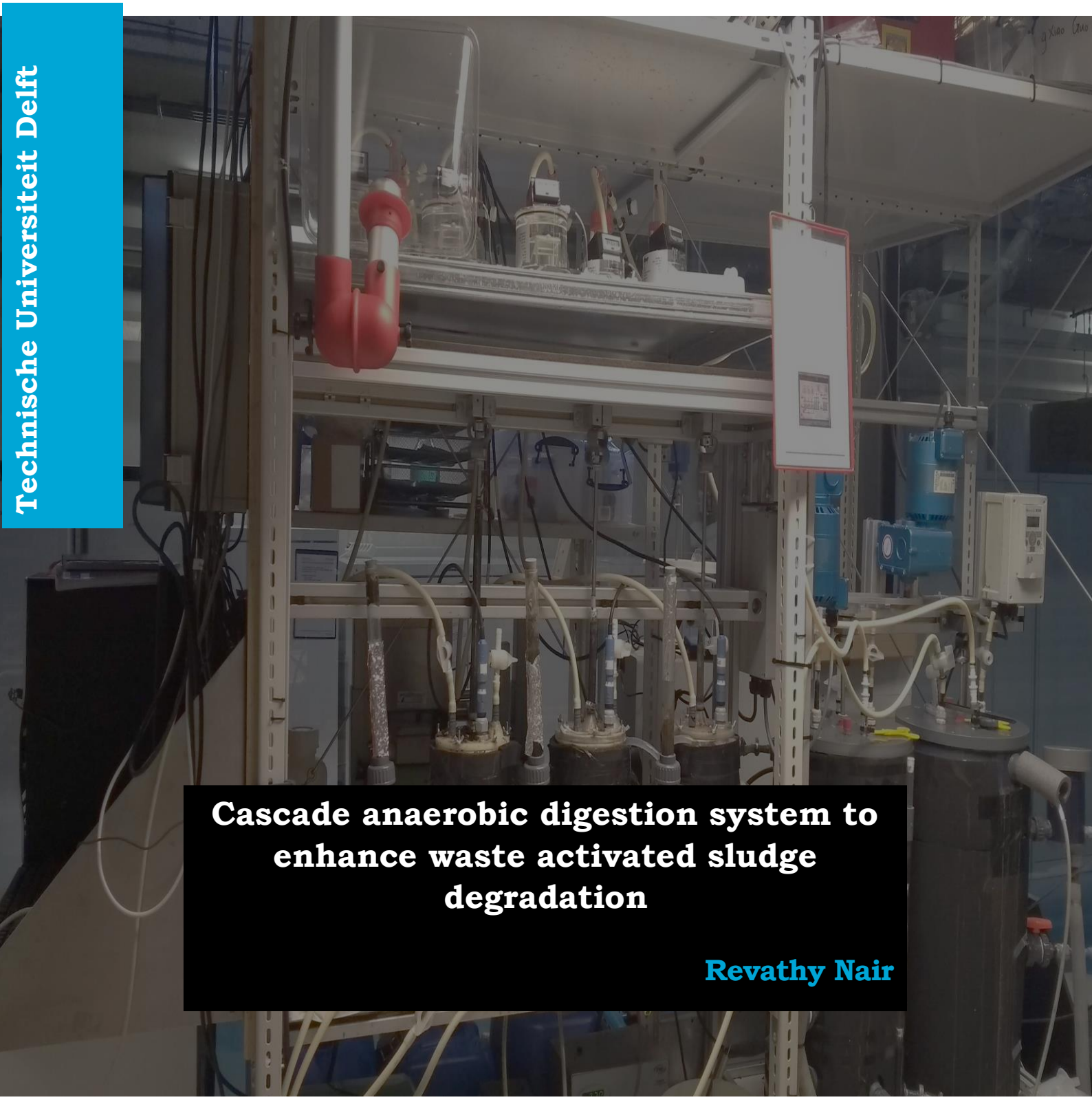
Technische Universiteit Delft



Cascade anaerobic digestion system to enhance waste activated sludge degradation

Revathy Nair

TU Delft Delft University of Technology

Challenge the future

Cascade anaerobic digestion system to enhance waste activated sludge degradation

by

Revathy Nair

4619226

in partial fulfilment of the requirements for the degree of

Master of Science
in Civil Engineering

at the Faculty of Civil Engineering & Geosciences of the Delft University of Technology.

to be defended publicly on February 22, 2019 at 11:30 AM.

Supervisor:	Prof.dr.ir. Merle de Kreuk	TU Delft
Daily Supervisor:	Ir. Hongxiao Guo	TU Delft
Thesis committee:	Prof.dr.ir. Jules van Lier	TU Delft
	Dr. Peter-Leon Hagedoorn	TU Delft
	Ir. Fabio Tonin	TU Delft

TU Delft Delft University of Technology

Abstract

Large amounts of residual waste activated sludge are produced as by-products during biological wastewater treatment processes. Anaerobic digestion is a widely accepted stabilisation method for waste activated sludge (WAS) treatment. However, the application of anaerobic digestion is limited by the long retention time and low degradation efficiency of compounds. Structural extra cellular polymeric substances (EPS) are metabolic products released by microorganisms that play an important role in the disintegration of sludge structure. The limitations in anaerobic digestion mentioned above pertain to the hydrolysis step in anaerobic digestion. The cascade reactor (cascade AD) system i.e. continuous stirred tank reactor (CSTR) in series, is a robust reactor system that is expected to enhance the hydrolysis step and to show a superior performance at low solid retention time compared to a conventional CSTR.

This research is aimed at observing the difference in performance of cascade AD and a conventional CSTR at shortened retention time. Various indicators were used to understand the performance enhancement of a cascade AD in comparison to a conventional CSTR. Moreover, the degradation of structural EPS by selected enzyme groups such as protease, cellulase and polygalacturonase were also studied.

The cascade AD showed better performance than a conventional CSTR at a retention time of 22 and 15 days. This improved performance was enabled by the smaller reactors in cascade AD that provided higher hydrolysis rate. Higher removal efficiency of protein, carbohydrate and structural EPS was observed in cascade AD. Improved ammonium and phosphate release were also indications of better performance of cascade AD. Although the mass balance in nitrogen was maintained in the reactor system, phosphorous mass balance indicated possibilities of precipitation.

The batch tests performed with the enzyme protease, cellulase and polygalacturonase were aimed at understanding the degradation of SEPS. It was inferred from the test that volatile suspended solids proved to be a better indicator for solubilisation compared to COD and ammonium concentration. Protease showed higher solubilisation compared to cellulase and polygalacturonase. Particle size distribution did not indicate a significant difference upon the addition of all three enzyme groups; indicating that a significant change in structure was not caused by the enzymes. Hydrolysis kinetics and SEPS degradation could not be derived from the test because of the variations in results based on the substrates used. Nevertheless, the tests proved to be useful in improving methodology for deriving hydrolysis kinetics of WAS.

In conclusion, the novel cascade AD showed better performance at shortened retention time. The system also showed stable performance despite the shortened retention time compared to a conventional CSTR. Thus, the stable performance suggests the opportunities to further lower the retention time in cascade AD.

Acknowledgements

With this MSc thesis, I am completing my master's in environmental engineering at TU Delft. I would like to take this opportunity to thank everyone who helped me during this period.

First of all, I would like to thank my thesis committee: Merle de Kreuk, Jules van Lier, Peter-Leon Hagedoorn, Fabio Tonin and Hongxiao Guo. I am very grateful to my supervisor Merle de Kreuk for providing me with this opportunity to join the sludge group. Her guidance, advice and timely discussions helped me to take forward my thesis and to widen my perspectives. I would also like to thank Jules van Lier and Peter-Leon Hagedoorn for all the suggestions and feedbacks. I am also thankful to Hongxiao Guo for patiently guiding me through all day to day work in lab. His guidance and all the numerous discussions pushed me to better standards. I would also like to thank everyone in the water lab for the wonderful atmosphere.

On a personal note, I would like to thank my parents, my sister and grandmother for always supporting and encouraging me. My friends Anjana, Amrita, Kamalesh and Shashank for always finding time for me. Also, to Ashwathi and Radesh for all the warm dinners and conversations after long days. I would also like to thank all my environmental engineering and water management friends for all their companionship without which last two years of study would not have been a memorable experience. Thank you all.

Revathy

Delft, February 2019

Table of Contents

List of figures

List of tables

Chapter 1

Introduction

Large amounts of residual waste activated sludge (WAS) are produced as by-products during biological wastewater treatment processes. As much as 10 million tons of dry sludge per year is being produced in the European union, which is equivalent to 60-90 g per population equivalent (Appels, Baeyens et al. 2008). Management of the residual WAS amounts to 50 % of the operating cost in a wastewater treatment plant (Yu, Wang et al. 2016). Therefore, effective management i.e. treatment and disposal of the residual sludge is crucial for the operation of the wastewater treatment plants. WAS ought to go through some treatment steps to reduce its volume, to improve its stability and minimize the associated health problems (Appels, Baeyens et al. 2008).

Anaerobic digestion (AD) is a widely accepted stabilisation method for WAS treatment because of its ability to reduce the organic fraction of the sludge, to remove pathogens and to recover energy in the form of methane and to improve its dewaterability. The main stages involved in anaerobic digestion are hydrolysis, acidogenesis, acetogenesis and methanogenesis where diverse groups of microorganisms such as acidogenic bacteria, hydrogen producing acetogenic bacteria, acetoclastic methanogens and hydrogenotrophic methanogens are involved. The process efficiency of anaerobic WAS digestion is limited due to the requirement of long sludge retention time and poor degradation rate owing to the complex nature of the sludge. These limitations are largely associated with the hydrolysis step, which deals with breaking down of the proteins and carbohydrates in particulate organic compounds into soluble products such as amino acids and sugars by extracellular enzymes produced by the microorganisms.

Studies have suggested that the rate of hydrolysis in anaerobic WAS digestion can be enhanced by the addition of enzymes. However, the economic viability of this approach is not yet clear owing to the increased operational costs (Parawira 2012). Enhancement of hydrolysis can also be achieved by the acceleration of enzymatic conversion rate of complex macromolecules in sludge, which is positively correlated to enzymatic activity (Kim, Nam et al. 2012). Enzymatic activity could be enhanced by increasing the substrate concentration according to Michalis Menten kinetics. In practice, enhancing the substrate concentration is made possible by increasing the organic loading rate (OLR). However, OLR may result in accumulation of volatile fatty acids (VFA), causing failure in a conventional AD system.

The cascade reactor system i.e. continuous stirred tank reactor (CSTR) in series enables maximising the OLR and thereby improving the enzymatic hydrolysis. Besides, this system physically separates the sensitive methanogenesis from the robust hydrolysis/acidogenesis. Therefore, the proposed treatment is expected to exhibit accelerated enzymatic hydrolysis and higher tolerance to potential volatile fatty acids (VFA) toxicity and more stability in anaerobic WAS digestion at high OLR/ low solid retention time (SRT).

Extracellular polymeric substances (EPS) are metabolic products released by microorganisms, playing a pivotal role in the formation of the sludge structure in activated sludge process (Guo, Wang, & Liu, 2016) lowering the diffusion rate of enzymes into sludge structure and therefore,

affect the hydrolysis of WAS during AD. However, studies on hydrolysing EPS lack specific details, such as the roles various types of organic matter have in EPS and which type of enzyme can disintegrate EPS efficiently. Felz, Al-Zuhairy et al. (2016), demonstrated that polymers in EPS can be classified as gel-forming and non-gel-forming based on its ability to form hydrogels with Calcium. The gel-forming EPS, which called structural EPS (SEPS), contributed to the formation of the sludge gel matrix. The enzymatic hydrolysis of SEPS could greatly influence the overall hydrolysis of sludge in anaerobic sludge digestion. The proposed cascade reactor system is expected to offer higher degradation of SEPS.

Therefore, the cascade WAS anaerobic digestion system is expected to perform better at lower SRT in comparison to a conventional CSTR because of its enhanced hydrolysis rate. The aim of this thesis is to validate the above hypothesis. As discussed above, SEPS plays an important role in the disintegration of sludge structure. A study on hydrolysis of SEPS by enzymes/enzyme groups could help to understand the mechanism of the decomposition of sludge structure.

1.1 Thesis Outline

The thesis is divided into the following chapters

Chapter 1: Introduction

Chapter 2: Background information and research questions: This chapter provides information about the phases in Anaerobic Digestion of WAS with emphasis on hydrolysis. This also includes details on different components in WAS and structural EPS. A detailed overview of the methods for enhancing the enzymatic activity using different additives and other possibilities are also presented. The research gap in this area of study and the research objectives that were derived are presented.

Chapter 3: Materials and methodology: In this chapter the characteristics of the proposed cascade treatment set up are provided. The details about performed measurements and analysis are also described here. The protocol for the batch experiments conducted as a part of the study are provided.

Chapter 4: Results and Discussion. The following results of the study are presented and discussed.

i. The performance of cascade AD in comparison to conventional CSTR
ii. The results from hydrolysis of WAS by enzyme groups with focus on SEPS

Chapter 5: Conclusion and Recommendation

.

Chapter 2

Background

The purpose of this section is to give the required background information for understanding this thesis. The fundamentals of anaerobic digestion are discussed in the first part, where hydrolysis is identified as the rate limiting step. Methods to enhance hydrolysis are subsequently discussed in this chapter. The role of EPS and its impact on hydrolysis are also discussed further.

2.1 Anaerobic digestion of waste activated sludge

AD is a widely accepted process for stabilisation of WAS reducing the organic fraction of the sludge and enabling the recovery of energy in the form of methane. AD is mainly composed of four steps i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 1). The steps involved in AD are discussed below.

2.1.1 Steps in anaerobic digestion

AD is a complex process with multiple steps and balance between these steps is crucial for the successive transformation of organic material into CO_2 and methane. AD is mainly composed of four steps i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 1).

Hydrolysis: During this step particulate organic compounds like proteins, carbohydrates and nucleic acids are converted into soluble materials such as amino acids and sugars by extracellular enzymes produced by hydrolytic bacteria. Consequently, these soluble products become accessible to acidogenic bacteria. Hydrolysis is generally considered as the rate limiting step in anaerobic digestion of particulate organics. (Bougrier, Albasi et al. 2006).

Acidogenesis: The soluble products formed during hydrolysis are further converted into VFA by acidogenic/ fermentative bacteria. Other products such as ammonia, CO_2, H_2S etc. are also formed as by-products of this conversion(Appels, Baeyens et al. 2008). This is considered to be the fastest conversion step in anaerobic digestion. The fermentative organisms can survive in a wide range of pH between 4 and 8.5 (Hwang, Jang et al. 2004).

Acetogenesis: The organic acids produced by fermentative bacteria are converted by acetogens into acetic acid, CO_2 and H_2. The partial pressure of hydrogen plays an important role in driving this conversion.

Methanogenesis: Methanogenesis is the final step in anaerobic digestion that leads to the production of methane. Methanogens reduce CO_2 using H_2 as electron donor or by acetate into CH_4 and CO_2. Decarboxylation of acetate is responsible for about 70% of methane production(Gerardi 2003). Methanogens are very sensitive to pH and can only thrive in a narrow range between pH of 6.5-7.2 (F. Bennett 2007).

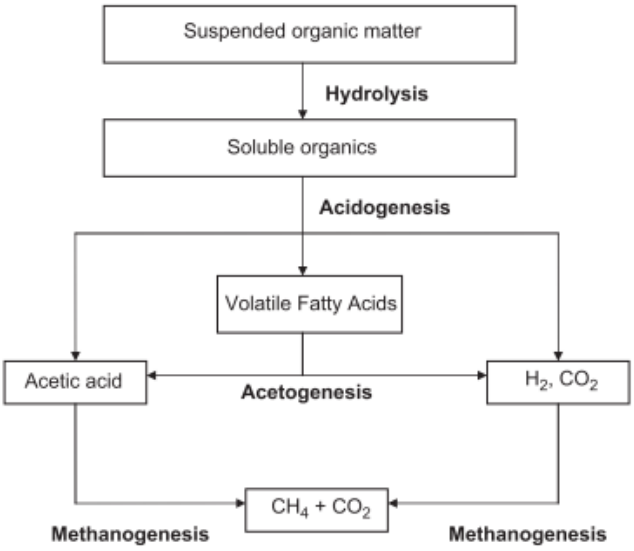


Figure 1: Steps in anaerobic digestion (Appels, Baeyens et al. 2008)

The microorganisms involved in anaerobic digestion are characterized as hydrolytic, fermentative, acetogenic and methanogenic bacteria. Hydrolytic bacteria are involved in hydrolysis step that breaks down complex particulate matter into soluble components. Acetogenic bacteria or acetogens are acetate forming organisms. This acetate, as had been discussed earlier, is the primary substrate for methanogenesis. However, acetogens cannot survive under high partial pressure of hydrogen. The balance in the hydrogen partial pressure is maintained by methanogens that consume hydrogen. Therefore, there exists a symbiotic relationship between the two type microbes. Finally, methanogens convert the products from the previous steps into methane and carbon dioxide (Jain, Jain et al. 2015).

Anaerobic digestion is a complex process with requires different operating environments to achieve the optimum conditions in various steps. As discussed earlier, methanogens are sensitive to pH changes. If the acid formation happens at a faster rate than methane conversion, the pH drops in the system which is not a favourable for methanogens. Therefore, a well-balanced digestion should enable an equilibrium between acidogenesis and methanogenesis. Thus, for an efficient process to take place the environmental conditions affecting AD such as temperature, pH, organic loading etc. should also be optimized.

2.1.2 Factors affecting anaerobic digestion

AD can be classified based on its operating temperature, operating parameters, reactor flow conditions i.e. plug flow, mixed etc. Several parameters such as pH, alkalinity, temperature and retention time can affect the overall rate of anaerobic digestion. Different microorganisms involved in the process have different optimum pH as discussed above. Methanogenic bacteria can counteract the pH drop caused by VFA in the form of alkalinity by CO_2, NH_3 and bicarbonate (F. Bennett 2007). The concentration of CO_2 in gas phase and bicarbonate based alkalinity plays an important role in maintaining pH (F. Bennett 2007). It has also been shown that a bicarbonate to VFA ratio of 1.4:1 is crucial to maintaining the stability of the process (Afvalwater 1985).

The retention time of the solids in the digestor plays an important role in determining the extent of the conversions. The solid retention time (SRT) indicates the amount of time spend by solids in the digester while hydraulic retention time (HRT) is the time spend by the liquid sludge(Appels, Baeyens et al. 2008). AD digesters without a recirculation SRT is equal to HRT.

SRT determines the stability and balance between different steps in digestion process. Short retention times would lead to washout of methanogens with the accumulation of VFA and insufficient breakdown of different compounds in sludge. AT 35° C the limiting values of minimum SRT were around 4 and 0.75 days for acetoclastic and hydrogenotrophic methanogen respectively(Rittmann and McCarty 2012). Therefore, generally SRT considerably higher than minimum SRT must be maintained to prevent washout of methanogens.

2.2 Extracellular polymeric substances (EPS)

Extracellular polymeric substances (EPS) are also another factor that affects the biodegradability of waste activated sludge. EPS are products released by microorganisms as part of metabolism i.e. secretions, cell lysis and hydrolysis products (Salama, Chennaoui et al. 2016). In activated sludge, microorganisms usually form aggregate such as flocs, granules and biofilms. (Salama, Chennaoui et al. 2016). EPS plays a significant role in determining the physico chemical properties of microbial aggregates such as structure, dewatering properties, surface charge, mass transfer and so on. Formation of gel like network to keep the bacteria together is another function attributed to EPS (Salama, Chennaoui et al. 2016)

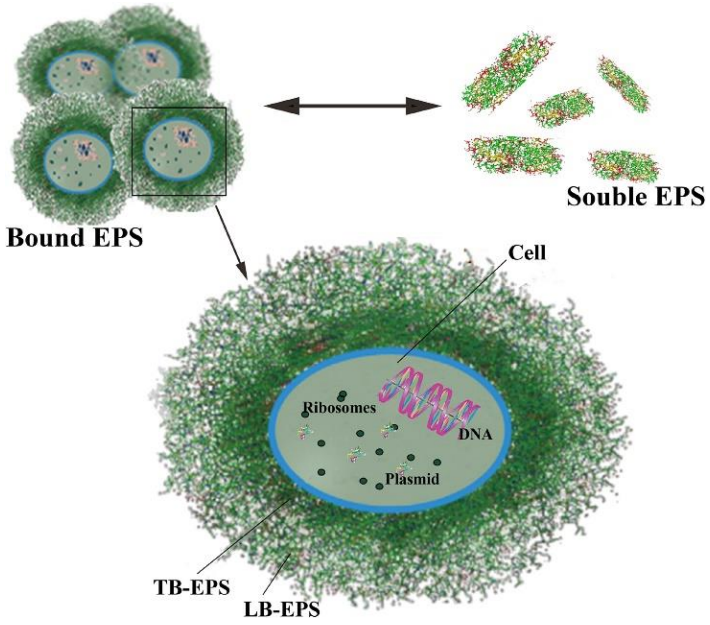


Figure 2: Structure of EPS adapted from Shi, Huang et al. (2017)

EPS consists of polysaccharides, proteins, humic acids, uronic acids, lipids, nucleic acids and inorganic materials like metals and ions. (Flemming and Wingender 2001). However, it was found to have been largely constituted by proteins and carbohydrates. In EPS of activated sludge, proteins were found to be predominant. The proteins formed electrostatic bonds with multivalent cations and were found to have a key role in the floc structure (Salama, Chennaoui et al. 2016). The macromolecular structure of EPS was found to be made of polymerisation of similar molecules (Table 1)(Salama, Chennaoui et al. 2016).The composition of EPS extracted from different microbial aggregates were found to be heterogenous. The different compositions measured could be ascribed to the different process parameters, culture and extraction method applied (Salama, Chennaoui et al. 2016).

Table 1: General composition of EPS. (Salama, Chennaoui et al. 2016)

EPS	*Principal components*	*Linkage between subunits*
Polysaccharides	*Monosaccharides, uronic acids, amino sugars*	*Glycosidic bonds*
Proteins	*Amino acids*	*Peptide bonds*
Nucleic bonds	*Nucleotides*	*Phosphodiester bonds*
(Phospho)lipids	*Fatty acids, glycerol, phosphate, serine, choline, sugars*	*Ester bonds*
Humic substances	*Phenolic compounds, simple sugars, amino acids*	*Peptide bonds, ether bonds*

EPS can be present both on the outside and inside the microbial aggregate(Sheng, Yu et al. 2010). EPS present outside the cells can be divided into bound EPS and soluble EPS (Figure 2). Soluble EPS are either weakly associated to cells or are dissolved in the solution. Whereas bound EPS forms a layer outside the cell wall. The structure of bound EPS could be represented as a 2-layer structure(Nielsen and Jahn 1999). Tightly bound EPS (TB-EPS) is the inner layer that is bound closely to the cell and loosely bound EPS (LB-EPS) is the outer layer which is a dispersed slime layer (Sheng, Yu et al. 2010).

Some highly hydrated EPS, which are subset of total EPS, are capable of forming a structural hydrogel matrix. These polymers are referred to as "structural EPS"(SEPS) (Felz, Al-Zuhairy et al. 2016). In the study by Lin, de Kreuk et al. (2010) these SEPS were found to be the dominant EPS contributing to the strong elastic structure of EPS in aerobic granular sludge. In another study by Lin, Sharma et al. (2013) gel forming structures were found to be part of the basic structure in granular and flocculent activated sludge. Anaerobic digestion was believed to weaken the structure of WAS during AD (Salama, Chennaoui et al. 2016).

2.3 Enhancing hydrolysis of waste activated sludge

Pre-treatment methods such as mechanical, chemical and biological have been used to improve hydrolysis. The pre-treatment steps are aimed at solubilizing or reducing the size of organic compounds making them more biodegradable (Bougrier, Albasi et al. 2006). However, the requirement for high input energy and related costs limits the applications of these treatment methods.

Some attempts have been made to enhance enzymatic hydrolysis by the usage of additives. The On the addition of bio surfactants (alkyl polyglycosides) and chemical surfactants (sodium dodecyl sulphate), EDTA accelerated the release of enzymes. The function of EDTA is to remove the cations and thereby disrupting the sludge matrix which leads to the release of enzymes(Wawrzynczyk, Recktenwald et al. 2007). The addition of EDTA was found to enhance the activities of amylase and protease (Kavitha, Adish Kumar et al. 2013). EPS can form enzyme-substrate complexes which would lead to the trapping of enzymes. Through the addition of biosurfactants, the TB-EPS gets converted into soluble EPS, leading to more release of enzymes and acceleration of hydrolysis rate and methane production (Kavitha, Jayashree et al. 2014). Meanwhile, studies have also used iron as an additive because of its role as cofactors for several key enzymes (Romero-Güiza, Vila, Mata-Alvarez, Chimenos, & Astals, 2016). The addition of zero-valent iron, which acts as on electron donor, showed increased activities of protease and cellulase by almost 90% (Feng, Zhang, Quan, & Chen, 2014). The

addition of Potassium mono persulphate, an oxidizer, also showed higher activities of protease and a-glucosidase (Luo et al., 2018).

Biological pre-treatment in the form of enzyme addition is an environmentally friendly alternative to the treatment methods discussed earlier. Enzymes offer a mild and specific method to enhance the biodegradability of the digested sludge (Parawira 2012). Enzymes are very specific in application and are effective based on operating time, dosage and enzyme types. However, the addition of enzymes are also often associated with higher costs (Eriksson, Börjesson et al. 2002). Moreover, enzymes being proteins that are biodegradable will degrade during the process of AD (Parawira 2012).

The enzyme activity is correlated with the amount of enzyme present and substrate concentration(Cornish-Bowden 2013). Therefore besides the dosage of such additives, the enzymatic activity could also be accelerated by increasing the substrate concentration according to Michaelis-Menten kinetics; when concentration of substrate is in the range of K_m (substrate concentration at which reaction rate is half of maximum rate) (equation 1), where S is the substrate concentration and V_{max} is the maximum rate (Figure 3). Higher substrate concentration could be made possible in AD by the use of reactors with smaller volume. This is discussed in the subsequent section.

$$\frac{dS}{dt} = \frac{Vm}{K_m + S} \qquad (1)$$

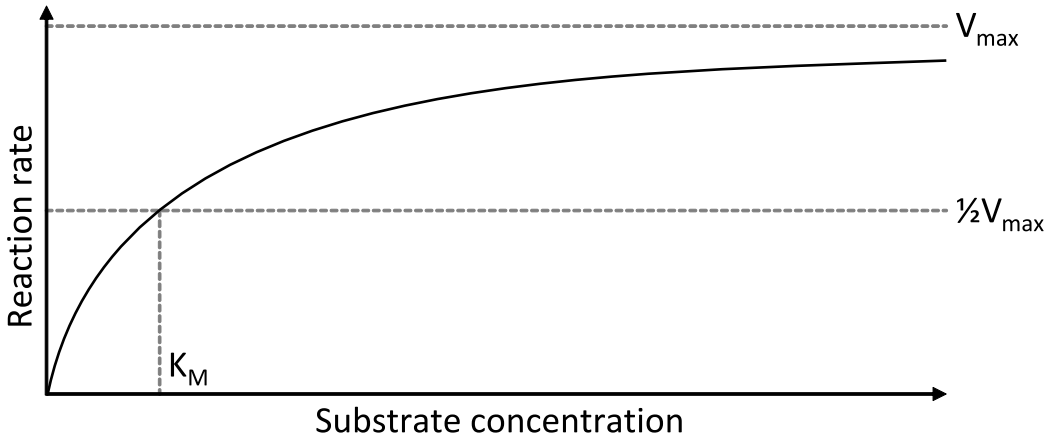


Figure 3:Michelis Menten kinetics (figure adapted from Wikipedia)

2.3.1 Optimizing reactor configuration for higher enzymatic activity

Continuous stirred tank reactor (CSTR) is a basic reactor configuration used in AD. In CSTR's complete and uniform mixing of the fluid particles are expected to take place. However, CSTR reactors are operated at solids retention times (SRTs) between 20-28 days. Moreover in reality, short-circuiting and presence of dead zones lowers the performance of CSTRs(Capela, Bilé et al. 2009). Such long retention times leads to the requirement of a high volume that would increase the costs (Metcalf and Eddy 2003). In contrast, plug flow systems can offer higher efficiency as the residence time for the substrate is more evenly distributed which makes mixing less of a problem (Metcalf and Eddy 2003). Plug flow can be achieved by connecting CSTRs in series.

As mentioned earlier, higher organic loading rate could provide higher enzymatic hydrolysis. To obtain higher substrate loading rate, in continuous reaction process, smaller reactors are employed in series at shorten hydraulic retention time (HRT). Low retention times are often required to maximize the enzymatic reaction rate (activity) (deGooijer, Bakker et al. 1996). Usually, short HRT leads to high concentration of volatile fatty acids (VFAs), an important intermediate in anaerobic digestion, which cause low pH where methanogenesis is inhibited in the conventional CSTR. This would eventually lead to failure of the process (Maspolim, Zhou, Guo, Xiao, & Ng, 2015). However, novel system of CSTRs in series (cascade) reactor system, approaching a plug flow, physically separates the fragile methanogenesis from the robust hydrolysis/acidogenesis. Also, the lower retention time in plug flow reactors enables the presence of higher quantity of enzymes to be present, which would also lead higher enzymatic hydrolysis. Therefore, it is expected to demonstrate a higher tolerance to VFAs toxicity and subsequently not only an accelerated enzymatic hydrolysis but more stable reactor performance than CSTR especially at high organic loading rate.

2.4 Research question and objectives

The literature study indicated the following research gap:

- *The option of improving the reactor design and process parameters to enhance enzymatic activity has not been well investigated in the anaerobic digestion of waste activated sludge.*
- *Although hydrolysis of total EPS by enzymes have has studied recently, the mechanism of hydrolysis of gel-forming EPS of the sludge by enzymes is largely unknown.*

Following the research gap, the two main research questions were formulated:

- ***To what extent differs the performance of cascade anaerobic WAS system from that of a conventional CSTR under shortened HRT?***
- ***How would the target enzyme groups influence the hydrolysis of structural EPS?***

*target enzyme groups: Protease, cellulase and polygalacturonase.

The following sub questions were expected to be answered through the course of the study:

1. How much of a difference was observed in sludge reduction, mass flow of COD, N and P between cascade system and CSTR?
2. If the cascade system achieves higher hydrolysis efficiency, how does this influence the acidogenesis and methanogenesis?
3. Which enzyme has the most important role in the hydrolysis of structural EPS?
4. How does the hydrolysis kinetics of structural EPS differ between different enzyme treatments?
5. Which parameters are more indicative of the hydrolysis occurring after the addition of enzymes?

Chapter 3

Methodology

3.1 3.1 Routine analysis of the set up

3.1.1 Description of the experimental setup

Four lab-scale continuous stirred tank reactors (CSTR) connected in series were operated in parallel to a single CSTR in this study. The temperature in each of the reactor were maintained within mesophilic range (35± 1°C) using a temperature-controlled water bath. The operational volumes of the first three reactors were 2.2 L, whereas R4 and reference CSTR were 15.4 L. 10% recirculation was provided from the third reactor to the first reactor to balance pH. The biogas production from the setup is measured by a biogas meter. During the start-up of the reactor antifoaming solution was added to R1. A mixing intensity of 40 rpm was applied for R1, R2, R3 and 65 rpm for R4 and reference CSTR. Figure 4 represents the scheme of the reactor.

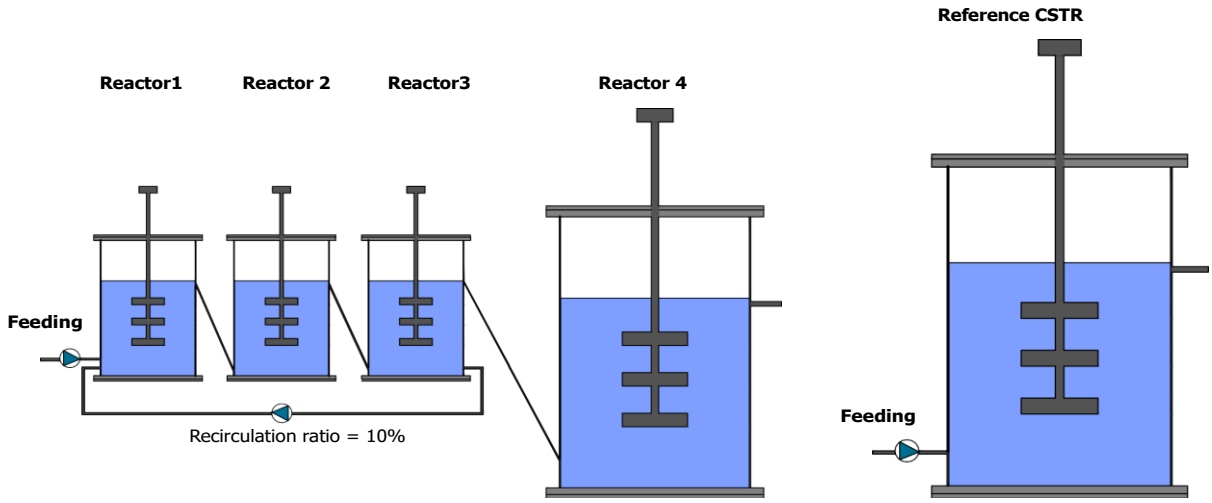


Figure 4: Scheme of the reactor system with cascade AD (left) and reference CSTR (right)

3.1.2 Operational and substrate conditions

The influent waste activated sludge was obtained from sewage treatment plant Harnaschpolder, Den Hoorn, Netherlands that was performing enhanced biological phosphorous removal. The influent sludge concentration was maintained at 4% w/v TS. In Stage 1 (48-156 days) of reactor operation, the HRT was maintained at 22 days followed by

HRT of 15 days (157- 220). In order to reduce the retention time, the flow rate of influent to reactor setup was changed accordingly in the second stage. The flow rate was increased from 1 L/day to 1.5 L/day during stage 2 of the reactor operation. The table below gives a summary of operating conditions of the reactors during the operational period.

Table 2: Operational conditions of cascade AD and reference CSTR

		HRT 22 days			***HRT 15 days***		
No.	***Working Volume (L)***	***pH***	***OLR (kg.COD/m3.d)***	***Operational duration***	***pH***	***OLR (kg.COD/m3.d)***	***Operational duration***
R1		*6.7 ± 0.0*	*23.31*		*6.6 ± 0.1*	*34.97*	
R2	*2.2*	*6.9 ± 0.0*	*21.20*		*6.7 ± 0.1*	*31.80*	
R3		*7.0 ± 0.0*	*19.02*	*108*	*6.8 ± 0.1*	*28.53*	*64*
R4	*15*	*7.5 ± 0.2*	*2.51*		*7.2 ± 0.1*	*3.77*	
Reference CSTR	*22*	*7.4 ± 0.2*	*3.33*		*7.1 ± 0.1*	*5.00*	

3.1.3 Performance indicators for reactor operation

Sampling was carried out at an interval of 5 days during start-up stage and on alternate days once the reactors are stable. Solids reduction (total solids and volatile solids) were tested according to standard methods (APHA). The amount of daily biogas generated from the reactors is recorded and biogas composition is assessed by gas chromatography. Chemical Oxygen Demand (COD), nitrogen, phosphorous, ammonia, and phosphate are determined using Hach Lange test kits. Gas chromatography (GC) with an FID detector (Agilent 7890A, USA) is used to measure various VFA and its concentration. The alkalinity is measured using digital titration apparatus with 0.1 M hydrochloric acid.

During regular operation, solids, COD, pH and VFA were measured once in 2 days. Once the reactors are stable, a complete nitrogen and phosphorous balance were also made. Proteins were measured using modified Lowry method (Frølund, Palmgren, Keiding, & Nielsen, 1996) with BSA as standard and polysaccharides were quantified using phenol-sulfuric acid using glucose as standard (Dubois et.al, 1956). Particle size distribution was made using (PSD) were performed using DIPA-2000 Eyetech particle analyser (Donner Technologies). ICP-MS (Inductively coupled plasma mass spectrometry) measurement was used to analyse the composition of cations in the reactor to account for precipitation.

3.1.3.1 Extraction of SEPS:

The EPS present in the sludge were extracted using mild-temperature Na_2CO_3 method by Felz, Al-Zuhairy et al. (2016). Sludge sample of 3g was taken from the reactors and was added to 0.5% w/v Na_2CO_3 solution made up to 50 mL. The solution was then stirred at 80°C for 35 mins which was followed by centrifugation at 4000g, 4 °C for 20 mins. The supernatant was discarded, and the remaining solid fraction was resuspended. The pH of the extracted solution was adjusted to 2.2 with 1 M and 0.1 M hydrochloric acid. This solution was centrifuged again at 4000g at 4°C for 20 mins. The remaining solids after discarding the supernatant was regarded as structural EPS (SEPS). SEPS was used for VS analysis and the measured content was reported as SEPS of the sludge (g/ $100g_{sludge}$).

3.2 Estimation of hydrolysis rate in the reactor

3.2.1 From COD balance

The hydrolysis rates in the reactors are to be theoretically calculated using the COD balance using the formula(Wu, Qin et al. 2015). Hydrolysis, acidogenesis and methanogenesis rates were estimated using SCOD, COD_{CH4}, COD_{VFA} are explained in equation (2), (3) and (4).

$$Hydrolysis\ rate\ \left(\frac{gCOD}{L.d}\right) = \frac{\left(\frac{massSCOD+massCOD_{CH4}}{d}\right)eff.-\left(\frac{massSCOD}{d}\right)inf.}{volume\ of\ the\ reactor} \quad (2)$$

$$Acidogenesis\ rate\ \left(\frac{gCOD}{L.d}\right) = \frac{\left(\frac{massCOD_{VFA}+massCOD_{CH4}}{d}\right)eff.-\left(\frac{massCOD_{VFA}}{d}\right)inf.}{volume\ of\ the\ reactor} \quad (3)$$

$$Methanogenesis\ rate\ \left(\frac{gCOD}{L.d}\right) = \frac{\left(\frac{massCOD_{CH4}}{d}\right)}{volume\ of\ the\ reactor} \quad (4)$$

where,

mass SCOD: weight of SCOD (g)
mass COD_{CH4}: CH_4 weight calculated as COD (g)
mass COD_{VFA}: VFA weight calculated as COD (g)
eff: effluent
inf: influent

3.2.2 From Batch test

Batch tests were performed to define hydrolysis rate coefficient which was estimated separately for each of the reactors. Batch tests were carried out in triplicates at 35°C in an automated methane potential test system (AMPTS). Equal quantity of the sludge from respective reactors were installed on the incubator shaker. Sampling was carried out at regular intervals to estimate the VFA accumulation. The composition and dosage of phosphorous buffer, macronutrients and micronutrients were referred from Zhang, Hu et al. (2014). As the aim of the test was to estimate hydrolysis rate, additional substrate was not added. The tests were stopped when the cumulative biogas production approaches an asymptote.

Hydrolysis rate would be estimated based on first order kinetics. By using the first part of the methane production curve, hydrolysis constant, k_h (d^{-1}) could be determined using a first order hydrolysis model.

$$\frac{dS}{dt} = -k_h t \quad (5)$$

where S is the substrate, k_h is the hydrolysis rate coefficient and t the time. As concentration of substrate is more difficult to determine, methane production was chosen as an indirect indication of hydrolysis of the substrate.

$$ln\frac{B_\infty - B}{B_\infty} = -k_h t \quad (6)$$

where B_∞ is the ultimate methane production corrected for VFA production and B is the methane production at a time, t (Angelidaki, Alves et al. 2009). To determine the hydrolysis rate coefficient equation 2 was solved in terms of Gompertz equation:

$$B(t) = B_O(1 - e^{-kt}) \quad (7)$$

where B(t) is methane production over time (ml CH_4), time (d); B_o is the maximum methane production (ml CH_4) and k is the kinetic constant rate (d^{-1}). Using the curve fitting tool in Origin Pro 2015 the fit was made.

3.3 Batch test to investigate the effect of chosen enzymes on the hydrolysis of waste activated sludge: development of method

The activated sludge used in this experiment was collected from secondary sedimentation tank Harnaschpolder, B stage Harnaschpolder and from Dokhaven WWTP, Rotterdam. The details about the sludge is provided in Table 4. The activated sludge was allowed to settle, and the supernatant was discarded. Three types of hydrolytic enzymes were used separately to target specific functional groups. Protease (Sigma, at least 500 U/g derived from Aspergillus oryzae) was used to target proteins, cellulase (Sigma, at least 1000 U/g derived from Aspergillus sp.) to target polysaccharides and polygalacturonase (Sigma, greater than 1 U/mg derived from Aspergillus niger) for uronic acid. Protease, cellulase and pectinase were dosed at 500 units/g.VSS.sludge. Enzyme dosage for the test was selected based on previous research on enzymatic hydrolysis of waste activated sludge (Table 3). Enzyme dosage of 500 units/g.VSS was chosen so that the availability of active sites would be limiting the respective reactions.

Table 3: Enzyme dosage used in literature

Reference	Enzyme dosage for Protease
Yang, Luo et al. (2010)	*400 units/g VSS*
Pei, Hu et al. (2010)	*250 units/g VSS*
Lü, Wang et al. (2016)	*490 units/ g VS*
This study	*500 units/gVSS*

100mL of well mixed sludge samples and enzyme were added to a 250 mL bottle. A control, without the addition of enzyme was also incubated for each test. The bottles were sealed with stoppers and flushed with nitrogen gas for 3 minutes and were installed in a temperature-controlled shaker at 35° C.

For the first test, the substrate was stored in the refrigerator (4° C) for about 10 days. First test was carried out as a trial measurement in order to fine tune the procedure for the test. Sampling was carried out regularly throughout the course of the experiment. One portion of sludge sample used for test-2 was subjected to aeration overnight and the other portion was maintained at anaerobic conditions. In test 2, the sampling was done in the beginning of the test at t= 6 hours and the end of test i.e. t=24 hours. Similar sampling duration was observed also for test-3.

Table 4: Details of the Batch test

	Source of substrate	***Duration of the test***	***Enzymes used***
Test -1	*Sedimentary Tank, Harnaschpolder*	*12 hours*	*Protease*
Test-2	*B-stage, Harnashpolder*	*24 hours*	*Protease, Cellulase*
Test-3	*B-stage Dokhaven*	*24 hours*	*Protease, Polygalacturonase*

The pH, COD, VSS (volatile suspended solids) and particle size distribution of the sludge are estimated before starting the test to visualize the effect of enzyme addition. SCOD and VSS were estimated at regular intervals by standard methods. Upon extraction, the samples were analysed immediately, so no further action of enzyme occurs.

Chapter 4

Results & Discussion

In this chapter, the results from monitoring the cascade AD in comparison to conventional CSTR at HRT of 22 and 15 days are discussed. More details about the other performance indicators such as COD removal, solids reduction etc. are also discussed here. Phosphate and ammonia release, protein and carbohydrate degradation in cascade AD and reference CSTR are also explained. To verify the improved hydrolysis occurring in cascade AD, hydrolysis ratio is presented and compared to reference CSTR. The variation degradation of structural EPS during cascade AD and the variation in degradation at lower retention time is also evaluated. Finally, the results from the addition of enzyme groups to sludge are also presented.

4.1 Performance of Cascade AD and Conventional CSTR under HRT of 22 and 15 days

The reactors were operated for a period of 172 days. The performance of the reactors from day 48 are provided in Figure 5 and Figure 6. During the entire operational period in this study, indicators such as total COD, soluble COD, NH_4-N, PO_4^{3-}, TS, VS, pH and VFA were monitored periodically.

Methane production of the reactors during the phase of study is shown in Figure 5. The parameters such COD removal, methane production and VFA concentration indicated stable performance in reference CSTR and cascade AD from day 79. From Day 79-150, stable reactor performance was observed. The summary of the performance of the reactor at this stage in presented in Table 5. The methane production at HRT of 22 days of Cascade AD was 16% higher than reference CSTR. Correspondingly, higher COD removal was also observed in cascade AD. Also, it was observed that around 60% of the removed COD in cascade AD was occurring in the first 3 reactors at a total HRT of 6.6 days.

TS content of 4% was fed to the reactor as influent. The TS in effluent of cascade AD and reference CSTR were respectively. The solids reduction (TS and VS) in cascade AD and reference CSTR are depicted in Figure 6. Volatile solids measurements are indicative of the amount of organic matter present in the sludge. VS reduction in a typical mesophilic full-scale anaerobic digester ranges from 13-27% at an HRT of 20-40 days (Bolzonella, Pavan et al. 2005). From Table 5, it can be seen that the VS removal in cascade AD and reference CSTR falls above the range given Bolzonella, Pavan et al. (2005).

In the second stage (Day 151 –220), HRT was reduced to 15 days. The lower HRT was achieved by increasing the flow rate resulting in higher OLR and lower retention time. After The increase in OLR caused the increase in methane production. The fluctuations observed in the flow rate of R1 and R2 was because of clogging of the biogas valve. To minimize the foaming produced by the higher loading rate, anti-foaming solution was added to R1, with the dosage of < 1% of influent TCOD. Methane production in reference CSTR indicated more fluctuations in performance throughout the operation in this stage. The summarised

performance of the reactor during this period of operation is provided in Table 5.By lowering the retention time, the difference in methane production between cascade and reference CSTR increased ($p< 0.05$). Similarly, the COD reduction also showed a significant difference in performance between cascade AD and reference CSTR. The significant difference in performance demonstrates the higher efficiency cascade AD at shortened retention time. The COD removal in conventional CSTR reduced by 40% when HRT was reduced to 15 days.

Besides the high COD removal, higher volatile solids reduction was also observed in cascade AD during stage 2 with respect to reference CSTR. The TS in the effluent of cascade AD and reference CSTR were 2.9% and 3.18% respectively. The VS reduction in cascade AD dropped from 39% to 35% while VS reduction in reference CSTR dropped from 35% to 28%. The performance of reference CSTR was observed to have higher drop in performance with respect to cascade AD. The performance of cascade AD was higher than the performance of conventional mesophilic digester (Bolzonella, Pavan et al. 2005) at a lower retention time.

Another indication of stability of both the systems were total VFA production (Appendix figure 1) and total alkalinity (Appendix figure 2). Higher amount of VFA accumulation occurred in R1 and R2. VFA in R4 and reference CSTR were low indicating that the VFA was consumed by methanogens. The VFA concentration stabilised after 70 days. It is apparent that accumulation of VFA had not occurred in the reactors. Furthermore, the pH in the reactors during the period of operation were stable (Table 2). During the operation of reactors, the alkalinity of cascade AD was higher than that of reference CSTR. The alkalinity produced in reactors was seen to increase along the cascade AD and the last reactor, R4 has the highest alkalinity. There existed significant difference between the alkalinity produced in cascade AD and reference CSTR ($p<0.05$) (Appendix figure 2). The changes in alkalinity are brought about by ammonium release and VFA removal (van Haandel 1994). So, an increase in VFA concentration would lead to decrease in alkalinity. Therefore, reactor 4 and reference CSTR with the least amount of VFA concentration and highest ammonium concentration (Figure 7) had the highest alkalinity. By lowering the retention time, VFA concentration in cascade AD and reference CSTR increased. From Appendix figure 2, it can be observed that the alkalinity produced in R2 and R3 decreased and the VFA concentration increased. Nevertheless, the current alkalinity produced was still sufficient to maintain the desired pH. While lowering the retention time further it is likely that the alkalinity would not be sufficient to maintain the pH. Then the recirculation ratio might have to be increased to maintain the pH.

In conclusion, it can be derived that the cascade AD indicated a better performance in comparison to a conventional CSTR. Although the performance at HRT of 22 days proved to be similar for both cascade AD and reference CSTR, significant difference in performance could be observed between the two after lowering the HRT. The effluent from cascade AD demonstrated lesser fluctuations and a more stable performance at HRT 15 days. However, the performance of both cascade AD and reference CSTR were still indicating some unsteadiness in performance at the end of this study. So, it is suggested that reactors should be operated at the same conditions for another HRT before making any further changes.

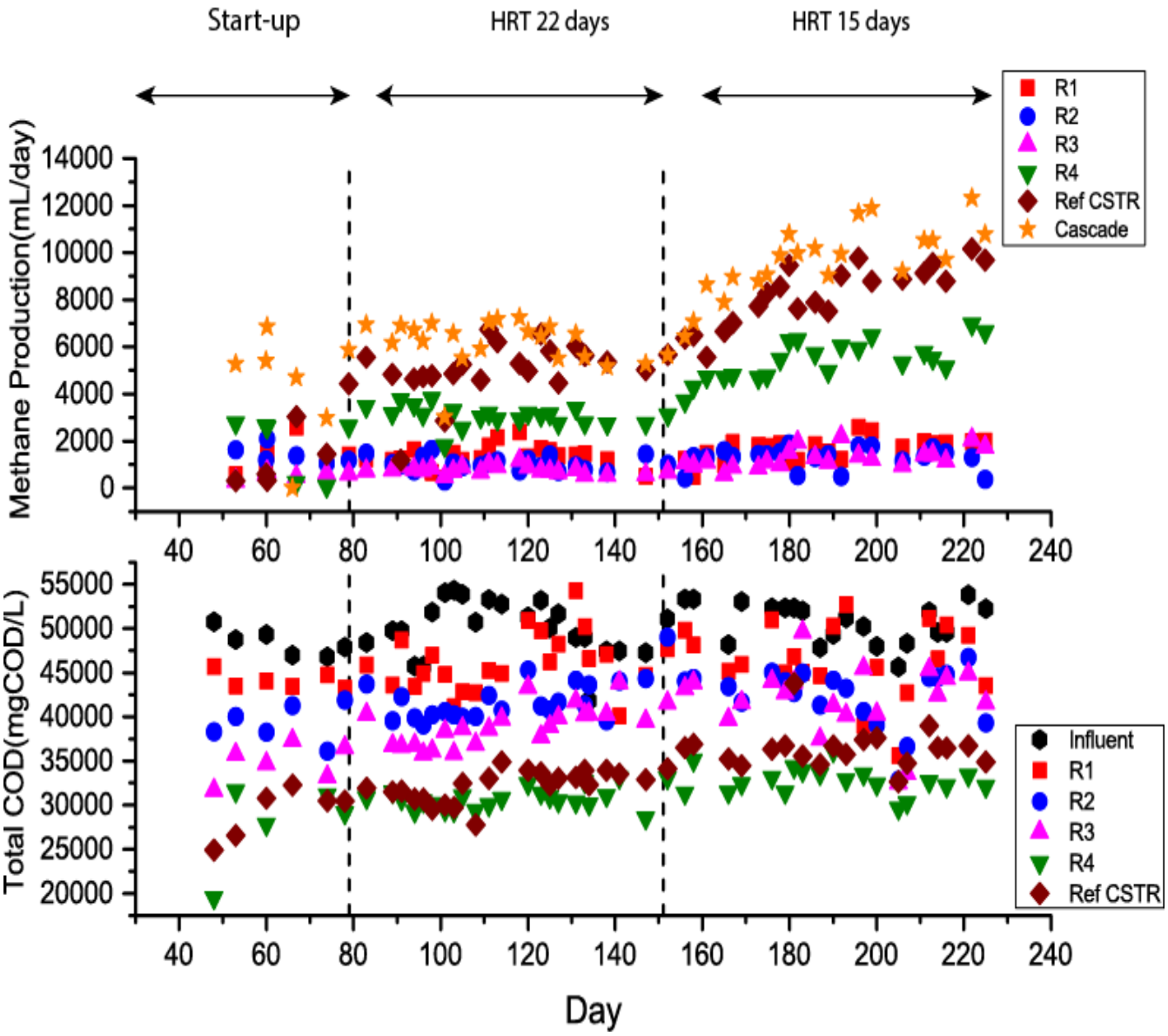


Figure 5: Methane production and TCOD in Cascade AD and Reference CSTR

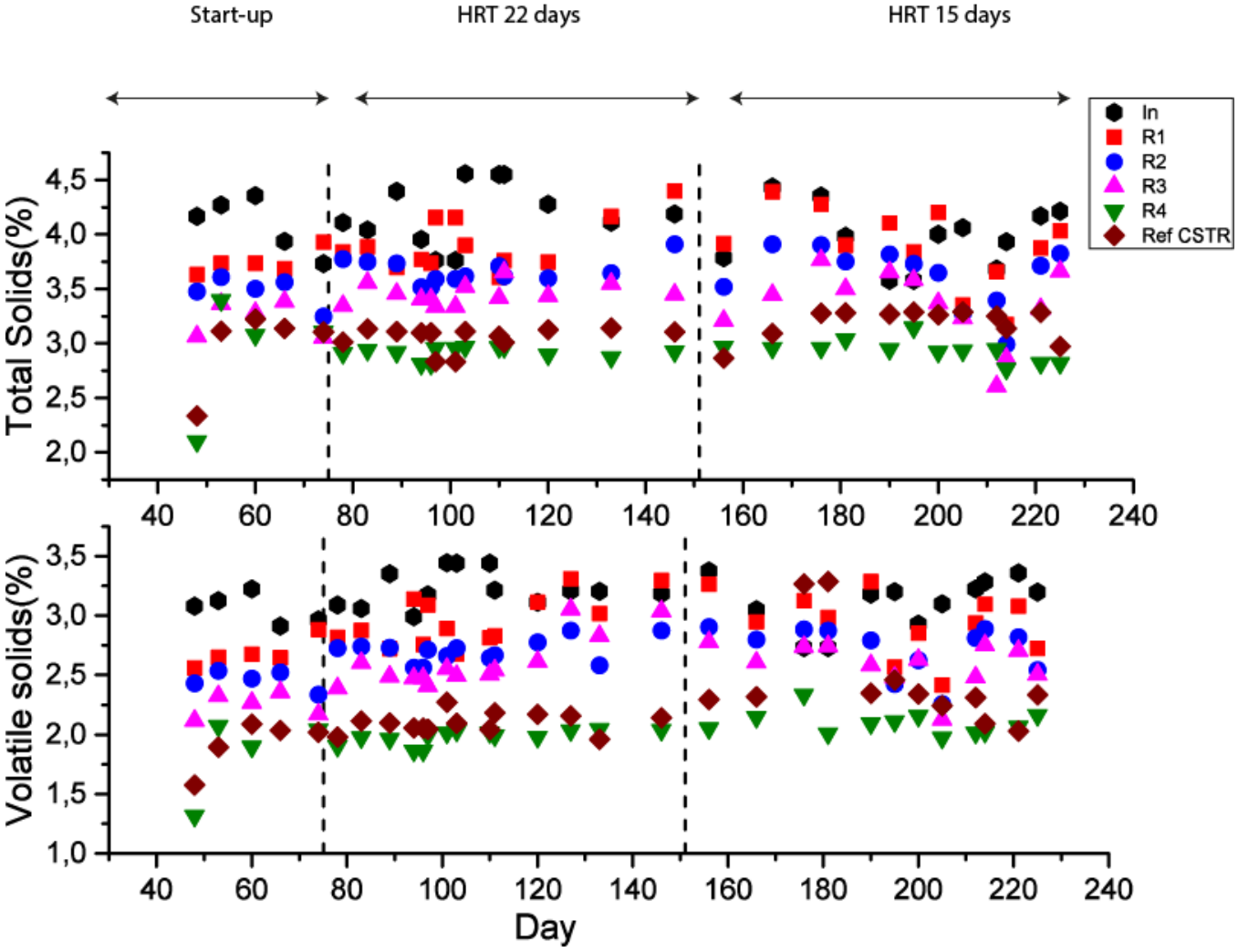


Figure 6: Total and Volatile solids in cascade AD and reference CSTR

Table 5: Performance overview of Cascade AD and reference CSTR at HRT of 22 and 15 days

	Parameters	***Cascade AD***	***Reference CSTR***
HRT 22 days	*Total COD removal (%)*	41 ± 5	31 ± 5
	Total solids removal (%)	30 ± 4	27 ± 4
	Volatile solids removal (%)	39 ± 6	35 ± 7
	Protein removal (%)	63 ± 7	54± 6
	Carbohydrate removal (%)	67 ± 2	65 ± 2
	Methane production (L/day)	6.56 ± 2.84	5.64 ± 2.62
HRT 15 days	*Total COD removal (%)*	40 ± 3	31 ± 5
	Total solids removal (%)	29 ± 4	14 ± 10
	Volatile solids removal (%)	35 ± 4	20 ± 11
	Protein removal (%)	37	38

Carbohydrate removal [1](%)	55	63
Methane production (L/day)	10.4 ± 0.9	8.9 ± 0.8

4.2 Effect of cascade AD on hydrolysis, acidogenesis and methanogenesis

The conversion rate based on COD for hydrolysis, acidogenesis and methanogenesis are shown in Table 6. During HRT of 22 days, the hydrolysis rate was seen to have been decreasing along cascade AD. The highest rate was seen among R1, R2 and R3 where higher organic loading rate had occurred. The higher rates imply that larger fraction of COD solubilization has occurred in R1, R2 and R3. The reference CSTR with lower OLR had significantly lower hydrolysis ratio. Higher hydrolysis rates were expected along cascade AD. The smaller reactor volumes enabled higher OLR. According to Michaelis Menten kinetics, higher substrate concentration would lead to higher enzyme activity. Hence, elevated hydrolysis rates were observed in cascade AD. On decreasing the hydraulic retention time to 15 days. The rate of hydrolysis increased in all the reactors as the organic loading rate increased with lowering the retention time. The highest increase in hydrolysis rate was seen in reactor 3 and reactor 4 as the biogas production in the two reactors had increased by 81% and 73% respectively. (Figure 5). It is also to be noted that as cascade AD is a plug flow with four reactors it is hard to point out the exact location of hydrolysis. It is also likely that the higher solubilization might have occurred in the previous rector. Nevertheless, it is apparent that an enhanced hydrolysis has taken place in the cascade AD in comparison to the reference CSTR.

Along with hydrolysis, acidogenesis and methanogenesis have also been higher in the smaller reactors in cascade AD. With the decrease in HRT, there was no significant increase ($p>0.05$) in VFA production in R1, R2 and R3 while a significant increase in acidogenesis rate could be observed in reactor 4 and reference CSTR of 69% and 36% respectively. Similar trend could be observed in methanogenesis as well. To conclude, cascade AD showed enhanced hydrolysis which has led to higher efficacy in the steps following anaerobic digestion as well.

Table 6: Rates of Hydrolysis, acidogenesis and methanogenesis

	gCOD/L-reactor/d					
	HRT 22 days			***HRT 15 days***		
	Hydrolysis rate	***Acidogenesis rate***	***Methanogenesis rate***	***Hydrolysis rate***	***Acidogenesis rate***	***Methanogenesis rate***
R1	*1.8*	*2.0*	*1.8*	*2.3*	*2.2*	*2.0*
R2	*1.3*	*1.4*	*1.5*	*1.9*	*1.2*	*1.3*
R3	*1.0*	*1.0*	*1.1*	*1.7*	*1.7*	*1.8*
R4	*0.6*	*0.6*	*0.6*	*1.3*	*1.0*	*1.0*
Reference CSTR	*0.6*	*0.7*	*0.7*	*1.1*	*1.0*	*1.0*

[1] The protein and carbohydrate measurements at HRT of 15 days were carried out only on Day 247.

4.2.1 Hydrolysis rate coefficient estimated from first order kinetics

The hydrolysis rate coefficient was estimated indirectly through the measurement of methane produced from the samples. The outcome from the test is presented in Appendix figure 6. The hydrolysis coefficient was calculated using curve fitting in Origin Pro. The calculated hydrolysis coefficients are provided in Appendix Table 3. It can be observed that the hydrolysis coefficient of CSTR was lower than R1, R2 and R3. Also, the hydrolysis rate coefficient was higher in the reactors with smaller volumes in comparison to R4 and CSTR.

Similar trend was observed even in the hydrolysis ratio estimated from COD. Therefore, it can be inferred that the hydrolysis of the smaller reactors improved by the occurrence of high organic loading rate.

4.3 Transformation of nitrogen, phosphorous, proteins and carbohydrates along cascade AD at HRT of 22 and 15 days

During the biodegradation of WAS, nitrogen and phosphorous were transformed from the particulate to dissolved phase. The ammonia release during AD in presented in Figure 7.. Ammonium is released from the anaerobic digestion of nitrogenous organic matter such as proteins and urea present in WAS (Kayhanian 1999). For that reason, ammonium concentration was expected to increase by AD. It can be observed that ammonium concentration in the effluent from cascade AD is higher than effluent from reference CSTR. As discussed in the previous section, cascade AD offers higher hydrolysis rates. During hydrolysis ammonium from nitrogenous organic matter is released. As expected, higher ammonia concentration could be seen in cascade AD in comparison to reference CSTR. After lowering the HRT, cascade AD showed significantly higher ammonium release with respect to reference CSTR. Moreover, the effluent concentration of ammonium from cascade AD was not altered by lowered retention time ($p>0.05$).

In cascade AD, 37% of particulate nitrogen was found to be hydrolysed while 31% was observed in reference CSTR. The concentration of particulate nitrogen was seen to decline along cascade AD. The total nitrogen balance along the reactors were seen to be maintained (Appendix figure 3). A difference in total nitrogen concentration was verified using a t test and it was observed that the difference in total nitrogen was not significant($p>0.05$).

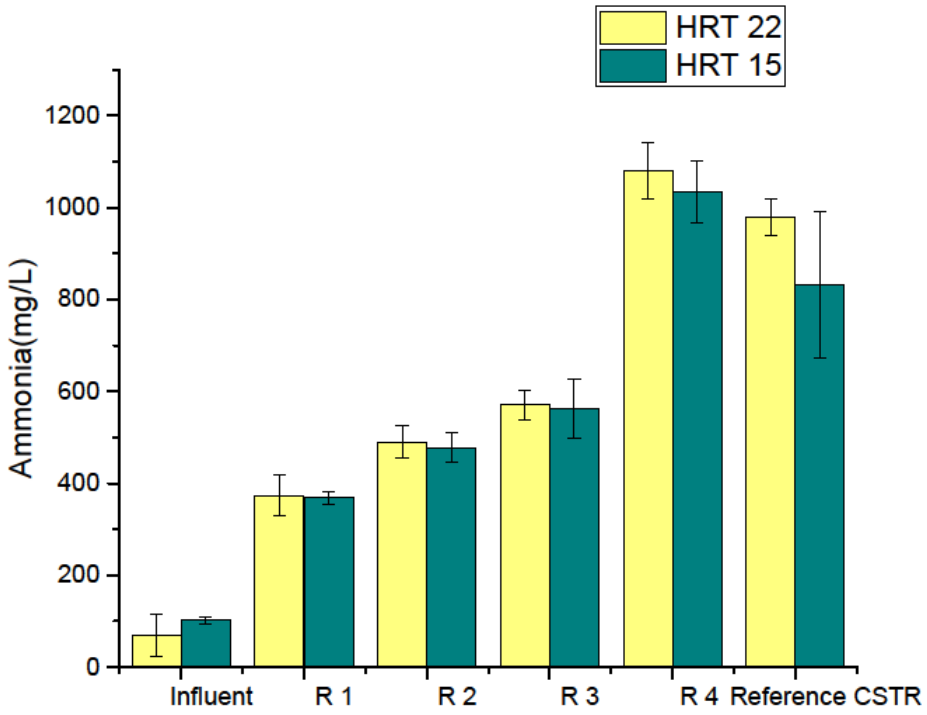


Figure 7: Release of Ammonium in cascade AD and reference CSTR at HRT 22 and 15 days

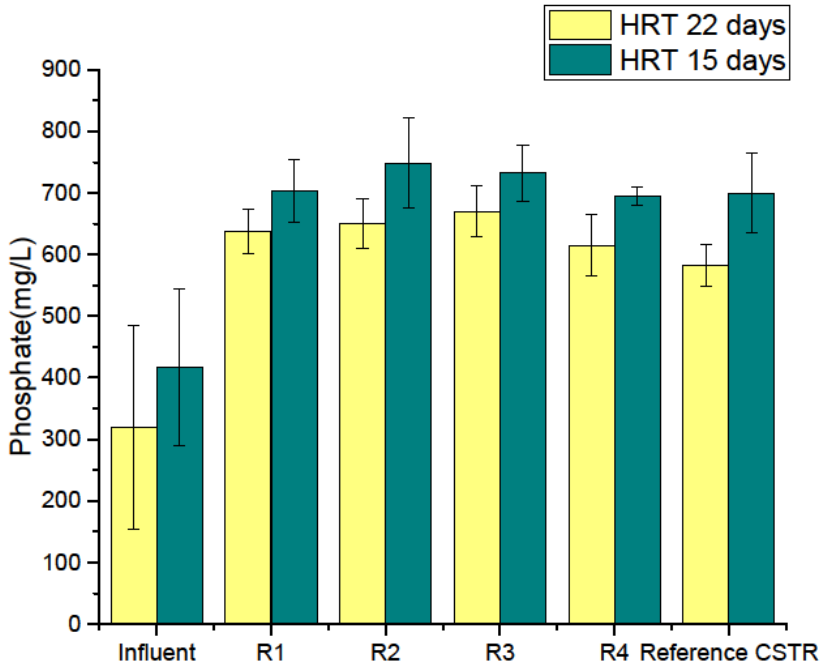


Figure 8: Phosphate release in cascade AD and reference CSTR at HRT 22 and 15 days

Phosphorous compounds present in the sludge in the form of organic and poly-phosphate. The ortho phosphate concentration was measured along the cascade AD and CSTR. The concentration of phosphate is presented in Figure 8. Phosphate release shows a similar trend in both cascade AD and reference CSTR. The PAO present in WAS are still active when it enters the reactors. So, they take up the VFAs produced in the reactors and store them as PHA by using the energy obtained from hydrolysis of polyphosphate. Therefore, a sharp increase in phosphate release was seen at the beginning of anaerobic digestion(Figure 8). The phosphate release in cascade AD and reference CSTR were similar after lowering the HRT. Therefore, an improved release of phosphate could not be observed in cascade AD. Similar trend in total phosphorous also can be seen along the cascade AD and reference CSTR at HRT of 22 days. By making the balance, it was observed that the difference between total phosphorous across the reactors was significant

The total phosphorous decreased along the cascade AD and reference CSTR. The presence of ions such as calcium, magnesium, iron, aluminium in influent could form compounds of low solubility when it combines with PO_4^{3-} . The formation of these compounds would result in the lower concentration of phosphate in the dissolved phase. The phosphate that is released may precipitate as metal phosphate like struvite ($MgNH_4PO_4 \cdot 6H_2O$). The precipitation occurs when the concentration of Mg^{2+}, NH_4^+ and PO_4^{3-} exceeds the solubility product of struvite (Marti, Bouzas et al. 2008). The solubility of struvite is also dependent on temperature and pH. To close the nitrogen and phosphorous balance, the concentration of metal ions was estimated along the reactor. The saturation index (SI) less than zero indicates that the solution is undersaturated. The SI values obtained from PhreeqC modelling are presented in Appendix Table 2. It was seen that SI value of struvite in cascade AD and reference CSTR were under saturated. However, the calcium concentration decreased along the reactor indicating possible precipitation (Appendix Table 2). Saturation index greater than 0 was seen for hydroxyapatite (HAP) ($Ca_{10}(PO_4)_6(OH)_2$). The Ca^{2+} ions present in the solution can form precipitate with ortho phosphate(Ren, Liu et al. 2008). The precipitation of HAP could explain the lower phosphate concentration along cascade AD and CSTR although current results do not indicate a significant difference in phosphate release in both the systems.

About 60% of the organic matter in the sludge is made up of proteins and carbohydrates (Neyens and Baeyens 2003). So, its degradation is critical for estimating the enhancement of anaerobic digestion. Figure 9 and Figure 10 shows the concentration of carbohydrate and protein during cascade AD and reference CSTR. At HRT of 22 days higher protein and carbohydrate removal efficiency were observed in cascade AD (Table 5). However, when the HRT was lowered cascade and protein and carbohydrate removal showed similar efficiency in both the systems. Although carbohydrate and protein removal efficiency were comparable in cascade AD and reference CSTR, COD removal in cascade AD was far more superior. The discrepancy could be verified by carrying out further measurements at the same HRT. The sampling for protein and carbohydrate analysis at HRT of 15 days was performed on Day 247. Therefore, to obtain a more representative value carbohydrate and protein concentrations needs to be estimated on multiple days. Nevertheless, it can be observed that the removal of proteins and carbohydrates have lowered at shorter HRT.

At HRT of 22 days, it was observed that the concentration of proteins decreased along cascade AD. Large fraction of protein removal had occurred in reactor 4. While in the case of carbohydrates, higher removal occurred at the beginning of cascade AD. This demonstrates that there was a delay in in protein degradation in comparison to carbohydrate. Upon lowering the retention time, a reduction in carbohydrate removal could affect removal efficiency of proteins also. Hence, it could be likely that on lowering the HRT, the protein degradation might get significantly affected. Protease is the enzyme responsible for enzymatic hydrolysis of proteins while carbohydrates are hydrolysed by cellulase. The breakdown of protein is

restricted as they require the breakdown of cell structure to be available for degradation. Faster hydrolysis of carbohydrates can also contribute to the slower degradation of proteins. Sugars formed during the hydrolysis of carbohydrate can cause repression in protease formation. (Breure, Mooijman et al. 1986). Thus, higher protein removal efficiency was observed only in R4 in cascade AD. It is likely that the degradation of carbohydrate which were part of EPS and cell structure, enabled better access of biomass to protein.

The particle size distribution in cascade AD is provided in Appendix figure 5. It could be observed that a significant difference in the particle size could not be observed for Cascade AD and reference CSTR. The WAS obtained from the WWTP contained polymer additions. The presence of polymer resisted a significant change in the structure. Therefore, only limited surface area of the substrate was available for the enzymes. Hence it is likely that WAS without the presence of polymers might show improved performance during anaerobic digestion.

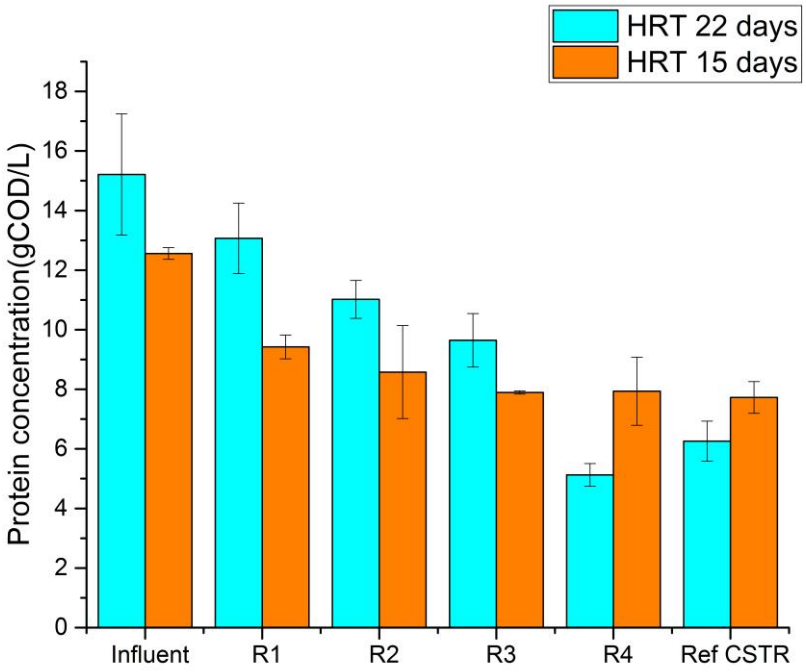


Figure 9: Protein concentration in cascade AD and reference CSTR

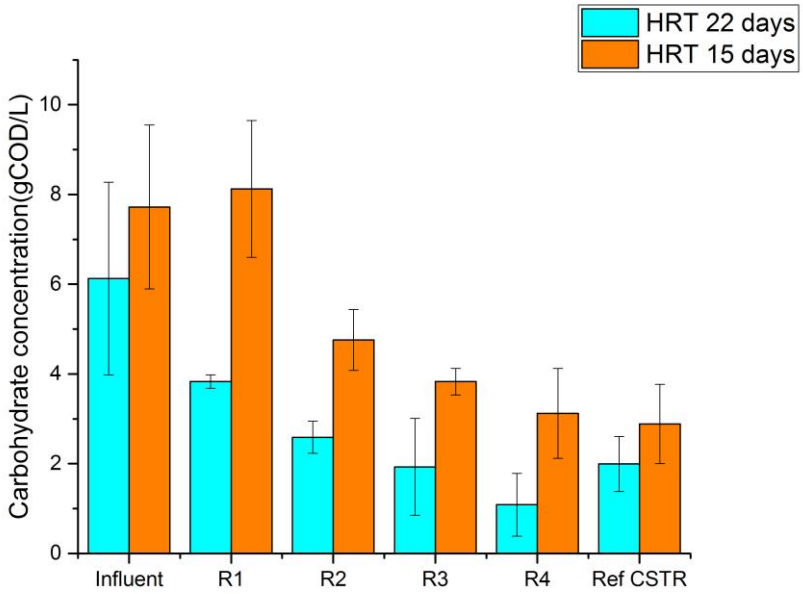


Figure 10: Carbohydrate concentration in cascade AD and reference CSTR

4.4 Degradation of structural EPS along Cascade AD system

The extracted structural EPS /100g of the sample is reported in Figure 11. As expected, the structural EPS content reduced along the subsequent steps of cascade AD system. cascade AD indicated a removal efficiency of 36% while the reference CSTR removed 26% of the structural EPS. From the previous section it was inferred that higher hydrolysis rates were occurring in cascade AD. The higher degradation of SEPS in cascade AD could be correlated to higher hydrolysis rate. As SEPS are integral in holding the aggregates together, the degradation of SEPS should be indicating higher hydrolysis.

After lowering the HRT to 15 days, the concentration of SEPS in the effluent of reference CSTR had increased significantly. Moreover, the SEPS content in cascade AD did not reduce significantly on lowering the HRT. It can also be observed that the SEPS degradation follows the same trend as protein removal. Studies have shown that proteins are a predominant content in the EPS of activated sludge (Sponza 2003). The pattern in the in SEPS removal indicates that most of SEPS degradation occurs in R4 and is therefore following the same pattern as protein removal. Thus Figure 11 suggests that the dominant content in the extracted SEPS amounts to protein. The concentration of cations, specifically calcium and magnesium were found to decrease along cascade AD and CSTR (Appendix Table 1). The cations play an integral role in maintaining the floc structure. As the concentration of cations in the bulk solution decreases due to precipitation, it is likely that more cations might leach into the bulk solution from the EPS further weakening the structure.

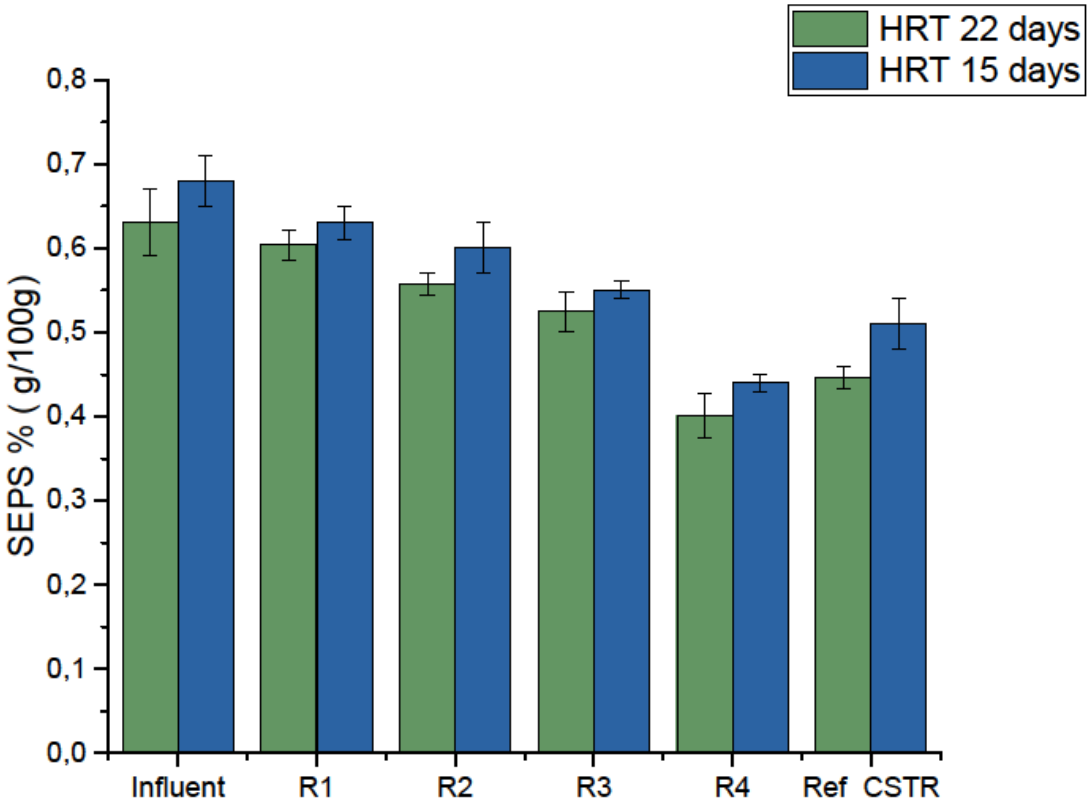


Figure 11: Degradation of structural EPS along Cascade AD and reference CSTR

4.5 Effect of three groups of enzymes on hydrolysis of waste activated sludge

The results from the addition of three groups of enzymes i.e. protease, cellulase and polygalacturonase on the hydrolysis of WAS is presented in this section. Based on the difference in the substrate used, variation was also seen in the effect of enzymes. The difference observed in the three tests were elucidated below.

The products from hydrolysis of WAS are mostly soluble monomers which were indicated by soluble COD (Appendix figure 7). In Test-1 (Table 4), the effect of protease on waste activated sludge hydrolysis was evaluated. As shown in Appendix figure 7, the soluble COD was found to decrease. After 6 hours of incubation, a marginal increase in soluble COD was seen. The addition of enzyme also contributes to the COD. So, a high initial COD was observed in the test. It was expected that the SCOD would indicate an increasing trend with incubation time as it provided more time for interaction between the enzyme and substrate. The substrate used in Test 1 was performing enhanced phosphorous removal. As a result, the phosphate removing bacteria present in substrate takes in lower fatty acids and store it as ploy-hydroxy alkenoates(PHA) during anaerobic conditions(Smolders, van der Meij et al. 1994). It can be speculated that soluble COD were used by PAO (phosphorous accumulating organisms) and GAO (glycogen accumulating organisms) present in the bio-P biomass for metabolism. Similar trend could be observed with VSS also (Appendix figure 8). It was believed that there existed an equilibrium between the breakdown of organics by enzymes and the consumption of soluble COD by PAO or GAO. However, by the end of 12-hour test, a net increase in SCOD/VSS could not be observed.

A follow up test that (Test-2 Table 4) was conducted by aerating the fresh substrate for about 6 hours also yielded similar results (Appendix figure 9). On comparing the performance of protease and cellulase, it could be seen that protease indicated a rapid decrease in soluble COD. This increase in COD indicates that VFA was taken up by the biomass for metabolism. Both the enzymes showed similar trend in COD and VSS (Appendix figure 10). However, the COD changes and VSS reduction are more pronounced for protease. Phosphate analysis did not show any significant increase in the ortho phosphate concentration on adding the enzymes.

To observe the COD solubilisation by enzymes without interference from phosphorus removing biomass, activated sludge from B-stage was used as substrate. An increase in soluble COD could be observed in Test 3. Almost double the increase in COD was observed for protease in comparison to polygalacturonase. Similarly, VSS reduction in protease was higher than polygalacturonase. VSS reduction reported by Yang, Luo et al. (2010) showed almost 39.7% for protease which is considerably higher than 13% observed in this study. However, the addition of polygalacturonase did not lead to higher VSS reduction and COD increase than blank. The lower COD solubilization by polygalacturonase in this test could also be because of the lower activity of enzyme at neutral pH (Sathiyaraj, Srinivasan et al. 2011).

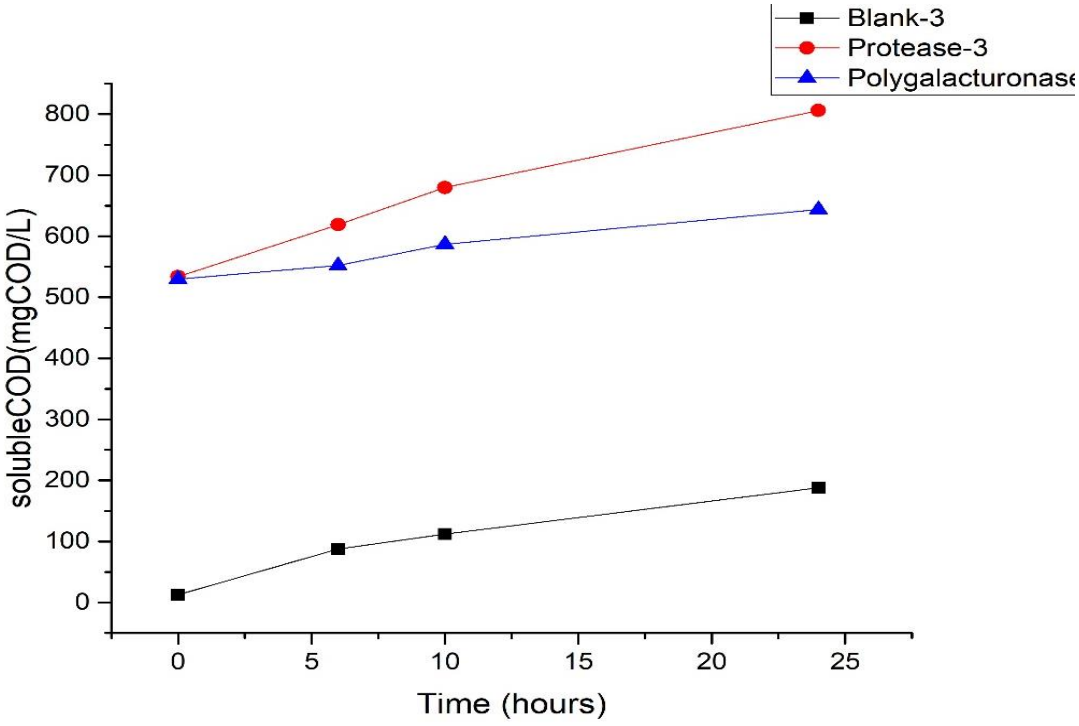


Figure 12: Soluble COD from enzyme batch -3 with B-stage non-bio-P sludge

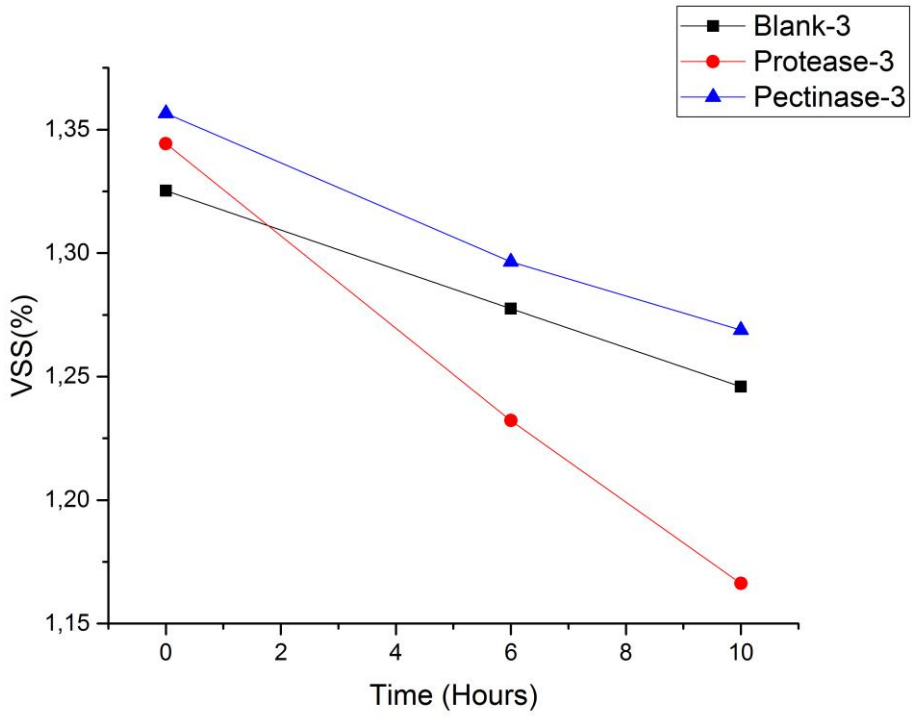


Figure 13: VSS reduction in enzyme batch test-3

Proteins are broken down into polypeptides and amino acid which in turn gets converted into organic acids. Ammonia and carbon dioxide are the end products of this reaction. So concentration of ammonia (measured as NH_4-N) can provide insight into enzymatic hydrolysis

of proteins in the substrate (Ji and Brune 2005). The concentration of ammonium after the addition of protease, cellulase and polygalacturonase was measured in the study. In test 2, the increase in NH_4-N concentration did not follow any trend (Appendix figure 12). NH_4-N production increased regardless of the enzyme used. Therefore, conclusive observations about the effect of protease and cellulase on NH_4-N concentration could not be made.

The addition of protease led to release of ammonium in test 3. Uronic acid derivatives are broken down by polygalacturonase. Uronic acid has a significant role to play in floc formation(Lü, Wang et al. 2016). From the test it can be seen that the addition of polygalacturonase did not cause a significant ammonium increase. Lü, Wang et al. (2016) obtained results that indicated no significant release of proteins were observed during addition of polygalacturonase. Although a significant release in polysaccharide was observed. However, polygalacturonase still had a key role in the floc formation of EPS. Therefore, it can be established that NH_4^+ concentration cannot be indicative of the hydrolysis occurring by polygalacturonase. From Figure 10, it could be seen that the ammonium release in blank solution was found to be higher than polygalacturonase.

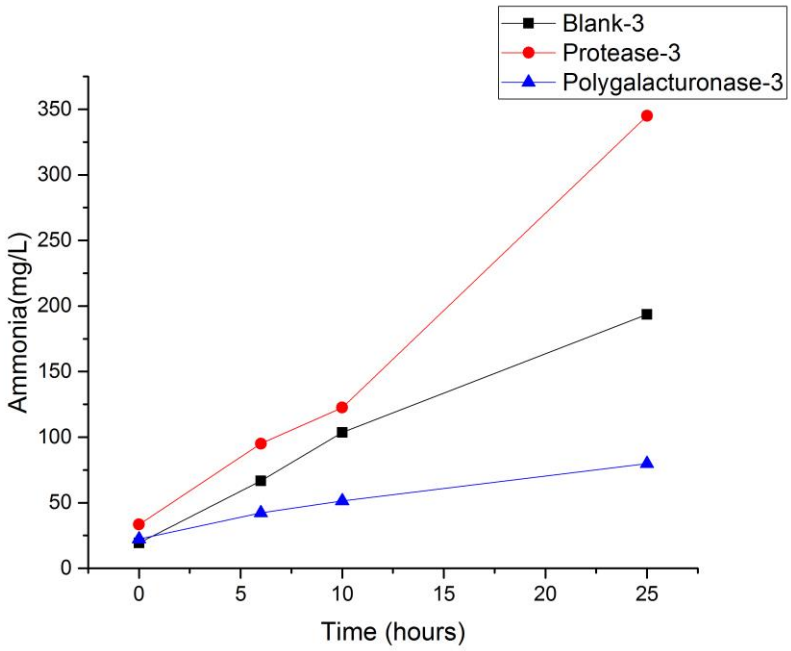


Figure 14: Ammonium release upon the addition of protease and polygalacturonase (Test-3)

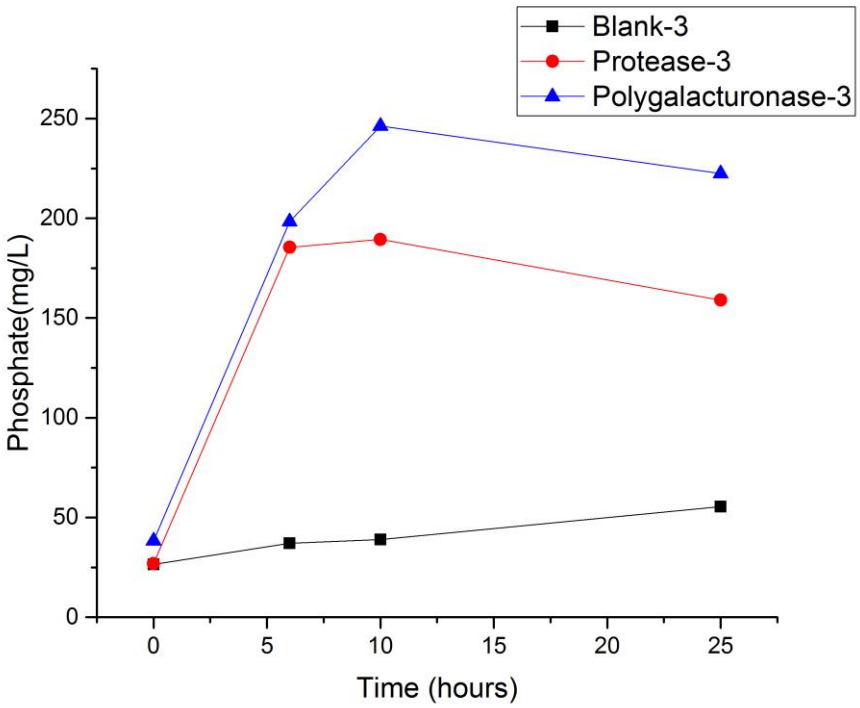


Figure 15: Phosphate release in enzyme batch test 3

The COD and solids reduction alone do not necessarily show the effect of these various enzyme groups on substrate. The sample extracted for soluble COD estimation must pass through a filter of pore size 0.45µm. All the particulate organics that were broken down by the enzymes need to necessarily get broken down to a size lower than pore size. Therefore, the measurement could also provide an underestimation of the effect of respective enzyme groups on waste activated sludge. Therefore, the variation in particle size distribution before and after the addition of enzymes were investigated.

The change in particle size has a substantial impact on the reaction rate and mass transfer in enzymatic hydrolysis. In test 1, a significant change in particle size distribution could not be observed (Appendix figure 13). The secondary sludge used had polymer additions in the earlier stages of treatment at WWTP. The polymers might have significantly reduced the accessibility of the enzymes to the substrate. It can be seen from Appendix figure 14 (particle size distribution) that addition of protease (in Test-3) did not cause a considerable shift in size distribution. Similar results were also observed by Lü, Wang et al. (2016) where significant dissolution of protein was not observed after the addition of protease. Polygalacturonase also did not cause a considerable shift in the particle size distribution. From the analysis from the two tests, particle size distribution did not show any significant change after enzyme addition. Therefore, it can be presumed that the enzymes used in the tests might not have caused a significant difference to the sludge structure. According to the results observed in this test, particle size distribution alone could not be an indicator of the hydrolysis. To visualize the complete picture, surface morphology analysis is also required.

The study indicated that release of SCOD ammonium and phosphate showed different trend for substrate that consisted PAO. This preliminary study also shows the likelihood of difference in enzymatic hydrolysis in cascade AD if non-phosphorous removing biomass would be used.

Chapter 5

Conclusions

The conclusion drawn by each category are presented below, followed by recommendations for future research. The research questions defined at the beginning of this report are briefly answered below.

1. *To what extent that the performance of cascade anaerobic WAS system differs from that in conventional CSTR under shortened HRT?*

In this study the performance of anaerobic digestion using a novel Cascade AD system was studied under lowered retention time. Various indicators were used to understand the performance enhancement of a cascade AD in comparison to a conventional CSTR. The overall performance of the cascade AD system was better than conventional CSTR at retention time of 22 and 15 days. COD removal and solids reduction in the cascade AD was significantly higher than reference CSTR. Moreover, the novel design of cascade AD was seen to maintain the performance at lower HRT also. Owing to the higher OLR provided by the lower reactor volumes in cascade AD, higher hydrolysis was observed in cascade AD. The hydrolysis rates decreased along the cascade AD and the hydrolysis rate stayed higher in cascade in comparison to conventional CSTR. Along with the improved hydrolysis, an improvement in acidogenesis and methanogenesis was feasible using cascade AD. Therefore, the cascade AD enhances the entire process of anaerobic digestion by improving hydrolysis.

Higher ammonia release, protein degradation and carbohydrate degradation could be observed in cascade AD. Higher carbohydrate removal was observed in R1, R2 and R3 but higher protein removal was observed in R4. The higher carbohydrate degradation must have improved accessibility to protein. The results in protein and carbohydrate removal was corroborated by SEPS degradation in cascade AD. Enhanced removal of structural EPS could be observed in cascade AD in comparison to CSTR. Nitrogen mass balance was maintained in both in cascade AD and reference CSTR. However, phosphorous mass balance indicated loss along cascade AD and CSTR. Moreover, the analysis of concentration of cations in the reactor systems indicated possibilities of precipitation as metal phosphates.

In conclusion the novel cascade AD system showed superior performance than a CSTR despite the lower retention time. The design of cascade AD enabled higher organic loading rate that promoted hydrolysis. Thus, the stable performance suggests the opportunities to further lower the retention time.

2. How would the target enzyme groups influence the hydrolysis of structural EPS?

The batch test was aimed at understanding the degradation of structural EPS of waste activated sludge after the addition of enzymes protease, cellulase and polygalacturonase. The hydrolysis kinetics was planned to be derived from COD and VSS to indicate the solubilization Although VSS also indicated similar trend in COD, it showed lesser variation. Therefore, VSS was inferred to be better suited for deriving the kinetics. The use of ammonium as an indicator for hydrolysis could be applied based on the enzyme used. The enzyme with the most significant role in hydrolysis of SEPS could not be derived from the test. In comparison to polygalacturonase, protease showed higher VSS reduction. The performance of protease against cellulase did not yield conclusive results in the study. Besides, the type of substrate used, which is bio P and activated sludge had a significant impact in deriving the kinetics. Although protease indicated marginally better solubilization, the solubilization by all three enzyme groups were significantly lower than previous research. The particle size distribution did not indicate a significant change upon the addition of enzymes protease and polygalacturonase. Therefore, it was inferred that the addition of aforementioned enzyme groups did not cause a significant change in the structure.

5.1 Recommendations:

The following the recommendations could be made based on this research:

- The study from cascade AD indicated that there is higher reduction of proteins and carbohydrates due to higher hydrolysis rate. In order to obtain a complete picture of the enhanced performance result from enzyme activity of protease and cellulase should be compared with the results from protein and carbohydrate analysis.
- There was a significant difference in trend for enzymatic hydrolysis results obtained between bio-P and non-bio-P sludge. Further analysis is required in sludge morphology to assess the difference occurring in the two types of substrates after enzyme addition.
- The solubilization caused by enzymes were significantly lower in this study. Use of enzymes with higher activity (units/mL) might provide better results.
- The estimation of kinetics using SCOD as an indicator needs to be verified further. The COD measurements from the enzymes showed substantial deviation in measurements. The likelihood of formation of various complexes with enzymes and composition of the enzyme needs to be investigated further before considering it as a parameter to derive hydrolysis kinetics.
- Use of multiple enzymes were shown to improve hydrolysis because of its synergistic effects. The synergistic effect of the enzymes on structural EPS needs to be explored further.

References

Afvalwater, S. T. O. R. (1985). Optimalisatie van de gistingsgasproduktie, Stora.

Angelidaki, I., M. Alves, D. Bolzonella, L. Borzacconi, J. L. Campos, A. J. Guwy, S. Kalyuzhnyi, P. Jenicek and J. B. van Lier (2009). "Defining the biomethane potential (BMP) of solid organic wastes and energy crops: a proposed protocol for batch assays." Water Science & Technology **59**(5): 927-927.

Appels, L., J. Baeyens, J. Degrève and R. Dewil (2008). "Principles and potential of the anaerobic digestion of waste-activated sludge." Progress in Energy and Combustion Science **34**(6): 755-781.

Bolzonella, D., P. Pavan, P. Battistoni and F. Cecchi (2005). "Mesophilic anaerobic digestion of waste activated sludge: influence of the solid retention time in the wastewater treatment process." Process Biochemistry **40**(3): 1453-1460.

Bougrier, C., C. Albasi, J. P. Delgenès and H. Carrère (2006). "Effect of ultrasonic, thermal and ozone pre-treatments on waste activated sludge solubilisation and anaerobic biodegradability." Chemical Engineering and Processing: Process Intensification **45**(8): 711-718.

Breure, A. M., K. A. Mooijman and J. G. van Andel (1986). "Protein degradation in anaerobic digestion: influence of volatile fatty acids and carbohydrates on hydrolysis and acidogenic fermentation of gelatin." Applied Microbiology and Biotechnology **24**(5): 426-431.

Capela, I., M. J. Bilé, F. Silva, H. Nadais, A. Prates and L. Arroja (2009). "Hydrodynamic behaviour of a full-scale anaerobic contact reactor using residence time distribution technique." Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology **84**(5): 716-724.

Cornish-Bowden, A. (2013). "The origins of enzyme kinetics." FEBS Letters **587**(17): 2725-2730.

deGooijer, C. D., W. A. M. Bakker, H. H. Beeftink and J. Tramper (1996). "Bioreactors in series: An overview of design procedures and practical applications." Enzyme and Microbial Technology **18**(3): 202-219.

Eriksson, T., J. Börjesson and F. Tjerneld (2002). "Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose." Enzyme and Microbial Technology **31**(3): 353-364.

F. Bennett, G. (2007). I.S. Turovskiy and P.K. Mathai, Wastewater Sludge Processing, Wiley–Interscience, Hoboken, NJ (2006) ISBN 0-471-70054-1 Price US$ 74.95, 366 pp.

Felz, S., S. Al-Zuhairy, O. A. Aarstad, M. C. M. van Loosdrecht and Y. M. Lin (2016). "Extraction of Structural Extracellular Polymeric Substances from Aerobic Granular Sludge." Journal of visualized experiments : JoVE(115).

Flemming, H.-C. and J. Wingender (2001). "Relevance of microbial extracellular polymeric substances (EPSs)-Part II: Technical aspects." Water Science and Technology **43**(6): 9-16.

Gerardi, M. H. (2003). The microbiology of anaerobic digesters, John Wiley & Sons.

Hwang, M. H., N. J. Jang, S. H. Hyun and I. S. Kim (2004). "Anaerobic bio-hydrogen production from ethanol fermentation: the role of pH." Journal of Biotechnology **111**(3): 297-309.

Jain, S., S. Jain, I. T. Wolf, J. Lee and Y. W. Tong (2015). "A comprehensive review on operating parameters and different pretreatment methodologies for anaerobic digestion of municipal solid waste." Renewable and Sustainable Energy Reviews **52**: 142-154.

Ji, R. and A. Brune (2005). "Digestion of peptidic residues in humic substances by an alkali-stable and humic-acid-tolerant proteolytic activity in the gut of soil-feeding termites." Soil Biology and Biochemistry **37**(9): 1648-1655.

Kavitha, S., S. Adish Kumar, K. N. Yogalakshmi, S. Kaliappan and J. Rajesh Banu (2013). "Effect of enzyme secreting bacterial pretreatment on enhancement of aerobic digestion potential of waste activated sludge interceded through EDTA." Bioresour Technol **150**: 210-219.

Kavitha, S., C. Jayashree, S. Adish Kumar, I. T. Yeom and J. Rajesh Banu (2014). "The enhancement of anaerobic biodegradability of waste activated sludge by surfactant mediated biological pretreatment." Bioresource Technology **168**: 159-166.

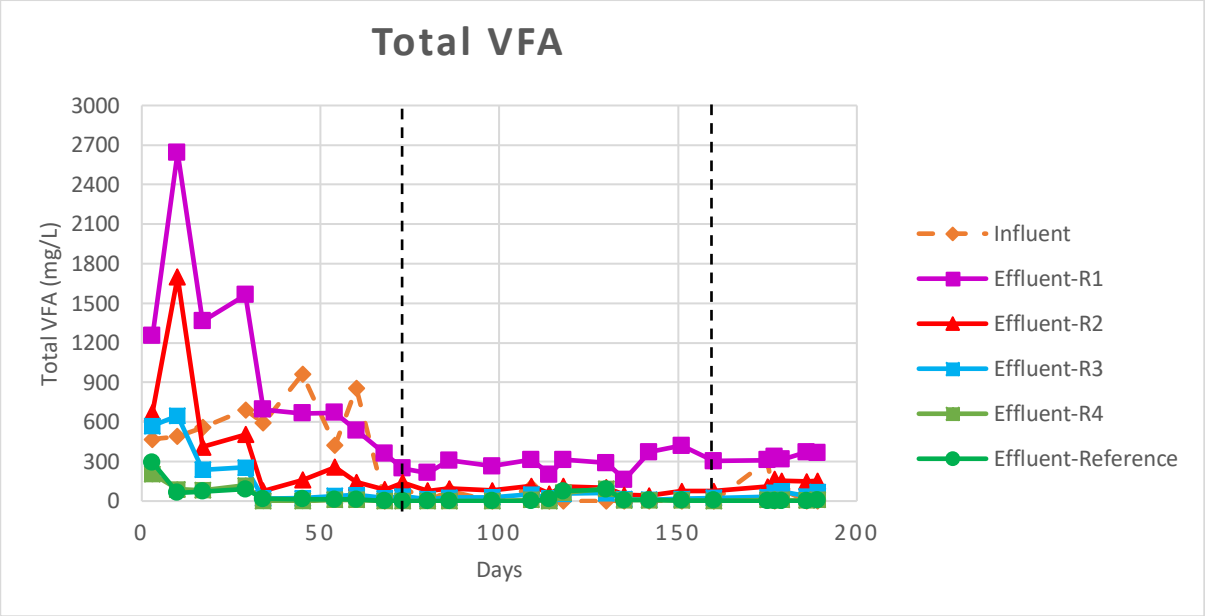
Kayhanian, M. (1999). "Ammonia inhibition in high-solids biogasification: an overview and practical solutions." Environmental Technology **20**(4): 355-365.

Kim, H. W., J. Y. Nam, S. T. Kang, D. H. Kim, K. W. Jung and H. S. Shin (2012). "Hydrolytic activities of extracellular enzymes in thermophilic and mesophilic anaerobic sequencing-batch reactors treating organic fractions of municipal solid wastes." Bioresource Technology **110**: 130-134.
Lin, Y., M. de Kreuk, M. C. M. van Loosdrecht and A. Adin (2010). "Characterization of alginate-like exopolysaccharides isolated from aerobic granular sludge in pilot-plant." Water Research **44**(11): 3355-3364.
Lin, Y. M., P. K. Sharma and M. C. M. van Loosdrecht (2013). "The chemical and mechanical differences between alginate-like exopolysaccharides isolated from aerobic flocculent sludge and aerobic granular sludge." Water Research **47**(1): 57-65.
Lü, F., J. Wang, L. Shao and P. He (2016). "Enzyme disintegration with spatial resolution reveals different distributions of sludge extracellular polymer substances." Biotechnology for Biofuels **9**(1): 29-29.
Marti, N., A. Bouzas, A. Seco and J. Ferrer (2008). "Struvite precipitation assessment in anaerobic digestion processes." Chemical Engineering Journal **141**(1): 67-74.
Metcalf and I. Eddy (2003). Wastewater engineering : treatment and reuse, Fourth edition / revised by George Tchobanoglous, Franklin L. Burton, H. David Stensel. Boston : McGraw-Hill, [2003] ©2003.
Neyens, E. and J. Baeyens (2003). "A review of thermal sludge pre-treatment processes to improve dewaterability." Journal of Hazardous Materials **98**(1): 51-67.
Nielsen, P. H. and A. Jahn (1999). Extraction of EPS. Microbial extracellular polymeric substances, Springer**:** 49-72.
Parawira, W. (2012). "Enzyme research and applications in biotechnological intensification of biogas production." Critical Reviews in Biotechnology **32**(2): 172-186.
Pei, H. Y., W. R. Hu and Q. H. Liu (2010). "Effect of protease and cellulase on the characteristic of activated sludge." J Hazard Mater **178**(1-3): 397-403.
Ren, T.-T., L. Liu, G.-P. Sheng, X.-W. Liu, H.-Q. Yu, M.-C. Zhang and J.-R. Zhu (2008). "Calcium spatial distribution in aerobic granules and its effects on granule structure, strength and bioactivity." Water Research **42**(13): 3343-3352.
Rittmann, B. E. and P. L. McCarty (2012). Environmental biotechnology: principles and applications, Tata McGraw-Hill Education.
Salama, Y., M. Chennaoui, A. Sylla, M. Mountadar, M. Rihani and O. Assobhei (2016). "Characterization, structure, and function of extracellular polymeric substances (EPS) of microbial biofilm in biological wastewater treatment systems: a review." Desalination and Water Treatment **57**(35): 16220-16237.
Sathiyaraj, G., S. Srinivasan, H.-B. Kim, S. Subramaniyam, O. Ran Lee, Y.-J. Kim and D.-C. Yang (2011). Screening and optimization of pectin lyase and polygalacturonase activity from ginseng pathogen Cylindrocarpon Destructans.
Shafee, T. (2015). Saturation curve for an enzyme reaction showing the relation between the substrate concentration and reaction rate. Wikimedia Commons. M. M. c. 2. Wikimedia Commons, Wikimedia.org.
Sheng, G.-P., H.-Q. Yu and X.-Y. Li (2010). "Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review." Biotechnology Advances **28**(6): 882-894.
Shi, Y., J. Huang, G. Zeng, Y. Gu, Y. Chen, Y. Hu, B. Tang, J. Zhou, Y. Yang and L. Shi (2017). "Exploiting extracellular polymeric substances (EPS) controlling strategies for performance enhancement of biological wastewater treatments: An overview." Chemosphere **180**: 396-411.
Smolders, G. J. F., J. van der Meij, M. C. M. van Loosdrecht and J. J. Heijnen (1994). "Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence." Biotechnology and Bioengineering **43**(6): 461-470.
Sponza, D. T. (2003). "Investigation of extracellular polymer substances (EPS) and physicochemical properties of different activated sludge flocs under steady-state conditions." Enzyme and Microbial Technology **32**(3): 375-385.
van Haandel, A. C. (1994). "Influence of the digested cod concentration on the alkalinity requirement in anaerobic digesters." Water Science and Technology **30**(8): 23-34.
Wawrzynczyk, J., M. Recktenwald, O. Norrlöw and E. S. Dey (2007). "Solubilisation of sludge by combined chemical and enzymatic treatment." African Journal of Biotechnology **6**(17).
Wu, L.-J., Y. Qin, T. Hojo and Y.-Y. Li (2015). "Upgrading of anaerobic digestion of waste activated sludge by temperature-phased process with recycle." Energy **87**: 381-389.
Yang, Q., K. Luo, X.-m. Li, D.-b. Wang, W. Zheng, G.-m. Zeng and J.-j. Liu (2010). "Enhanced efficiency of biological excess sludge hydrolysis under anaerobic digestion by additional enzymes." Bioresource Technology **101**(9): 2924-2930.

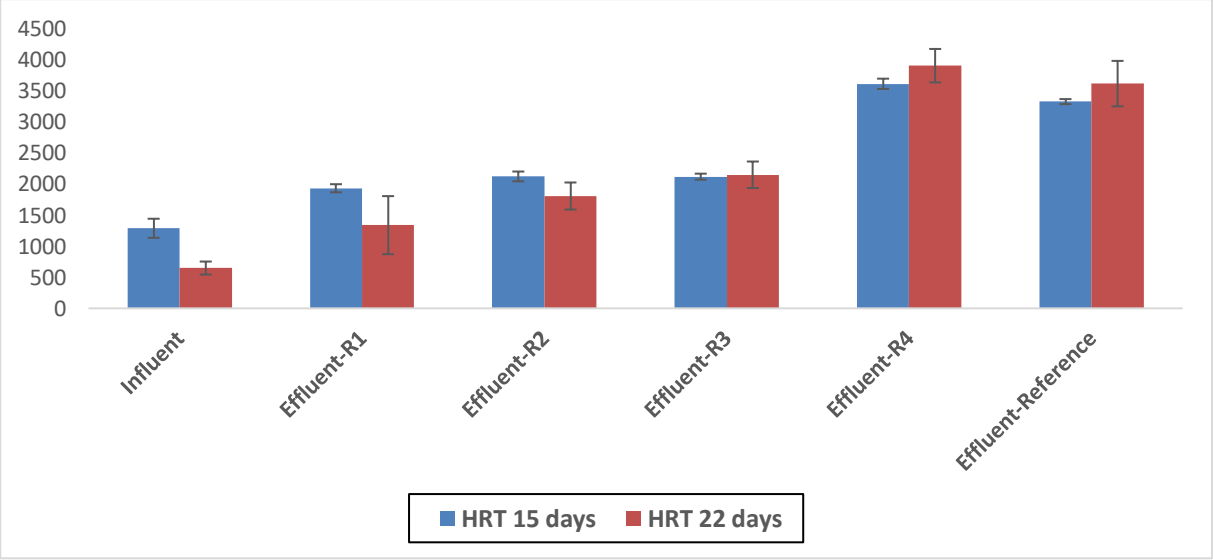
Yu, H., Z. Wang, Z. Wu and C. Zhu (2016). "Enhanced waste activated sludge digestion using a submerged anaerobic dynamic membrane bioreactor: performance, sludge characteristics and microbial community." Sci Rep **6**: 20111.
Zhang, X., J. Hu, H. Spanjers and J. B. van Lier (2014). "Performance of inorganic coagulants in treatment of backwash waters from a brackish aquaculture recirculation system and digestibility of salty sludge." Aquacultural Engineering **61**: 9-16.

Appendix

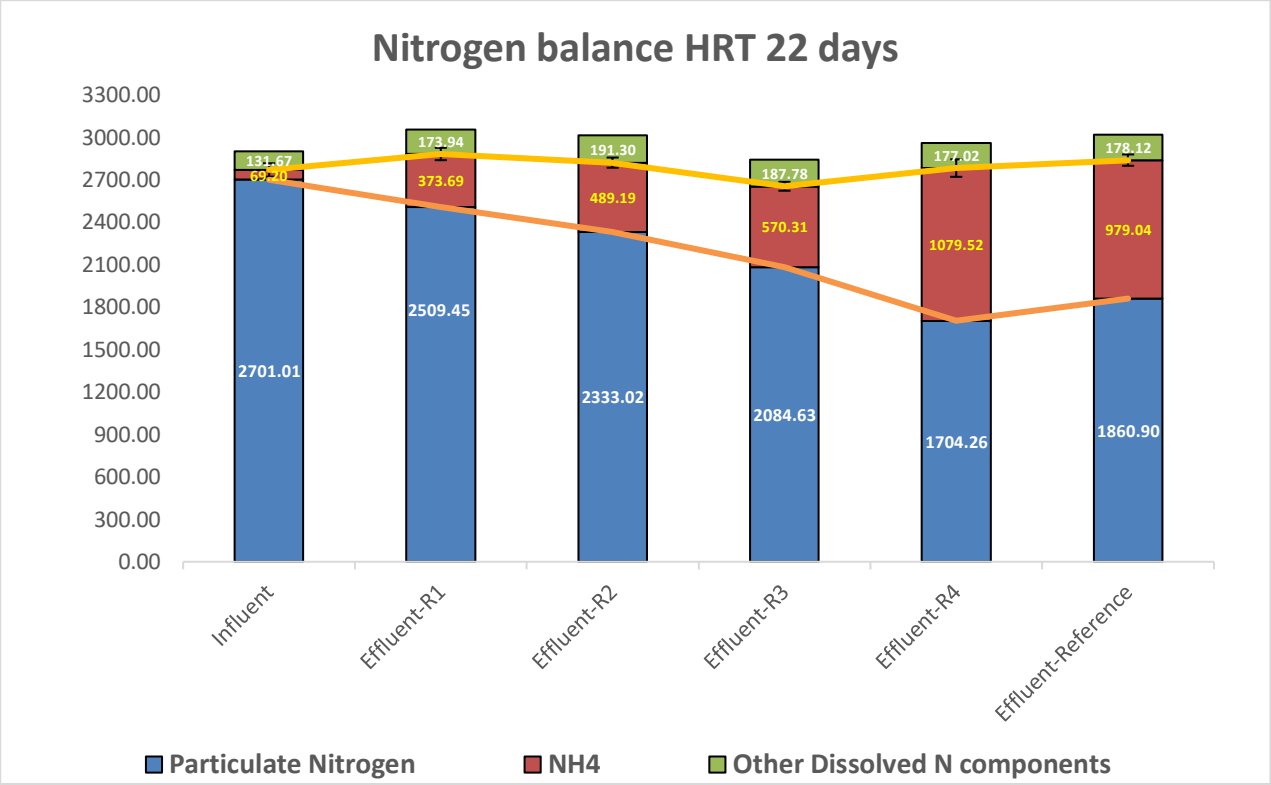
A. Performance of cascade AD and reference CSTR at HRT of 22 and 15 days



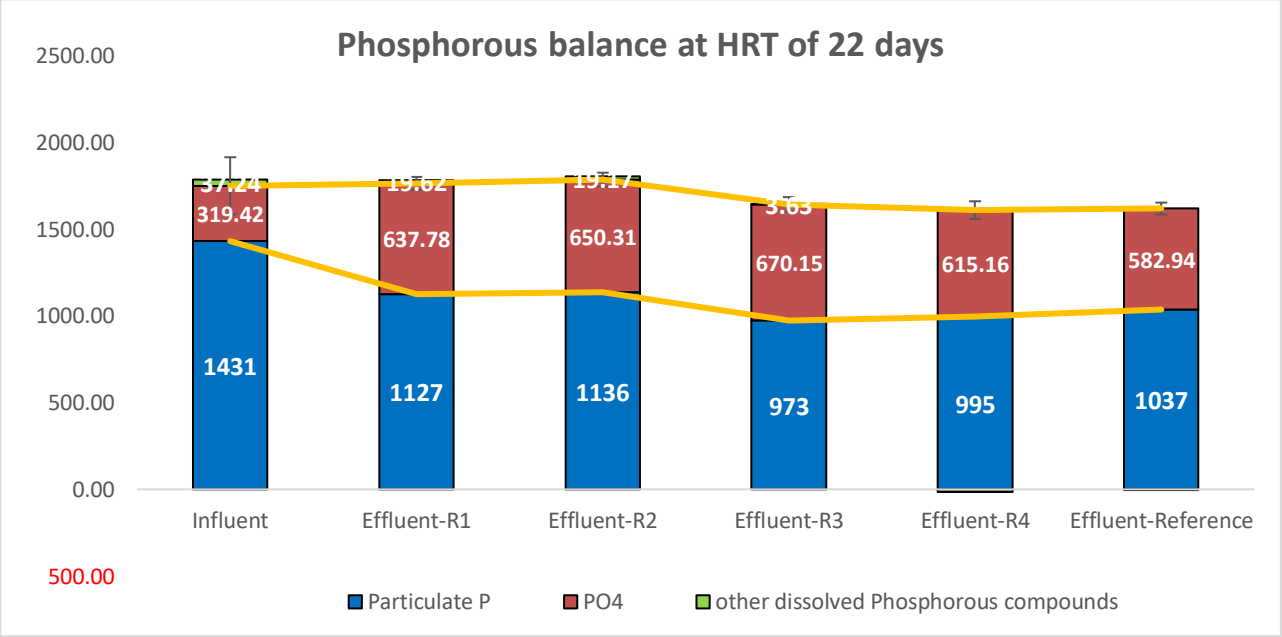
Appendix figure 1: VFA production in cascade AD and reference CSTR



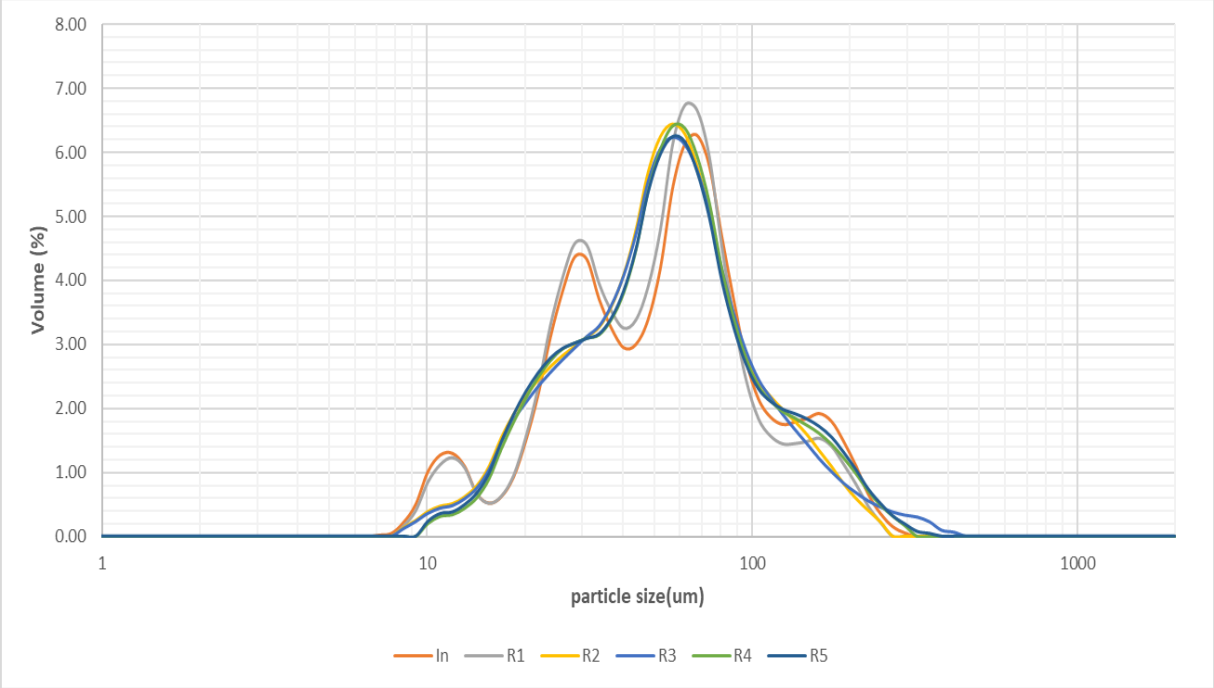
Appendix figure 2: Alkalinity in cascade AD and reference CSTR



Appendix figure 3: Nitrogen balance at HRT of 22 days



Appendix figure 4: Phosphorous balance at HRT 22 days



Appendix figure 5:Particle size distribution of cascade AD and reference CSTR at HRT of 22 days

Appendix Table 1: Concentration of cations obtained from ICP-MS at HRT of 15 days

	Sodium (mg/L)	***Magnesium (mg/L)***	***Potassium (mg/L)***	***Calcium (mg/L)***
In	97.6	121.6	389.7	94.8
R1	97.0	32.6	518.3	53.8
R2	96.4	7.5	545.0	41.2
R3	93.9	13.0	543.1	37.5
R4	98.7	7.3	616.4	21.5
Ref CSTR	92.1	2.9	562.4	19.6

Appendix Table 2: Saturation index at HRT= 15 days. Results obtained from Phreeqc modelling

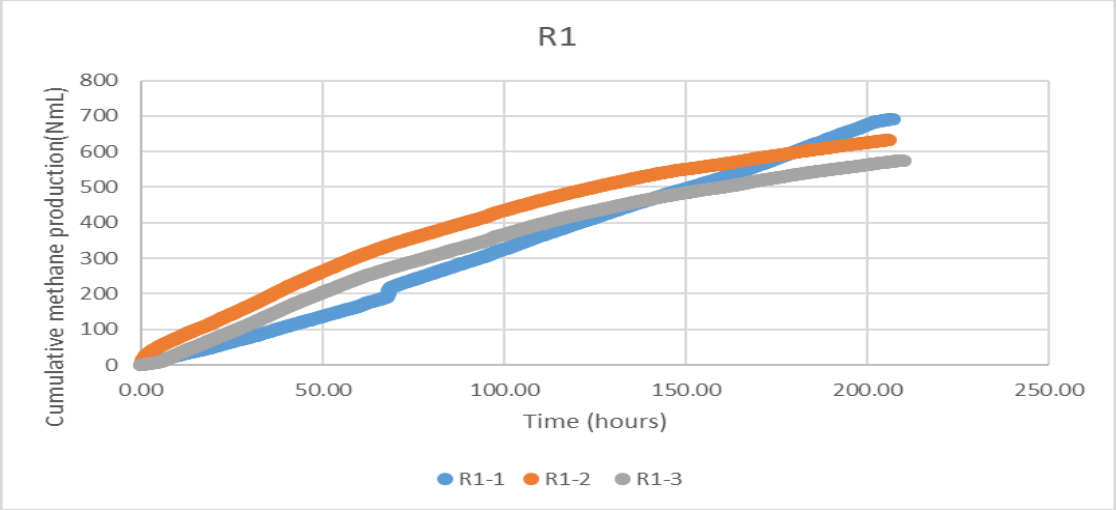
	R1	***R2***	***R3***	***R4***	***Reference CSTR***
Struvite	*-0.58*	*-0.98*	*-0.59*	*-0.13*	*-0.59*
Hydroxyapatite	*5.5*	*5.44*	*5.44*	*5.77*	*5.33*

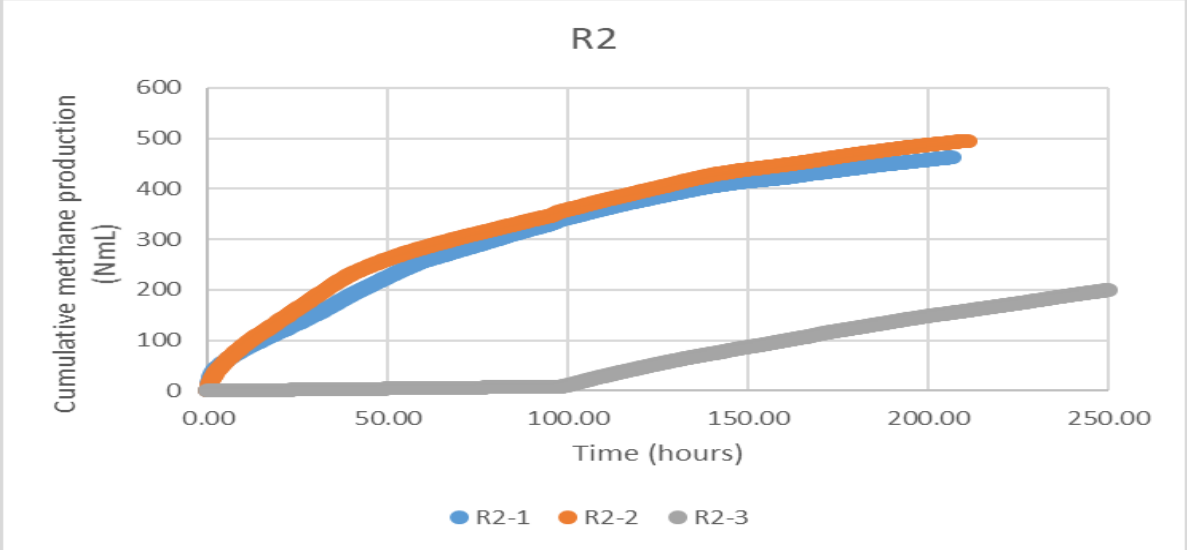
Appendix Table 3: Hydrolysis rate coefficient estimated from batch test

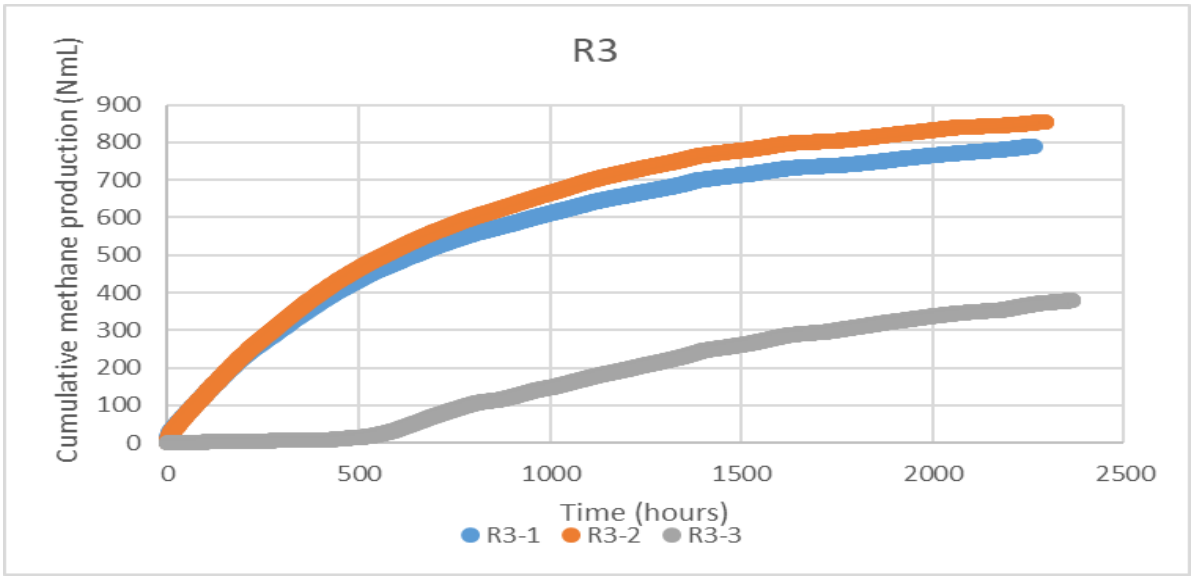
	Hydrolysis rate coefficient (d^{-1})
R1	*0.17±0.05*
R2	*0.30±0.03*

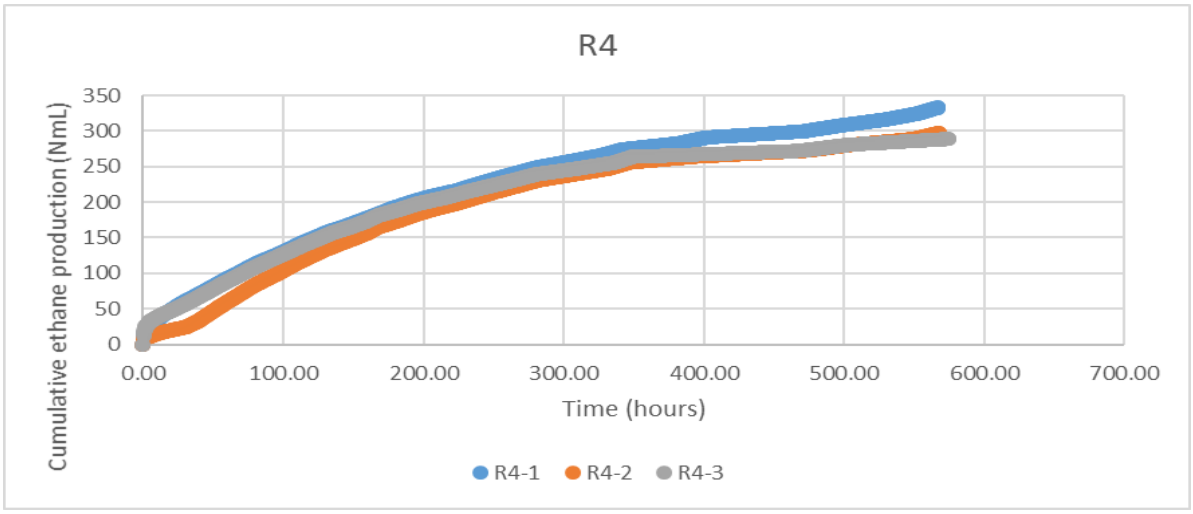
R3	*0.15±0.00*
R4	*0.12±0.02*
Reference CSTR	*0.13±0.06*

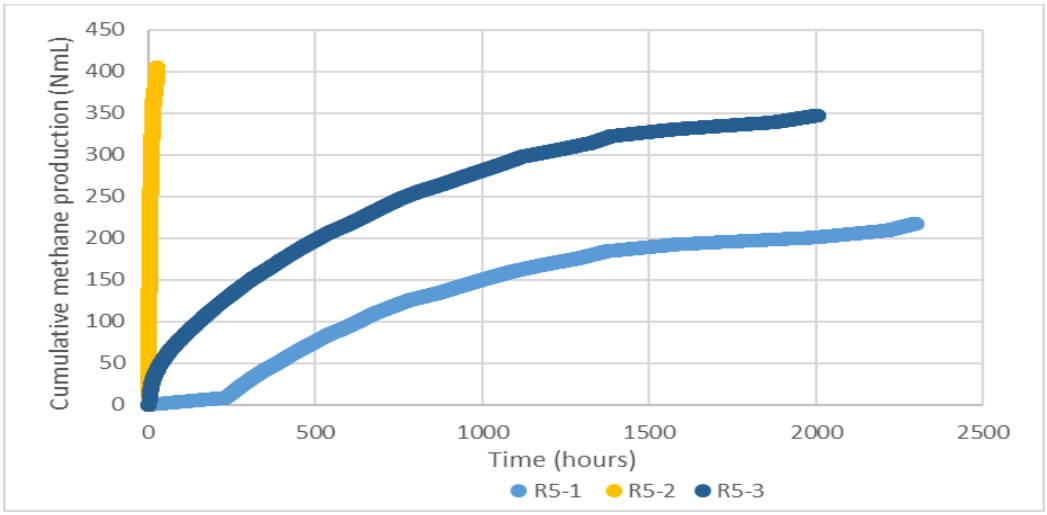
Methane production from batch test to estimate hydrolysis coefficient











Appendix figure 6 : Methane production from batch test

B. Appendix B: Batch test to investigate the effect of chosen enzymes on the hydrolysis of waste activated sludge: development of method

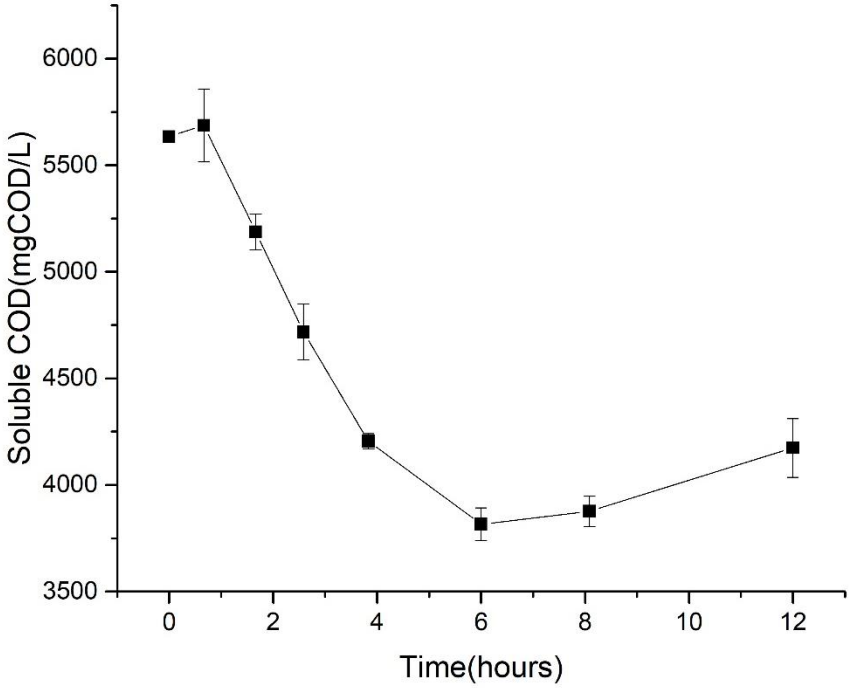
Data regarding the batch tests with the enzymes, protease, cellulase and polygalacturonase are provided here

Appendix Table 4: pH for enzyme batch test-2

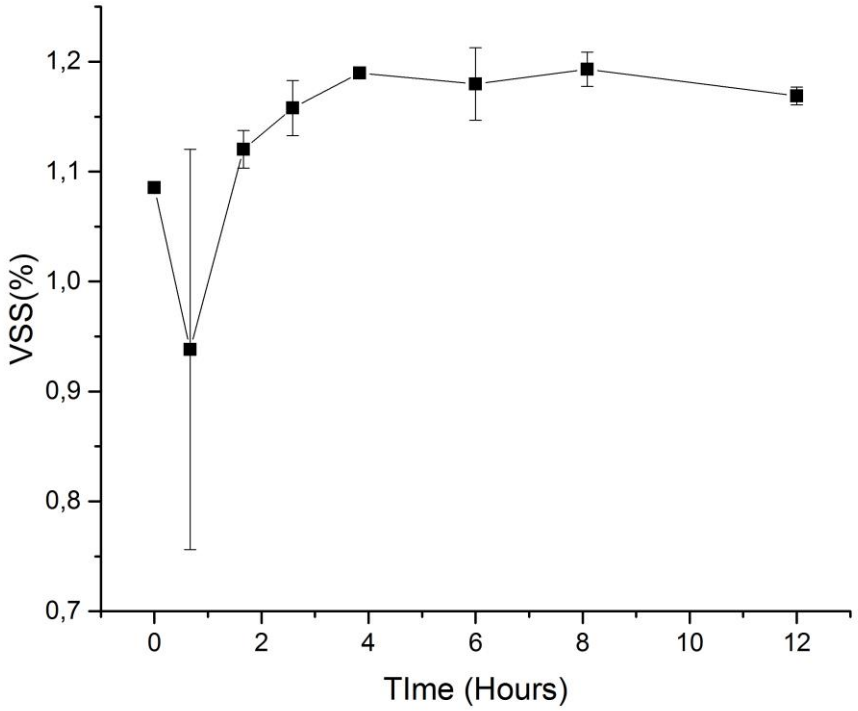
Enzyme Batch Test-2			
pH	***Before enzyme addition***	***After enzyme addition***	***t=25***
Protease	*6.772*	*6.747*	*5.399*
Protease aerated	*7.621*	*7.145*	*5.433*
Cellulase	*6.774*	*6.818*	*5.184*
Cellulase aerated	*7.619*	*7.56*	*5.133*
Anaerobic	*-*	*-*	*6.421*
Aerated	*-*	*-*	*6.363*

Appendix Table 5:pH for enzyme batch test 3

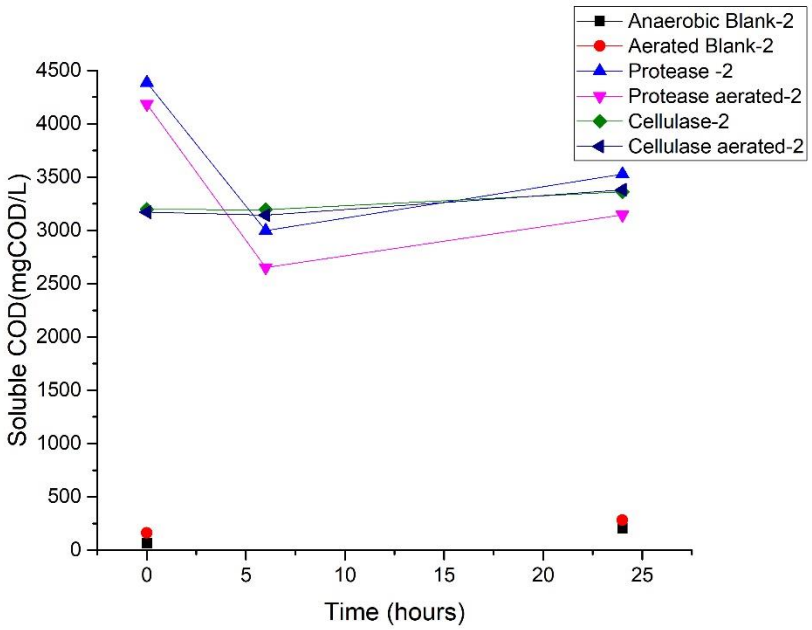
Enzyme Test-3					
	Before enzyme addition	***After enzyme addition***	***t=6 hours***	***t=10 hours***	***t=26 hours***
Blank	*6.954*		*6.852*	*6.797*	*6.724*
Protease	*6.958*	*6.893*	*4.956*	*5.224*	*5.791*
Pectinase	*6.957*	*6.875*	*4.686*	*4.436*	*5.206*



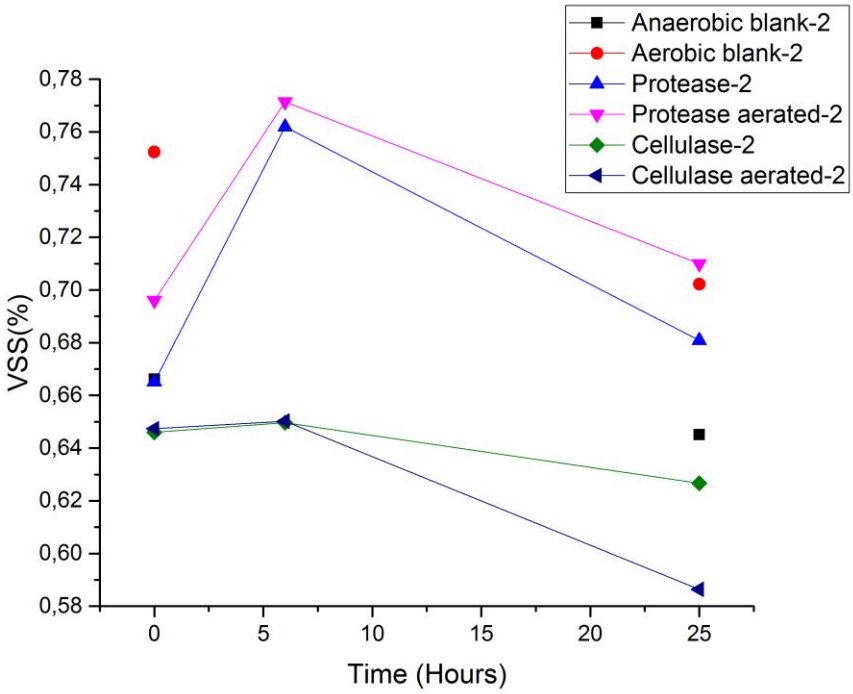
Appendix figure 7: Soluble COD increase in Enzyme Batch Test-1 with sludge from secondary sedimentation tank



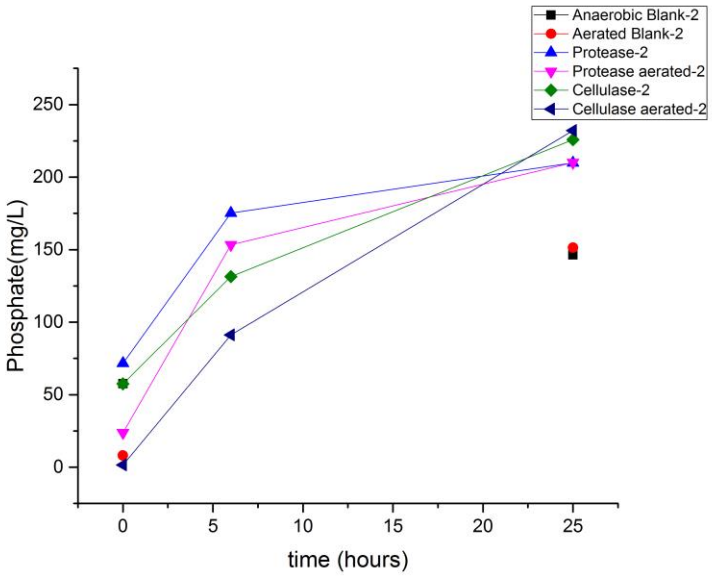
Appendix figure 8: VSS in Enzyme Batch Test-1 with sludge from secondary sedimentation tank



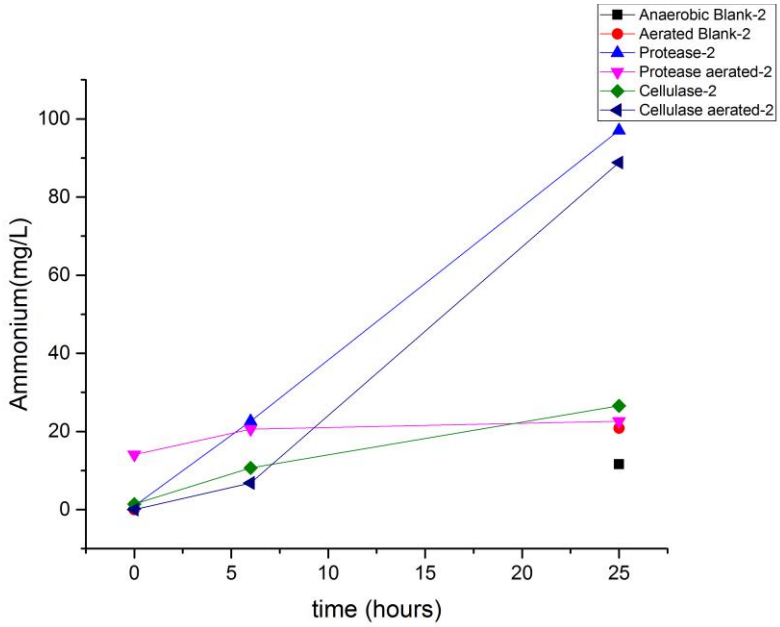
Appendix figure 9: Soluble COD in enzyme batch test-2



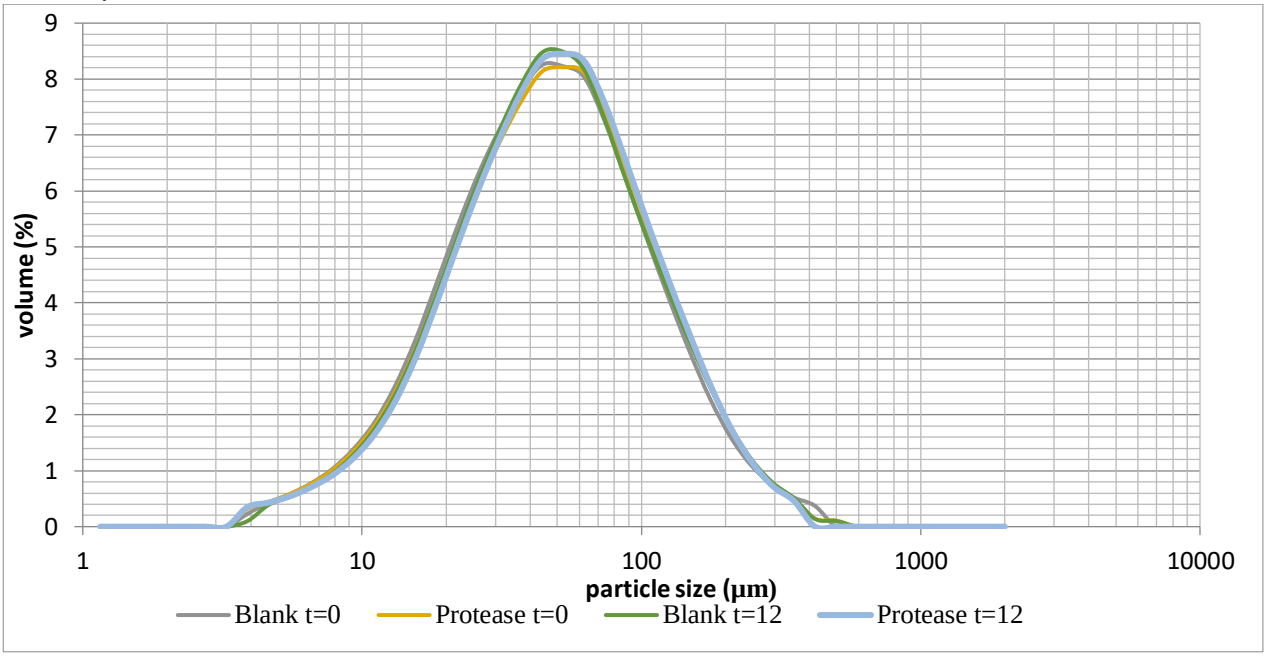
Appendix figure 10: VSS in enzyme batch test-2



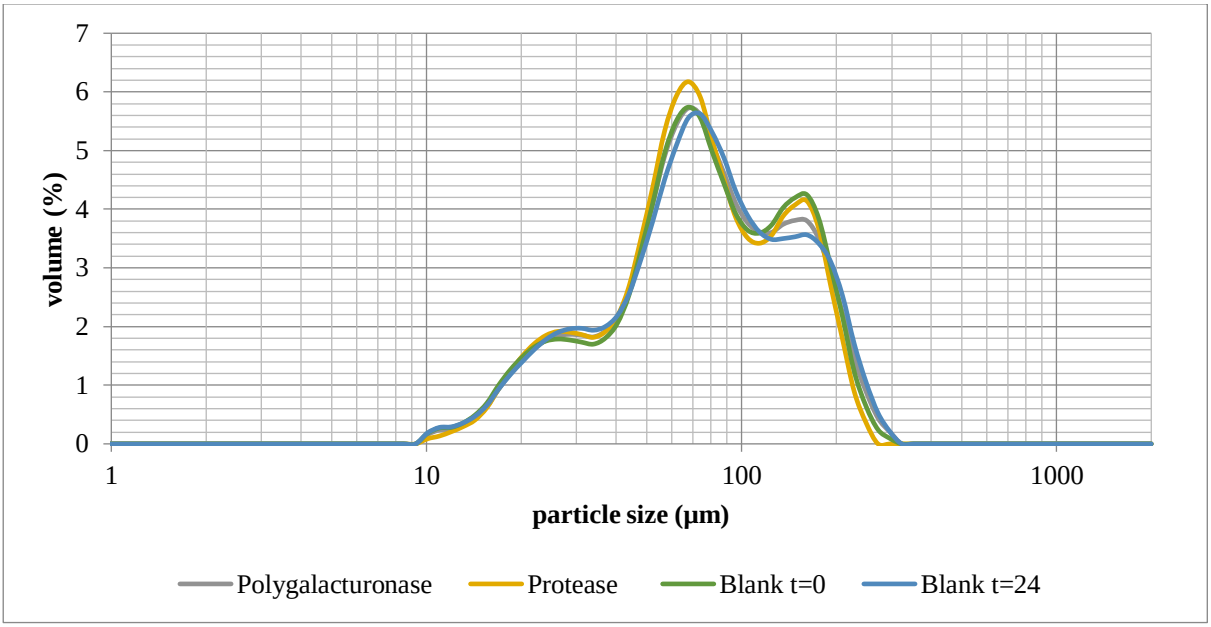
Appendix figure 11: Phosphate release from enzyme batch test-2



Appendix figure 12: Ammonia release in enzyme batch test 2



Appendix figure 13: Particle size distribution enzyme batch test 1



Appendix figure 14: Particle size distribution enzyme batch test 3