPS reduction (Lee et al., 2008) and lower solids concentration confer lower cake resistance. On the other hand, low apparent effectiveness of immobilized enzymes (Fig. 6-2) could be due to the significant presence of proteins in the gel layers (0.92 ± 0.11 mg COD/L/cm²), giving rise to a higher gel resistance that possibly counteracted and neutralized the reduction in cake layer resistance.

6.3.3. Particle and molecular size distributions

Fig. 6-5: PSDs of sludge suspensions sampled on (a) Day 12 and (b) Day 26 of each phase during the continuous filtration experiment.

By assessing the PSD profiles of the sludge suspensions (Fig. 6-5), similar particle sizes were noticed on the 12th day of each phase, whereas more distinct differences were discerned on the 26th day. Volume-weighted means on Day 26 of Phases I to III were 113, 104 and 91 μm respectively, indicating declines in particle sizes possibly

owing to the enzymatic solubilization of organic particulates and EPS (Ayol et al., 2007). The latter, as evidenced by the reduction in bound EPS concentrations (Fig. 6-3), could lead to deflocculation and exacerbate fouling (Sheng et al., 2010). Fortunately, the PSD curves only shifted slightly with no colloidal particles below 0.45 μm detected (Fig. 6-5(b)), suggesting that deflocculation was not aggravated at the current dose (0.08% w/v). Nevertheless, Watson et al. (Watson et al., 2004) observed more severe deflocculation after supplementing proteases, cellulases and β -glucosidases (10% w/v each) to a methanogenic bioreactor, alluding to the need for dose optimization.

Figs. 6-6(a) and (b) illustrate the PSDs of suspended cake layers and the MSDs of solubilized gel layers obtained from the continuous filtration experiment, respectively. Analyses of the cake and gel layers revealed larger particle and molecular sizes following enzyme augmentation. Volume-weighted means of the cake layers were 164, 224 and 172 μm , and intensity-weighted means of the gel layers were 281, 355 and 393 nm, as calculated from the size profiles of the control, immobilized enzymes and free enzymes respectively. The results were contrary to initial expectation, whereby enhanced enzymatic hydrolysis should reduce the foulant sizes, suggesting the presence of a strong competing effect. Agglomeration of foulants is conceivably the result of prolonged consolidation of fouling layers, reinforced by hydrophobic and electrostatic interactions with the exogenous enzymes (Massé et al., 2006). The implication of larger particle sizes is lower cake resistance, according to the Carmen Kozeny equation. As cake formation predominantly governed the filterability in AnMBRs (Jeison et al., 2008; Liu et al., 2012), decline in cake resistance may partially explain the fouling reduction observed (Fig. 6-2).

Fig. 6-6: (a) PSDs of suspended cake layers and (b) MSDs of solubilized gel layers extracted from fouled membranes during the continuous filtration experiment.

Fig. 6-7: SEM images of (a,b) virgin membranes and fouled membranes from the batch filtration experiments involving: (c) 20X diluted enzymes and (d) its control, (e) 50X diluted enzymes and (f) its control, and (g) immobilized enzymes and (h) its control.

The SEM images (Fig. 6-7) illustrate the surface morphologies of fouling layers formed during the batch experiments. SEM images from the continuous experiment were not

shown, as it was difficult to ensure and maintain the structural integrity of the thick cake layers during sample preparation. It should be highlighted that cross comparisons between different experimental sets are inappropriate due to possible temporal variations in sludge characteristics. For example, fouling layers in Figs. 6-7(c) and (d) consist of fissures and elongated gaps, whereas others compose predominantly of circular voids and pores. The virgin membranes (Figs. 6-7(a) and (b)) consist of an intricate network of valleys and ridges, which could potentially entrap foulants. Compared with its control (Fig. 6-7(d)), fouled membrane from the experiment utilizing 20X diluted enzymes (Fig. 6-7(c)) showed wider and longer fissures. Similarly, fouled membranes from the studies applying 50X diluted enzymes (Fig. 6-7(e)) and immobilized enzymes (Fig. 6-7(g)) displayed more porous and less consolidated fouling layers compared to their respective controls (Figs. 6-7(f) and (h)). All the images of fouled membranes generally indicated larger hydraulic channels for advective flow following enzyme supplementation.

To characterize the major foulants, FTIR spectra of fouled membranes (Fig. 6-8) obtained from the continuous filtration experiment were examined. Peaks near 1660 cm^{-1} (N-H bending), 1540 cm^{-1} (N-H in plane) and 1235 cm^{-1} (C-N stretching) are indicative of proteins, belonging to the amines I, II and III groups respectively (Meng et al., 2010; Lin et al., 2011; Kim and Jang, 2006). The broad peak near 1100 cm^{-1} is attributed to the symmetric and asymmetric C=O stretching, suggestive of carbohydrates (Kimura et al., 2005). Peak in the vicinity of 1400 cm^{-1} is due to the symmetrical stretching of -COO^- , which is typically associated with amino acids (Meng et al., 2010). Bands in the “fingerprint” region ($<1000\text{ cm}^{-1}$) are representative of the sulfate and phosphate functional groups (Lin et al., 2011). All FTIR spectra revealed the presence of proteins (1529 and 1631 cm^{-1}) and carbohydrates (1026 cm^{-1}) as foulants, possibly the macromolecular constituents of EPS and SMP. Additionally, amino acids (1382 cm^{-1}) and inorganic materials ($<1000\text{ cm}^{-1}$) were also present. Peak intensities of amines I and II, relative to C=O peak, seem to increase following the application of free enzymes, suggesting the enzymatic hydrolysis of carbohydrates or

the presence of residual exogenous enzymes. The former reduces the intensity of C=O peak, whereas the latter increases the intensities of amines I and II peaks.

Fig. 6-8: FTIR spectra of cake layers formed during the continuous filtration experiment.

Spatial distributions of proteins and carbohydrates along the depth of cake layers obtained from the continuous filtration experiment were visualized using CLSM, as shown in Fig. 6-9. It should be noted that the EPS matrices underneath the top cake layers may not be adequately represented in the 3D images, due to mass transfer limitation of fluorescent dyes (Meng et al., 2010). Hence, discretion must be exercised when analyzing deeper cake layers, due to the rapid decline in fluorescence intensity with increasing depth. In this study, the fluorescent signals were lost after scanning the top 40-60 μm of the stained bio-cakes.

Fig. 6-9: CLSM images of cake layers obtained from the continuous filtration experiment involving (a) no enzymes (control), (b) immobilized enzymes, and (c) free enzymes. 2D images of cake layer surfaces and 3D reconstructed images are illustrated on the left and right panels respectively. Proteins and carbohydrates were stained green and red respectively.

The CLSM images (Fig. 6-9) indicate the predominance of proteins (green signal) relative to carbohydrates (red signal) within the cake layers, which is agreeable with the

EPS and SMP results (Fig. 6-4). Following enzyme supplementation, less proteins and carbohydrates were discernible in the cake layers (Figs. 6-9(b) and (c)) relative to the control (Fig. 6-9(a)), as reflected by their fluorescence intensities, which also coincides well with the EPS and SMP trends (Fig. 6-4). In terms of areal coverage, EPS encompassed 40.5% and 30.9% of the cake layer surfaces in the studies applying immobilized and free enzymes respectively, which were lower than the 46.3% coverage in the control. Therefore, lower coverages (Fig. 6-9) and contents (Fig. 6-4) of EPS in the bio-cakes, probably the result of enzymatic catalysis, could offer a plausible explanation for the reductions in filtration resistance observed (Fig. 6-2).

6.4. Conclusions

This study examined the effects of exogenous enzymes, supplemented in the form of free or immobilized hydrolases, on membrane fouling in AnMBRs. Enhancement of filterability by the former was evident throughout the entire study, judging from the reductions in operational TMP. On the other hand, the latter lost their effectiveness when utilized for an extended period of 20 days, whereby TMP reached a similar plateau value of ~40 kPa relative to the control. During the continuous filtration experiment, chemical analyses of the cake layers indicated EPS reductions of 30.6% and 46.0%, and SMP reductions of 34.2% and 42.9%, following the supplementation of immobilized and free enzymes respectively, implying the enzymatic catalysis of macromolecular foulants formed on the membranes. The observed reductions in EPS and SMP were further corroborated by CLSM imaging of bio-cakes stained for proteins and carbohydrates, which illustrated lower fluorescence intensities and surface coverages compared to the control. Since FTIR analysis revealed the presence of protein and carbohydrate as the main foulants, the proposed strategy of introducing hydrolases to specifically target these compounds appeared effective and feasible. Furthermore, solids accumulation in AnMBRs prevalent at high sludge retention times could be consequently minimized. Nevertheless, slight deflocculation was observed following enzyme supplementation, suggesting the need to avoid excessive dosages.

6.5. References

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CHAPTER 7: EFFECTS OF SLUDGE-DERIVED ENZYME EXTRACTS ON THE PERFORMANCE OF AN ANAEROBIC MEMBRANE BIOREACTOR TREATING RAW SEWAGE

7.1. Introduction

The two major impediments to the application of anaerobic membrane bioreactors (AnMBRs) for the treatment of particulate-containing wastewaters, such as raw sewage, are membrane fouling and slow hydrolysis kinetics. The former is associated primarily with extracellular polymeric substances (EPS) (Le-Clech et al., 2006), while the latter exacerbate solids accumulation in AnMBRs. Enzyme augmentation, in the form of hydrolases, is a potential solution to these problems involving macromolecular degradation. The hydrolysis of organic particulates and EPS can be enzymatically enhanced, thereby abating solids accumulation and membrane fouling. However, economic consideration largely hindered the application of commercial enzymes in wastewater treatment. To overcome this limitation, cheaper alternatives were sought, of which resource recovery from waste contributes directly to the notion of sustainable wastewater treatment.

Hydrolytic enzymes can be recovered from wasted sludge via physical disintegration techniques such as ultrasonication (Nabarlatz et al., 2012) and Dyno-Mill disruption (Jung et al., 2002), or via chemical means such as non-ionic detergent, cation exchange resin (CER) and ethylenediaminetetraacetic acid (Gessesse et al., 2003). Chemical methods require additional procedures for separating additives and product enzymes, and are therefore usually not preferred. Among the physical approaches, ultrasonication seems attractive due to efficient volatile solids (VS) destruction and small footprint requirement (Khanal et al., 2007). Ultrasonication generates pressure fluctuations, weakens intermolecular cohesion and results in the violent collapse of cavitation bubbles (Harisson, 1991). The cavitation forces induce sludge disintegration, therefore reducing the outlay associated with downstream sludge treatment, which accounts for

up to 50-60% of total operating costs (Banu et al., 2011). The corresponding saving is directly proportional to the amount of solids removed. Improvements in COD solubilization by 4-42% and VS reduction by 6-57% were observed in nine separate studies pretreating waste activated sludge (5-30 g TS/L) ultrasonically (Khanal et al., 2007).

Enzymatically active extracts were successfully recovered from various sludge sources via ultrasonication. Nabarlantz et al. (2012) ultrasonicated 0.5-1.2 g VSS/L of activated sludge under an intensity of 3.9 W/cm^2 for 30 mins and recovered approximately 3.5 and $21 \mu\text{mol g}^{-1} \text{ VSS min}^{-1}$ of protease and lipase respectively. Yu et al. (2009) extracted 1.1 and $15 \mu\text{mol g}^{-1} \text{ VSS min}^{-1}$ of protease and amylase respectively from activated sludge by applying a specific energy of 83 kJ/g TSS. Whiteley et al. (2002) detected 1.1 absorbance unit (at 440 nm) $\text{mg}^{-1} \text{ protein h}^{-1}$ of protease activity in the supernatant after subjecting methanogenic sludge to 2.2 kJ/g TSS of sonication (Whiteley et al., 2002).

Although these positive outcomes support the conception of supplementing AnMBRs with recovered enzymes, complication exists due to the co-existence of soluble EPS and other extra- and intra-cellular compounds in the extracts. These biopolymers, particularly those larger than 300 kDa, tend to be poorly biodegradable (Eskicioglu et al., 2006; Noguera et al., 1994). Although these recalcitrant compounds are significantly retained by fouled microfiltration (MF) membranes (Aquino et al., 2006), their excessive accumulation exacerbates membrane fouling (Le-Clech et al., 2006). To partially circumvent this problem, either biomass acclimatization to these compounds should be promoted, or ultrasound-induced cell lysis should be minimized.

These results lead to the hypothesis that the enzyme extract from ultrasonicated sludge can catalytically enhance AnMBR performance, unless its EPS content is not promptly biodegraded. In this study, we aimed to optimize the ultrasonic extraction using a full

factorial design, followed by an assessment of the impact of supplementing enzyme extracts on the performance of an AnMBR treating synthetic raw sewage.

7.2. Materials and Methods

7.2.1. Optimization of ultrasonic extraction

Enzyme recovery and sludge solubilization were optimized by varying the applied intensity (2.0-4.6 W/cm²) and duration (2-30 mins), and analyzed statistically (Design Expert 8.0, Stat-Ease, USA) using a 2² full factorial design with duplicated center points. The former corresponded to ultrasonic densities of 0.39-0.91 W/mL. The experimental bounds were selected with reference to other studies on enzyme recovery and EPS extraction from activated sludge (Comte et al., 2006; Nabarlantz et al., 2012). After identifying the main and interactional effects from the half-normal plot, their coefficients in the model equation were estimated from the gradients of the effect plots. Statistically significant effects were identified and retained using analysis of variance, and the corresponding responses were averaged to determine the constant term. A base 10 logarithm transformation was applied to the glucosidase equation, in the form of log₁₀ (specific glucosidase activity + 0.01), as suggested by the Box-Cox plot. The optimal condition was ascertained by varying the factors simultaneously to attain the highest response. All the experiments and analyses were performed on the same day to minimize the variability in sludge characteristics and to ensure comparability of results. Under each test condition, 100 mL of anaerobic sludge was obtained from the laboratory scale AnMBR (Fig. 1), immersed in an ice bath and treated using an ultrasonic processor operating at 20 kHz (VC 750, Sonics and Materials, USA). The remaining sludge suspension was centrifuged at 4,250g for 20 mins at 4 °C, and the supernatant was recovered as the enzyme extract for further analyses.

7.2.2. AnMBR setup and operation

The same AnMBR setup, synthetic feed and operating condition described in Chapter 5 were adopted in this study. The entire experimental period was divided into three phrases lasting 28 days each: control (Phase 1) and test (Phases 2 and 3) studies involving the supplementation of anaerobic and aerobic sludge extracts respectively. Prior to executing Phrase 1, the AnMBR had been operated continuously for approximately 5 months to achieve steady-state, thus forming a baseline for comparison. During Phases 2 and 3, 100 mL and 500 mL of wasted sludge were ultrasonicated daily under the optimized condition respectively, and the soluble extracts returned to the AnMBR via the influent line. These were done after sludge and permeate sampling. Returned activated sludge and anaerobic digester sludge were obtained from Ulu Pandan Water Reclamation Plant, Singapore. The former was batch cultivated in a 20 L carboy under a solids retention time (SRT) of 20 days, and fed with the OECD synthetic sewage at an organic loading rate of 1.12 kg COD/m³/d. All results were expressed as mean $\pm$ standard deviation, unless otherwise stated.

7.2.3. Analytical methods

Protease and amylase activities were quantified according to the methodologies described in Chapter 5. α -glucosidase activity was measured colorimetrically using an enzymatic assay (EC.3.2.1.20, Sigma Aldrich, USA). One glucosidase unit ($U_{\text{glucosidase}}$) refers to the liberation of 1 μmol of glucose from p-nitrophenyl α -D-glucoside per min at pH 6.8 and 37 °C.

Analysis of biogas composition was carried out using a gas chromatography fitted with two thermal conductivity detectors (GC 7890A, Agilent, USA). The front detector was employed to detect CO₂, O₂, CH₄ and N₂ using helium as the carrier gas at a flowrate of 15 mL/min, which were resolved by customized CP-Molsieve 5A (321067859-4, Agilent, USA) and Hayesep C (321067859-3, Agilent, USA) columns connected in

series. The back detector was used to detect H₂ using CP-Molsieve 13X column (321067859-5, Agilent, USA). The temperatures of the injector port and detectors were 120 °C and 150 °C respectively. Calibration was performed with a standard gas mixture comprising of 20% CO₂, 10% H₂, 10% N₂ and 60% CH₄ (National Oxygen, Singapore). Volatile fatty acids (VFA) were analyzed by another gas chromatography equipped with a flame ionization detector (GC 7890A, Agilent, USA). DB-FFAP column (123-3233, Agilent, USA) was used to resolve the VFA using helium as the carrier gas at a flowrate of 6.5 mL/min. The injector port and detector were operated at 260 °C. Calibration was performed with a standard VFA mixture (IT Technologies, Singapore) containing 10 mM each of acetic, butyric, caproic, formic, heptanoic, propionic and valeric acids. 0.9 mL of sample was acidified with 0.1 mL of formic acid (10% v/v) prior to analysis.

Excitation-emission matrix (EEM) scans were performed using a fluorescence spectrometer (LS 55, PerkinElmer, USA). EEM spectra were obtained by varying both the excitation and emission wavelengths from 230 to 550 nm at 10 nm intervals. The excitation and emission slit widths and scanning speed were 10 nm and 1000 nm/min respectively. The Raman scattering peak intensity of deionized water at excitation and emission wavelengths of 350 and 400 nm respectively was recorded prior to analysis and the differences between all values were less than 5%. Samples were also monitored for deviations from near-neutral pH to minimize the changes in fluorescence properties due to protonation or deprotonation. Dilution was performed if the maximum fluorescence intensities exceeded 1,000 arbitrary units (Chen et al., 2003).

Bioreactor sludge, permeate and sludge extract were sampled 2-5 times per week for quantitative analyses. Bioreactor supernatant was derived from the sludge suspension by centrifugation at 4,250g for 20 mins at 4 °C (Sorvall Legend Mach 1.6, Thermo Scientific, USA), followed by filtration using a 0.45 µm Nylon syringe filter (F2602-1, Scientific Procurement, Singapore). The Standard Methods (APHA, 1998) were adopted for the measurement of total suspended solids (TSS), volatile suspended solids

(VSS) and chemical oxygen demand (COD) concentrations. Details of other analytical procedures and methods are reported in the preceding chapters.

7.3. Results

7.3.1. Optimization results

Full factorial design was applied to statistically assess the interactive effects of ultrasonic intensity and duration on enzymatic activities and solubilized COD from AnMBR sludge, as reported in Table 7-1. The steady-state TSS and VSS were approximately 2,500 mg/L and 2,200 mg/L respectively, corresponding to a COD equivalent of 3,124 mg/L. Normalizing the solubilized COD (360-2,328 mg/L) by the initial insoluble COD (3,124 mg/L), significant VSS solubilization of 11-74% was observed, possibly owing to the pronounced energy inputs of 19,200-648,000 kJ/kg TSS at low solids concentration. Protease and amylase were the predominant enzymes recovered from the AnMBR sludge. The proteinaceous nature of the synthetic feed might induce significant protease expression by the hydrolytic bacteria, hence partially accounting for the highest proportion of extracted protease (69-84%) detected in all experiments.

The optimal ultrasonic condition was determined to be 3.3 W/cm² and 30 mins, which yielded the highest enzymatic activities and sCOD according to the model predictions (Table 7-2). High desirability of 0.811 was attained, which is an indication of the significance of the determined response, the highest being a value of 1. The models suggest that duration is more important than intensity for efficient enzyme extraction and COD solubilization, as similarly pointed out by Erden and Filibeli (2009), possibly owing to strong influence of the former on specific energy inputs within the confines of this study. For example, an increase in ultrasonic duration from 2 to 30 mins corresponds to a 15-fold rise in specific energy, but only a 2.25-fold increase by

adjusting ultrasonic intensity from 2 to 4.6 W/cm². Therefore, their relative predominance is significantly dependent on the selected ranges.

Table 7-1: Enzyme activities and sCOD extracted under the various ultrasonic conditions. Specific enzyme activities (expressed as $\mu\text{mol min}^{-1} \text{g}^{-1} \text{VSS}$) were indicated in parentheses.

Condition		Glucosidase activity	Amylase activity	Protease activity	sCOD
Intensity (W/cm ²)	Duration (min)	(U _{glucosidase} /mL)	(U _{amylase} /mL)	(U _{protease} /mL)	(mg/L)
2	2	0 (0)	0.225 (99)	0.538 (244)	360
2	30	0.015 (6.81)	0.252 (110)	1.238 (562)	2328
4.6	2	0 (0)	0.269 (116)	0.607 (275)	1248
4.6	30	0.006 (2.72)	0.259 (113)	1.410 (640)	2256
3.3	16	0.004 (1.81)	0.272 (119)	0.653 (296)	1488
[#] 3.3	16	0.004 (1.81)	0.212 (93)	0.642 (291)	1044

[#] Repeated center point.

Table 7-2: The estimated coefficients in the model equations correlating extracted enzyme activities or solubilized COD with ultrasonic duration and intensity.

	Ultrasonic duration (min)	Ultrasonic intensity (W/cm ²)	Constant (-)
Specific glucosidase activity ⁺ ($\mu\text{mol min}^{-1} \text{g}^{-1} \text{VSS}$)	+ 1.40	-	- 0.42
Specific amylase activity ($\mu\text{mol min}^{-1} \text{g}^{-1} \text{VSS}$)	-	-	+ 108
Specific protease activity ⁺ ($\mu\text{mol min}^{-1} \text{g}^{-1} \text{VSS}$)	+ 170	-	+ 384
sCOD ⁺ (mg/L)	+ 744	-	+ 1454

⁺Statistically significant model ($p < 0.05$).

7.3.2. Effects of enzyme extracts on AnMBR performance

Stable COD removal efficiency of $93.0 \pm 0.9\%$ during the control period suggests the attainment of steady-state. Following the recycling of sludge extract containing $2,294 \pm 122$ mg COD/L back into the AnMBR, COD removal improved slightly to $94.2 \pm 1.2\%$ (Fig. 7-1). Additionally, the permeate quality did not deteriorate and remained around 35.1 ± 6.8 mg COD/L. On the other hand, COD removal fell to $91.5 \pm 1.8\%$ when dosed with aerobic sludge extract consisting of $1,703 \pm 181$ mg COD/L, corresponding to a rise in permeate COD to 46.6 ± 9.9 mg COD/L.

In view of the inappreciable build-up of bioreactor sCOD following supplementation (Fig. 7-1), the majority of the soluble extracts were most likely biotransformed into methane. As illustrated in Fig. 7-2, biogas production increased inappreciably from 2.0 ± 0.2 to 2.1 ± 0.6 L/d after recycling the AnMBR sludge extract, but increased more substantially to 2.3 ± 0.3 L/d when dosed with aerobic sludge extract. Methane and carbon dioxide compositions were not affected by the supplementation, remaining in the ranges of $87.1 \pm 2.1\%$ and $12.9 \pm 2.1\%$ respectively for the 11 samples quantified weekly. Taking into account the additional COD contributed by the sludge extracts, the methane yields (expressed as L CH₄/g COD removed) were estimated to be 0.34 ± 0.03 L/g, 0.33 ± 0.09 L/g and 0.34 ± 0.05 L/g during Phases 1 to 3 respectively. A different trend was discerned for VFA production, which declined marginally from 8.4 ± 3.9 to 7.2 ± 3.7 mg COD/L, followed by a slight increase to 9.6 ± 6.3 mg COD/L. Acetic and propionic acids constituted the majority of the VFA throughout the experimental period.

Fig. 7-1: Temporal profiles of sCOD in bioreactor and in permeate, and COD of sludge extracts supplemented to AnMBR.

Although the biological performance of the AnMBR was largely unaffected, the TMP profile (Fig. 7-3) indicated an exacerbation of membrane fouling after supplementing with aerobic sludge extract. Temporal TMP results were scaled by subtracting off initial TMP. According to Gao et al. (2011), fouling development could be classified into three distinct stages: (1) an initial rapid TMP increase, (2) an extended period of slow TMP rise, and (3) sudden TMP jump. Similar TMP profiles were observed during Phases 1 and 2, whereby the scaled TMPs fluctuated in the range of 25.1 ± 2.5 kPa after the initial TMP spike. TMP jump was noted only from Day 76 to 83 (Phase 3), increasing at a rate of 1.0 ± 0.4 kPa/d, which reflected severe membrane fouling by aerobic sludge extract at the present dosage.

Fig. 7-2: Evolution of VFA concentration (expressed as COD equivalent) and biogas production over time.

Fig. 7-3: TMP profile of the AnMBR over time, operated without and with the supplementation of anaerobic and aerobic sludge extracts. Dotted lines represent average TMPs during stage 2 fouling, and arrow indicates the onset of TMP jump.

7.3.3. EPS and SMP concentrations

Fig. 7-4: Comparison of the concentrations of (a) bound EPS and (b) SMP extracted during Phases 1 to 3. Error bars represent standard deviations from 9 samples without, and 14 samples with the supplementation of sludge extracts. Samples were taken weekly.

Bound EPS and SMP can provide insights to the filtration behaviors observed in Fig. 7-3 due to their considerable fouling propensities (Le-Clech et al., 2006). As illustrated in Fig. 7-4, bound EPS concentrations declined slightly from 27.0 ± 3.6 mg COD/g TSS to 20.1 ± 4.9 and 25.5 ± 2.3 mg COD/g TSS following the supplementation of anaerobic and aerobic sludge extracts respectively. SMP concentrations also fell moderately from

35.7±7.8 mg COD/L to 29.1±6.8 mg COD/L after recycling the anaerobic sludge extract. Conversely, SMP concentrations increased to 42.0±9.1 mg COD/L after providing with aerobic sludge extract.

7.3.4. Size exclusion chromatography

Size exclusion chromatograms (Fig.7-5) were differentiated into three molecular fractions according to Lyko et al. (2008): (1) macromolecular fraction (>4 kDa), (2) humic fraction (1-4 kDa), and (3) low molecular fraction (<4 kDa). Low molecular compounds were absent in the anaerobic sludge extract, but predominantly present in the aerobic counterpart (Fig. 7-5(a)). This fraction was easily biodegraded as these peaks were not found in the bioreactor supernatant (Fig. 7.5(c)). Macromolecular and humic peaks centered at around 300 and 3 kDa respectively were detected instead, which also appeared in the permeate (Fig. 7-5(b)). Similarly, macromolecular compounds in the size range of 4-1000 kDa were identified in the permeate chromatograms of both the control and the anaerobic sludge extract.

Only compounds larger than 1000 kDa were retained efficiently judging from their absence in the permeate (Fig. 7-5(b)). From the chromatograms of the extracted foulants (Fig. 7-5(d), (e), (f)), the presence of macromolecular and low molecular compounds was evident, of which the latter elicited higher detector responses. The molecular size distributions of foulants within the cake and gel layers were similar, but distinctly different from that of the extracted EPS from bulk sludge.

Fig. 7-5: Size exclusion chromatograms of (a) sludge extracts, (b) permeate, (c) bioreactor supernatant, (d) bound EPS of bulk sludge, (e) bound EPS of cake layer, and (f) SMP of gel layer. Samples for the chromatographic analyses of (b), (c) and (d) were withdrawn on Day 8, 36 and 64.

7.3.5. Excitation-emission matrix

Fig. 7-6: Excitation-emission matrix spectra of (a) aerobic sludge extract, (b) bound EPS of cake layer, and (c) SMP of gel layer sampled during Phase 3. Figures (d) and (e) illustrate the controls for (b) and (c) respectively.

Since the supplementation of aerobic sludge extract deteriorated permeate quality (Fig. 7-1) and exacerbated membrane fouling (Fig. 7-3), EEM fluorescence spectroscopy

was employed to characterize its constituent compounds. Two main peaks (H and F), located at excitation/emission wavelengths of 450/520 nm and 250/465 nm respectively, could be identified from the EEM spectra of the sludge extract (Fig. 7-6(a)). According to the regional classification by Chen et al. (2003), Peaks H and F represent humic acid-like compounds and fulvic acid-like materials respectively. Less intense peaks were also observed at short excitation wavelengths (<250 nm) and short emission wavelengths (<350 nm), which were associated with simple aromatic proteins. Other than the above-mentioned peaks, Peak S appeared in the EEM spectra of bound EPS and SMP extracted from the fouling layers (Figs. 7-6(b)-(e)), which belonged to the soluble microbial byproduct-like group exhibiting intermediate excitation wavelengths (250-280 nm) and short emission wavelengths (<380 nm). The spectra of the bound EPS extracted from the bio-cake during the control and test periods were rather similar (Figs. 7-6(b) and (d)), except that Peak H in the latter red-shifted by 5-10 nm along the emission axis. A red shift is associated with an increase of carbonyl, hydroxyl, alkoxyl, amino or carboxyl functional groups in the fluorophores (Liu et al., 2011). No distinct difference was discerned between the EEM spectra shown in Fig. 7-6(c) and Fig. 7-6(e), suggesting similar gel layer compositions unaffected by the supplementation.

7.4. Discussion

Disposal of excess sludge constitutes as much as 50-60% of the total operating costs in a wastewater treatment plant (Egemen et al., 2001). Considering an associated outlay of 17-112 Euros per tonne of dried sludge (Verrecht et al., 2010), the observed VSS reductions of 11-74% by ultrasonic treatment could translate into substantial savings. By further treating the solubilized sludge anaerobically, 1,718 kWh of electricity per tonne of VSS can be generated (Fuchs et al., 2003), which is equivalent to an additional saving of 172 Euros per tonne, hence conceivably fulfilling the cost requirement of 235 Euros per tonne for ultrasonication (Dewii et al., 2006). Furthermore, crude protease and amylase with commercial values of US\$5/kg (Zhang et al., 2011) and US\$10/kg (Zhang, 2011) respectively are recoverable from the wasted sludge.

Other than the potential cost savings, recycling of anaerobic sludge extract led to a slight improvement in COD removals (Fig. 7-1), and reductions in bound EPS and SMP concentrations (Fig. 7-4), by virtue of the catalytic effects of the recovered enzymes. Enhanced enzymatic hydrolysis of organic particulates and soluble macromolecules to monomers smaller than 1 kDa facilitates faster cellular uptake of these substrates (Burgess and Pletschke, 2008). Other than the feed constituents, macromolecular EPS are also the target of enzymatic degradation (Pei et al., 2010; Molebela et al., 2010), as similarly observed in this study. Bound EPS are solubilized into biomass associated products (BAP), adding to the pool of SMP, followed by complete hydrolysis into monomers (Laspidou and Rittmann, 2002).

On the contrary, higher dose of aerobic sludge extract adversely affected treatment (Fig. 7-1) and membrane (Fig. 7-3) performances. Additional organics from the sludge extract could induce shock load, which possibly engendered the accumulations of COD (Fig. 7-1), VFA (Fig. 7-2) and SMP (Fig. 7-4) in the bioreactor. Yoon et al. (2004) investigated the recycling of ultrasonic pretreated sludge initially containing 7,500 mg TSS/L back into an 8.5 L MBR daily, equivalent to an additional sCOD of ~1 g/L. Additional sCOD values were calculated using available data by normalizing sCOD of sludge extracts with bioreactor volumes to facilitate comparison. An increase in permeate COD from 46 ± 7 to 79 ± 24 mg/L and a 10-fold longer TSS filtration time were reported, relative to the control MBR. The latter suggests higher sludge filtration resistance after ultrasonic pretreatment, as similarly noted by Gonze et al. (2003) in a dead-end configuration. On the other hand, the recycling of less thermo-chemically pretreated sludge, corresponding to an additional sCOD of 67-145 mg/L daily, did not induce any undesirable effects (Banu et al., 2011). Conversely, the daily supplementation of ozone pretreated sludge, equivalent to an additional 123 mg sCOD/L, led to a 2-fold reduction in the rate of TMP rise, which was ascribed to a more porous bio-cake (Hwang et al., 2010). Since a similar amount of additional sCOD (~170 mg/L) was applied in this study, quantity of COD could not explicate the

observed discrepancies, suggesting the influence of other factors on MBR performance after disintegrated sludge recycling. It is also worth mentioning the distinct difference of recycling extracts containing residual solids in their studies, which seemingly had little impact on MBR performances, therefore alluding to the significance of the soluble fraction.

The physico-chemical properties of the sludge extracts may be a pertinent factor influencing their biodegradability and filterability. More SEC peaks were discerned for the aerobic sludge extract relative to the anaerobic counterpart (Fig. 7-5(a)), especially after 20 mins of retention time (<35 kDa), which represented poorly membrane-retained compounds (Fig. 7-5(b)) that compromised the permeate quality (Fig. 7-1). Macromolecular compounds larger than 30 kDa could be rejected by fouled MF membranes in AnMBRs (Aquino et al., 2006). Among the intermediate and low molecular weight compounds, the humic fraction with a peak at around 2 kDa (Fig. 7-5(b)) was identified as the recalcitrant SMP that persisted in the permeate. The humic substances were probably derived from the sludge extract, which predominantly consisted of humic and fulvic acids-like substances (Fig. 7-6(a)). These compounds are biologically resistant and incapable of being retained by MBR membranes due to their small molecular sizes (Tang et al., 2010). Nevertheless, membrane rejection of 6-18% was observed by Ng et al. (2006) in four pre-anoxic MBRs installed with MF polyolefin membranes, possibly due to humic acid aggregation via hydrophobic interaction (Yuan and Zydney, 1999). In this study, humic and fulvic acids were also found in the cake and gel layers respectively (Figs. 7-6(b) and (c)), partially contributing to fouling. Nevertheless, the contribution by the humic fraction was probably less compared to that by the macromolecular SMP (Fig. 7-4(b)), which were significantly retained (Fig. 7-5(c)). These accumulated SMP can constitute pore blockage and/or form gel layer, which are difficult to remove by back transport mechanisms (Meng et al., 2009). The two fractions may also form carbohydrate-humic acid complexes via cationic bridging and synergistically aggravate fouling (Lyko et al., 2007).

Since the specific energies applied in this study exceeded the minimum threshold of 1-3 kJ/g TS required to induce cell breakage (Bougrier et al., 2005; Lehne et al., 2001), it seems highly probable that the enzyme extracts consisted of intracellular materials and cell wall constituents. The latter comprise heteropolysaccharides and D-configured amino acids, which are poorly biodegradable (Aquino et al., 2006). These compounds can be detected by the protein and carbohydrate assays, therefore possibly contributing to the pool of accumulated SMP (Fig. 5). Nonetheless, the extent of SMP accumulation could be attenuated by providing sufficiently long residence time (~11 days) and acclimatization period (>100 days) (Schiener et al., 2010; Aquino et al., 2006). The former was conferred by membrane retention (Fig. 7-5), whereas the latter requirement suggested partial microbial acclimatization in this study.

In spite of the detrimental effects of dosing aerobic sludge extract, biogas production generally registered a positive response (Fig. 7-2). The excess biogas could be derived from the biomethanization of the monomeric compounds present in the aerobic sludge extract (Fig. 7-5(a)), which were easily biodegraded (Fig. 7-5(c)). Enhanced enzymatic hydrolysis of macromolecular compounds larger than 1000 kDa, as inferred from the lower SEC peaks relative to the control (Fig. 7-5(c)), might also contribute to the biogas generation. The methane yields before and after ultrasonic treatment were around 0.34 L/g, indicating the efficient bioconversion of sludge extracts into methane. VFA accumulation up to 24.4 mg COD/L observed on Day 80 (Fig. 7-2) suggested slight process imbalance without inhibiting methanogenesis. Ahring et al. (1995) also found that individual VFA concentrations less than 50 mM (3.2 g COD/L as acetic acid) did not reduce methane yield.

In this study, ultrasonication was carried out in an ice bath to minimize thermodenaturation of enzymes at high local temperatures, as similarly adopted by Yu et al. (2008) and Nabarlatz et al. (2012). As a guideline, temperature increases by 1 °C every 38 seconds under an ultrasonic density of 0.11 W/mL without heat dissipation (Khanal et al., 2007). This phenomenon inevitably sets an upper limit to the supplied

energy, unless temperature control is implemented. Yan et al. (2010) determined the upper bound to be 30 kJ/g of dried sludge, above which enzyme activities declined. In the attempt to maximize enzyme recovery and sludge solubilization, this threshold value was exceeded, therefore necessitating the regulation of temperature. The presumed benefits of preserving the enzyme activities of anaerobic sludge extract include higher COD removals (Fig. 7-1), improved biogas productions (Fig. 7-2), and less SMP accumulation (Fig. 7-4(b)). Alternatively, the recovered enzymes can be purified for other industrial applications (Nabarlatz et al., 2012). Although enzyme recovery seems desirable, further economical evaluation and operational optimization have to be performed before upscaling to an industrial scale. If determined to be unfeasible, sludge sonication with the aim of maximizing COD solubilization may be considered instead.

Two major competing factors are likely implicated in affecting membrane filterability after the addition of sludge extracts, namely (1) concentrations and compositions of extracted SMP, and (2) enzyme activities of sludge extracts. Reducing the fouling propensity and/or improving the catalytic efficiency of the enzyme extracts can potentially mitigate fouling. The former is possible by increasing SRT moderately, reducing organic loads, avoiding stressful conditions, and adding adsorbents or coagulants (Meng et al., 2009). In this study, the supplementation of anaerobic sludge extract did not lead to any observable reduction in TMP (Fig. 7-3), which is likely due to equivalent contributions by both factors.

7.5. Conclusions

Enzymatically active extracts were successfully recovered from anaerobic and aerobic wasted sludges via ultrasonication, and the optimal ultrasonic condition was determined to be 3.3 W/cm² and 30 mins using a full factorial design. Both intracellular and extracellular enzymes were conceivably extracted considering the ultrasonic intensities applied, which could induce cell breakage. Using the optimized protocol,

indigenous enzymes were extracted from wasted sludge and supplemented daily into an AnMBR. Average biogas production increased after ultrasonic treatment. The surplus biogas was probably derived from the biomethanization of extracted monomeric compounds and/or the enzymatic hydrolysis of macromolecular substrates larger than 1000 kDa. COD removal efficiencies improved moderately following the recycling of anaerobic sludge extract, but declined after dosing with its aerobic counterpart. The former was conceivably the catalytic effect of the recovered enzymes, whereas the latter could be ascribed to shock loading or presence of recalcitrant compounds in the sludge extract. Physico-chemical characterization of the aerobic sludge extract allowed the identification of humic and fulvic acids-like substances as possible culprits due to their poor biodegradability. However, the presence of these small-sized molecules might not explicate the membrane fouling aggravation observed. The accumulation of macromolecular SMP could likely exacerbate fouling, leading to a rapid increase in TMP. Contrarily, membrane permeability was not deteriorated by the recycling of anaerobic sludge extract.

7.6. References

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CHAPTER 8: CONCLUSIONS AND RECOMMENDATIONS

8.1. Conclusions

The effects of enzyme augmentation, in the form of crude hydrolases, on the performance of an AnMBR treating raw sewage were investigated. Direct treatment of screened raw sewage containing organic particulates using AnMBRs obviates the need for primary sedimentation and separate sludge digestion, therefore reducing the footprint requirement of sewage treatment. However, solids accumulation is expected under typical operating conditions due to complete solid retention and slow hydrolysis kinetics. Another prevalent problem is membrane fouling, which necessitates higher operating pressure to maintain a desired flux. By enhancing the hydrolysis of organic particulates and macromolecular foulants, solids accumulation and membrane fouling can be potentially alleviated respectively.

Within the confines of this study, anaerobic hydrolysis of organic particulates was optimal at pH 7.0, 35 °C and 0.7% (w/v) of enzyme dose. Operating at near neutral pH is advantageous as hydrolysis and methanogenesis can occur simultaneously in a single-stage bioreactor without compromising either process considerably. Higher temperature and enzyme dosages led to faster rates of particulate hydrolysis. There is a practical upper bound to the former, typically within the mesophilic range, as it is economically unfeasible to heat large volumes of sewage. On the other hand, enzymes may be catalytically reused, therefore representing a potential approach to enhancing hydrolysis. Furthermore, low-cost crude enzymes can be utilized due to the contaminated nature of wastewaters.

However, excessive dosages of enzymes could detrimentally affect the physicochemical characteristics of anaerobic sludge. Reductions in sludge settleability occurred at enzyme dosages higher than 0.34 g/g TSS, as evidenced by sludge stratification during the SVI analysis. This is likely due to the enzymatic hydrolysis of bound EPS, which induced sludge deflocculation. Nevertheless, it is noteworthy that

deflocculation was not exacerbated in this study, as D_f and PSD of sludge suspensions revealed insignificant changes in the floc structures and sizes respectively. Although sludge settleability is not a major concern in AnMBRs, deflocculation can aggravate membrane fouling as a result of the release of colloidal substances into the supernatant. Therefore, this study suggests that enzymes should be supplemented moderately to avoid excessive deflocculation.

Small doses of crude enzymes (0.9-18 mL/g influent COD) were introduced daily into an AnMBR, resulting in reduced solids accumulation and enhanced biogas production. Declines in average TSS and VSS by 19% and 22% were observed following enzyme supplementation, owing to the enzymatic hydrolysis of organic particulates. These hydrolytic enzymes are unlikely to act on active cells due to the complexity of cell wall structures. Uncompromised microbial activities were corroborated by COD removal efficiencies, which increased slightly from $94.5 \pm 0.5\%$ to $95.3 \pm 0.6\%$ after enzyme augmentation. Enhanced solids hydrolysis coupled with excellent COD removals resulted in a significant increase in biogas production from 0.27 to 0.34 L/g of influent COD. Additionally, membrane retention of exogenous enzymes was noticed using size exclusion chromatography, possibly owing to reductions in nominal pore sizes after fouling.

Hydrolytic enzymes were covalently immobilized onto virgin membranes, in an attempt to mitigate membrane fouling and prolong enzyme life-span. Multi-point attachment of enzymes onto a support can improve their conformational stability. However, immobilized enzymes lost their effectiveness when utilized for an extended period of 20 days, which could be ascribed to loss of catalytic abilities and/or formation of consolidated bio-cake. On the other hand, fouling mitigation by free enzymes was consistently observed throughout the entire study. Enzymatic degradation of macromolecular foulants and particle agglomeration in the bio-cake were determined to be the mechanisms of fouling alleviation.

An alternative to commercial crude enzymes is the recovery of indigenous enzymes from wasted sludge. In this study, hydrolases were ultrasonically extracted from various wasted sludge using the optimized condition of 3.3 W/cm² and 30 mins, and supplemented daily into an AnMBR. Marginal improvements in COD removal and biogas production were observed following the recycling of AnMBR sludge extract. Subsequently, 5-fold higher dose of enzyme extract from activated sludge was introduced to further augment the hydrolytic activities in the AnMBR. In a sewage treatment facility, wasted activated sludge can be obtained from an aerobic basin. However, although biogas production increased concomitantly, COD removal efficiency declined and severe fouling occurred. EEM and SEC analyses allowed the identification of the humic substances and macromolecular SMP in the sludge extract as possible culprits. Therefore, lower dose of AnMBR sludge extract is preferable, unless the extraction of undesirable SMPs from its aerobic counterpart could be minimized.

Nonetheless, cost remained a pertinent issue governing the applicability of enzymes for wastewater treatment. Enzyme inactivation in the bioreactor environment necessitates frequent replenishment of enzymes, which compels a reassessment of the dosing regimen. Complemented by online monitoring and automated control, periodic dosing can be applied to alleviate solids accumulation and membrane fouling when required. Enzyme cost is likely to decline over time due to advancements in protein engineering and fermentation technology, therefore improving the feasibility of low-dose/high-frequency dosing regimen.

8.2. Recommendations

This study presents an interesting approach to resolve the pertinent issues facing the application of AnMBR for raw sewage treatment, namely solids accumulation and membrane fouling. Before upscaling the technology, further studies should be conducted to investigate the following aspects:

- Different methodologies to enhance the activities and prolong the life-spans of the crude hydrolases in wastewater media. Suitable immobilization techniques can be explored to achieve this objective.
- Devise a concoction of hydrolytic enzymes that can achieve the highest efficiency without any adverse effects. Other hydrolytic enzymes worth considering are cellulase, phosphatase, glucosidase and peptidase.
- Study the feasibility and optimization of periodic dosing whenever necessary, such as under shock loading conditions. Therefore, enzymes can be conserved during normal operation.
- Discover other techniques to efficiently recover enzymes from wasted sludge, while minimizing the extraction of undesirable SMPs. Other sustainable sources of enzymes should also be explored.
- The effects of enzyme augmentation on the microbial community dynamics in AnMBRs. In particular, the community shifts of hydrolytic bacteria in response to exogenous hydrolases will be interesting to study.
- Consider the incorporation of a solid-state fermenter upstream of the AnMBR for the continuous production of crude enzymes. This is a low-cost alternative to obtaining crude enzymes from other commercial companies.
- Assessments of life-cycle costs and economic analyses of the proposed strategies in the contexts of AnMBR operation and sewage treatment. Due to the low profit margin of public utilities, any additional outlays should be regulated within the bounds of the cost savings that enzyme augmentation offers.