ENHANCEMENT OF THE FORMATION OF MULTILAYERED ANAEROBIC GRANULES IN UASB REACTORS

MR. NASRUL HUDAYAH

ID: 53920101

**A THESIS SUBMITTED AS A PART OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN ENVIRONMENTAL TECHNOLOGY**

**THE JOINT GRADUATE SCHOOL OF ENERGY AND ENVIRONMENT
AT KING MONGKUT'S UNIVERSITY OF TECHNOLOGY THONBURI**

2ND SEMESTER 2013

Enhancement of the Formation of Multilayered Anaerobic Granules in UASB Reactors

Mr. Nasrul Hudayah
ID 53920101

A Thesis Submitted as a Part of the Requirements
for the Degree of Doctor Philosophy
in Environmental Technology

The Joint Graduate School of Energy and Environment
At King Mongkut's University of Technology Thonburi

2nd Semester 2013

Thesis Committee

(Assoc. Prof. Dr. Pawinee Chaiprasert)	Advisor
(Dr. Benjaphon Suraraksa)	Co – advisor
(Assoc. Prof. Dr. Sirintornthep Towprayoon)	Member
(Dr. Somkiet Techkarnjanaruk)	Member
(Prof. Dr. Chongrak Polprasert)	External examiner

Thesis Title: Enhancement of the Formation of Multilayered Anaerobic Granules in UASB Reactors

Student's name, organization and telephone/fax numbers/email

Mr. Nasrul Hudayah
The Joint Graduate School of Energy and Environment (JGSEE)
King Mongkut's University and Technology Thonburi (KMUTT)
126 Pracha Utit Rd., Bangmod, Tungkru, Bangkok 10140, Thailand
Telephone: 0-8767-30821
Email: nasraul_01@yahoo.com

Supervisor's name, organization and telephone/fax numbers/email

Assoc. Prof. Dr. Pawinee Chaiprasert
Division of Biotechnology, School of Bioresources and Technology
King Mongkut's University of Technology Thonburi (Bang Khun Tian)
83 Moo 8 Thakham, Bangkhuntian, Bangkok 10150, Thailand
Email: pawinee.cha@kmutt.ac.th

Co - supervisor's name, organization and telephone/fax numbers/email

Dr. Benjaphon Suraraksa
Excellent Center of Waste Utilization and Management
National Center for Genetic Engineering and Biotechnology (BIOTEC)
King Mongkut's University of Technology Thonburi (Bang Khun Tian)
83 Moo 8 Thakham, Bangkhuntian, Bangkok 10150, Thailand
Email: benjaphon@biotec.or.th

Topic: Enhancement of the formation of multilayered anaerobic granules in UASB reactors
Name of student: Mr. Nasrul Hudayah **Student ID:** 53920101
Name of supervisor: Assoc. Prof. Dr. Pawinee Chaiprasert
Name of co-supervisor: Dr. Benjaphon Suraraksa

ABSTRACT

The formation of multilayered anaerobic granules mainly consists of nucleation and maturation phases. This study was aimed to construct initial granule nuclei with high syntroph microbial activities and enhance the formation of mature granule by stimulating EPS production. Three UASB reactors were used as R1 (control reactor, no cationic polymer addition), R2 (dynafloc 8265 addition) and R3 (chitosan addition). Mixed VFA (acetate: propionate: butyrate = 2:1:1 based on gram COD) was used as main substrate during nucleation phase. The addition of cationic polymers were aimed for shortening nucleation time. After nucleation phase, early maturation phase was started by switching mixed VFA to glucose for stimulating EPS production without polymer addition.

The addition of chitosan significantly decreased the zeta potential value of microbial aggregates in R3 from -26.4 to -10.5 mV at day 0 and 58, respectively. This zeta potential value affected on fast nucleation time in R3 which was observed at day 58. Nuclei ratio and average diameter size of microbial aggregates of R3 at day 58 were approximately 55.1% and 115 μm, respectively. Granule ratio in each reactor was low at day 58 in which the highest of granule ratio was observed in R3 as 8.2%. During maturation phase, granule ratio in R3 significantly increase as 17.6, 30.8 and 34.2% at day 74, 88 and 118, respectively. Higher nuclei ratio in R3 at day 58 was good as starting point for granule formation during early maturation phase. EPS produced during early maturation phase trapped nuclei with other nuclei to form large aggregates or granule. The microbial adaptation to mixed VFA during nucleation phase successfully increased the activity ratio between methanogen (acetoclastic methanogens) to non-methanogens (glucose-degrading microorganism). However, glucose enhanced the activity of GDM resulting on decrease of activity ratio between methanogens and non-methanogens activities during early maturation phase. The balance between methanogens and non-methanogens was adversely affected, especially in R1 and R2.

The structure of the microbial aggregate in R3 at day 58 was stronger and more compact compared to those in R1 and R2, which were weak and loose. Filamentous microorganisms were dominant inside the microbial aggregate of R1. While, the dominant microorganism inside microbial aggregates of R2 and R3 were mixed microorganism (cocci-, rod- and filament- shaped microorganism). Self-aggregation of microorganism may occurred in R1 and the addition of cationic polymers in R2 and R3 trapped mixed microorganism to form nuclei. At day 118, the morphology of microbial aggregates in each reactor was improved due to the effect of EPS. Microbial aggregates of R3 were very similar with the structure of mature granule which showed smoother surfaces and more spherical shapes.

The microbial distribution in the microbial aggregate of each reactor at day 58 showed that *Archaea* was dominant over *Eubacteria* in that aggregates which indicated that the methanogen population was larger than that of the acetogens due to the adaptation to mixed VFA. However, it cannot be determined that the layer of microorganism was formed in this phase because mixed microorganism groups, *Archaea* and *Eubacteria*, were still randomly dispersed in microbial aggregates of each reactors. At the end of early maturation phase (day 118), microbial distributions in granule of R3 were better than that at day 58. Red *Archaea* clumps, presumptive methanogens, was mostly located at middle and inner part of aggregates. Those *Archaea* clumps were surrounded by green *Eubacteria*, presumptive acidogenic or acetogens microorganism. It can be concluded that green *Eubacteria* clumps around red *Archaea* clumps were acetogens microorganism because the distance between VFA degraders (acetogens microorganism) and methanogens was close each other. Those position allowed the possibility of syntroph relationships inside microbial aggregate of R3. These microbial distribution in microbial aggregate of R3 was probably the main cause for better ACM activity and reactor performances during early maturation phase.

Keywords: Anaerobic granule; multilayered; syntroph; nuclei; cationic polymers

ACKNOWLEDGEMENTS

All praise is due to Allah SWT, The Most Gracious and the Most Merciful, to Whom I dedicated my life and all my knowledge-seeking activities. He is the prime cause that makes this journey of inquiry possible and this piece of work completed. I pray to him that He may accept this sincere contribution to human knowledge and that He may forgive me of its errors and failings.

I would like to express my gratitude to my advisor, Assoc. Prof. Dr. Pawinee Chaiprasert, and my co-advisor Dr. Benjaphon Suraraksa for their excellent support, understanding, continuous guidance, patience, and encouragement. They extended it throughout the time of research contributing a great deal to success. I would like to thank my thesis committee members, Prof. Dr. Chongrak Polprasert, Assoc. Prof. Dr. Sirintornthep Towprayoon and Dr. Somkiet Techkarnjanaruk for their valuable suggestions and supports.

As an international student, I enjoy and appreciate the warmth and attention from staff members of KMUTT, staff members and friends in Excellent Center of Waste Utilization and Management (ECoWaste), all my Thai friends and colleagues, Indonesian friends in KMUTT and PERMITHA. Their friendliness and friendship have helped me to cope and to adapt with the new environment I encountered during my study in Thailand.

Last, but not least, loving thanks also goes to my dear family, whose members are now living in different parts of the world but have never given up supporting me. I am indebted to my beloved parents, bapak H. Mohammad Suyoni and ibu Hj. Urifah, my first and all-time teachers of life who always love and care for me uncoditionally. Special thanks also go to my brother Dicky Afrizal and my sister Ifani Nur Azizah for their continuous attention and praying.

ABBREVIATIONS

ACM : Acetoclastic methanogens
AHR : Anaerobic hybrid reactor
BDM : Butyrate-degrading microorganism
CLSM : Confocal laser scanning microscopy
COD : Chemical oxygen demand
DLVO : Derjaguin Landau Verwey Overbeek
EGSB : Expanded granular sludge blanket
EPS : Extracellular polymeric substance
FISH : Fluorescence in situ hybridization
FITC : Fluorescence isothiocyanate
F/M : Food to microorganism
GDM : Glucose-degrading microorganism
HRT : Hydraulic retention time
ICA : Internal circulation anaerobic
LB-EPS : Loosely bound extracellular polymeric substances
OLR : Organic loading rate
PDM : Propionate-degrading microorganism
R1 : Reactor control (no polymer addition)
R2 : Reactor with dynafloc 8265 addition
R3 : Reactor with chitosan addition
SEM : Scanning electron microscope
SMA : Specific methanogenic activity
SNMA : Specific non-methanogenic activity
SS : Suspended solids
SVI : Sludge volume index
TB-EPS : Tightly bound extracellular polymeric substances
TS : Total solids
TVA : Total volatile acids
UASB : Upflow anaerobic sludge blanket
VFA : Volatile fatty acids
VSS : Volatile suspended solids

CONTENTS

CONTENTS (Cont')

CONTENTS (Cont')

CONTENTS (Cont')

CONTENTS (Cont')

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Cont')

LIST OF FIGURES (Cont')

CHAPTER 1
INTRODUCTION

1.1 Rationale

Anaerobic digestion is recognized as the most attractive treatment for organic wastewater, because it proves to be an excellent process for waste stabilization by recovering both energy and compost. It has been recognized as the most reliable and economically feasible in industrial scale of wastewater treatment for its simple and compact technology with high COD removal efficiencies and low sludge production [1]. For reason of energy recovery, anaerobic digestion produce methane as energy source which is able to generate approximately 1.5 kWh electric energy per kg COD removed (assumption of 40% electric conversion efficiency from methane energy) [2, 3]. In anaerobic digestion, complex organic matters in anaerobic digestion is rapidly hydrolyzed and then fermented by acidogenic microorganism into volatile fatty acids (VFAs) which are then utilized by acetogenic microorganism to produce acetate, hydrogen and carbon dioxide as suitable substrates for methanogens to produce methane and carbon dioxide [1, 4, 5].

Anaerobic digestion technologies based on granular sludge bed, e.g. upflow anaerobic sludge blanket (UASB) reactor, expanded granular sludge blanket (EGSB) reactor and internal anaerobic (ICA) reactor, and anaerobic hybrid reactor (AHR), are favorable wastewater treatment technologies for most industries. These technologies rely on microbial self-aggregation into active and compact granules as the main effective factor of reactor operation [1, 6-11]. Anaerobic granule is considered as a dense microbial community that consist of millions of microorganism per gram of biomass in which complex degradation is conducted by complex interactions among microorganisms in granules [12]. Two main characteristics of anaerobic granule which caused anaerobic granule become superior to other sludge type are its high settling ability and high specific microbial activity [12-14]. High settling ability of anaerobic granules is important factor for retaining anaerobic granules inside reactor during increase of upflow liquid loading rate and biogas production inside anaerobic reactor. The developments of microbial communities, i.e., syntrophic microorganism relationships, in anaerobic digestion could enhance the efficiency of substrate degradation due to the optimum position of substrate supply and assimilation among microorganism inside anaerobic granules [15-17]. The formation of anaerobic

granule is a multistep process involving physicochemical and biological forces that can result in a multilayered structure of anaerobic granules [12].

A multilayered anaerobic granule is recognized as an anaerobic granule that consists of several layers in which each layer of granule is occupied by specific microorganisms. Outer, middle and inner layers of multilayered anaerobic granules are typically occupied by hydrolytic and fermentative or acidogenic microorganism, syntroph microorganism and methanogen, respectively [15, 17, 18]. The structure of multilayered anaerobic granules provides good syntrophic relationship among microorganism inside granule which then allows efficient substrates degradation. Multilayered anaerobic granule is favored as starting inoculum in anaerobic reactors for its effective substrate degradation and distribution inside granules, microbial inter-closed relationships and resistance from toxic substances. However, this type of granule is generally found at reactor which has already been operated for 1-2 years and is strongly depend on the type of substrate [15-17, 19, 20]. For that reason, mass culturing of anaerobic granules is needed to provide good anaerobic granules which can decrease the start-up period of industrial scale anaerobic reactor and cost of anaerobic granules seeds. Larger volume of wastewater with higher organic loads are able to be treated by anaerobic reactor which is inoculated by multilayered anaerobic granule than flocculent sludge.

The availability of mature multilayered anaerobic granules for industrial scale reactors is limited due to high purchasing, handling and transportation costs. The international cost of good-quality matured granule sludge is EUR130 – 150 per cubic meter [21]. The additional cost is commonly needed for the possibilities of process failure such as excessive losses of granular sludge and re-inoculation. Due to several main drawbacks of available mature anaerobic granule, non-granular sludge or digested sewage sludge is considered as potential seeding sludge for new reactor operations [8, 12, 22, 23]. Nevertheless, reactor start-up with digested sludge is very slow and sludge is easy to wash out from reactors due to low microbial activities and settling ability, respectively. Generally, start-up period which is related to the formation of anaerobic granules can be approximately 2-8 months [24]. Previous research for overcoming these problems had been conducted for shortening reactor start-up and granulation process, such as the addition of cationic polymers and multivalent ions, extracellular polymer substances (EPS) production and the application of high hydrodynamic condition inside reactors [7, 10, 22, 23, 25-32]

Research related to the formation of multilayered anaerobic digestion mostly investigated the characteristics of naturally formed multilayered anaerobic granules. Less research has considered the investigation of the formation of multilayered anaerobic granules from digested anaerobic sludge. The formation of multilayered anaerobic granule generally involves two main phases namely granule nuclei formation (nucleation) and maturation phases. The characteristics and type of initial microorganism population in inoculum is one important factor for constructing multilayered anaerobic granule. Syntroph-enriched methanogenic consortia was found as best for starting microbial groups in granulation process [33]. However, the initial separated-enrichment of microorganism to specific substrate was time consuming (approximately one year adaptation of microorganism to specific substrate). The drawback factors of digested sludge to build multilayered anaerobic granule were possible to be solved by simultaneous adaptation of microorganism to specific substrate (syntroph specific substrate) and the addition of cationic polymers at the same time during reactor operation.

The main objectives of this study were to enhance the formation of multilayered anaerobic granules from digested sludge by the addition of natural and synthetic cationic polymers and the microbial adaptation to syntroph specific substrate during UASB reactor operation and also to investigate the stability of that multilayered anaerobic granule. In order to achieve this objective, nuclei formation (nucleation) of syntroph nuclei was shortened by the addition of synthetic and natural cationic polymers with syntroph specific substrate feeding and then followed by nuclei maturation by stimulating the production of extracellular polymeric substances (EPS) to entrap nuclei or microorganisms that leads on the structure strengthening of anaerobic granules.

1.2 Research objectives

The objectives of this research are:

1.2.1 To construct the initial granule nuclei of syntroph enriched microorganisms by the addition of natural and synthetic polymers

1.2.2 To enhance the formation of early mature granules by stimulating EPS production

1.2.3 To observe the physicochemical and microbial stabilities of anaerobic granules during the operation of the anaerobic reactor

1.3 Scopes of research work

1.3.1 Sludge sizing under 100 μm is used as the initial microorganism for anaerobic granulation.

1.3.2 Natural and synthetic polymers were represented by chitosan and polyacrylate (dynafloc 8265), respectively.

1.3.3 Nuclei was defined as a microbial aggregate larger than 100 μm. Then, nucleation was development process of microbial aggregates to larger size than 100 μm.

1.3.4 EPS production for enhancing the nuclei maturation was stimulated by switching the substrate into glucose

1.3.5 Early maturation phase was defined as the development of nuclei to be larger aggregates than 500 μm and microbial compositions inside granules.

1.3.6 The microbial compositions were limited on the presence of *Eubacteria* and *Archaea* groups by fluorescence in situ hybridization (FISH) for initial seed sludge. The syntroph composition, as well as *Eubacteria* and *Archaea* in granules, was analyzed by confocal laser scanning microscope (CLSM).

1.4 Expected results

1.4.1 Understanding the formation of granule nuclei with and without the addition of polymers.

1.4.2 The stimulation of EPS production as possible to enhance or fasten the maturation of granules.

1.4.3 The mature granule from maturation phase has high syntroph specific microbial activities and unique multilayered structures.

CHAPTER 2
THEORIES

In developing countries, such as Indonesia and Thailand, large amounts of organic wastewater from domestic and industries can be a serious problem for the environment if they are not properly treated. Biological treatment is more preferred for treating that organic wastewater than physicochemical treatment due to its several advantages such as treatment efficiency. There are two kinds of biological treatment, i.e., aerobic and anaerobic biological treatment. Anaerobic biological treatment or anaerobic digestion is more beneficial than aerobic biological treatment in several aspects as shown below.

2.1 Anaerobic digestion

Anaerobic digestion is considered to be a biological process in which complex organic substrates degraded into biogas, e.g. methane and carbon dioxide, as the final product by microbial consortia in anaerobic conditions. Degradation processes consist of multiple biological processes namely, hydrolysis (breakdown of complex and insoluble organics into small molecules that can be transported into microbial cells), acidogenesis (conversion of sugars, amino acids and fatty acids to organic acids, hydrogen and carbon dioxide), acetogenesis (conversion of several organic acids into acetic acid and hydrogen) and methanogenesis (methane and carbon dioxide production as final products of anaerobic digestion) [1]. Biological anaerobic treatment or anaerobic digestion has been implemented worldwide to treat industrial or domestic wastewater for the reason that it offers several advantages than biological aerobic treatment [1]. The major advantages of anaerobic digestion are:

- Low cost operation, because of less energy required for the reactor operation.
- Useful energy as biogas production
- Ability to be operated at high loading rates
- Low sludge production
- Stable excess sludge
- Possibility to be combined with other post treatment

Beside its fundamental advantages, anaerobic digestion also has several drawbacks that affect on the application of this technology, such as high susceptibility of microorganisms,

especially acetogens and methanogens, to toxic compounds and slow start-up periods due to a lack of knowledge about microbial growth conditions [1]. Several conditions in anaerobic reactor must be accomplished in order to achieve good reactor performances. According to Lettinga (1995), those important conditions are high retention of viable sludge in the reactor, sufficient contact between viable bacterial biomass, high reaction rates and absence of serious transport limitations, and the dominance of favorable conditions for all required microorganism inside anaerobic digestion system. Those important conditions were commonly observed from anaerobic granule.

2.2 Anaerobic granulation

It is important to know and understand the critical points of anaerobic digestion in order to succeed the operation of anaerobic digestion. One of those critical points is the immobilization of anaerobic microorganism or anaerobic granulation. Anaerobic granulation naturally occurs in the upflow anaerobic sludge blanket (UASB), internal circulation anaerobic (ICA) and expanded granular sludge blanket (EGSB) type reactors [1, 15, 34, 35]. Anaerobic granulation process is completely natural process in UASB reactor [1]. Microorganism conglomeration or aggregation involves physical, chemical and biological processes resulting self-immobilization of microorganism under hydrodynamic conditions inside reactors.

Microbial aggregates can be distinguished among 3 types, i.e. flocs, pellets and granules. Flocs or flocculent sludge is defined as conglomerates with loose structures that form one homogenous macroscopic layer upon settling. Pellets are aggregates with denser structure than flocs and still visible as separate materials after settling. While, granules are microbial aggregates which are denser, have well-defined granular appearance with special shape and can resist on hydrodynamic pressure inside reactor. According to Hulshoff Pol et al., (2004), microbial granules have main advantages than flocculent sludge, such as;

- Superior settling characteristics that allow granules to be resistant on high pressure inside reactors,
- High specific methanogenic activity, since the presence of well-defined microbial consortia to efficiently degrade complex substrates,

- Toxic resistance, since the most sensitive microorganisms (methanogen) are located at the inner part of granules, while the least sensitive microorganisms are commonly at the surface or middle layer of the granules.

2.3 Anaerobic granulation models

Many models describing anaerobic granulation has been proposed by previous researchers. However, there is still no consensus about the valid mechanism of anaerobic granulation. Schmidt and Ahring (1996) reported that granular sludge could be characterized as a spherical biofilm. Hulshoff Pol. et al. (2004) has reviewed the model of anaerobic granulation in three groups, namely physical, microbial and thermodynamic approaches. The development of biofilm and granulation have several similarities which can be divided into 4 main steps:

a). *Cell transport* to the surface of inert materials or other cells (substratum)

b). *Initial reversible adsorption* to the substratum by physicochemical forces

c). *Irreversible adhesion* of the cells to the substratum

d). *Cell multiplication* and granule development

2.3.1 Cell transport to the substratum

There are several mechanisms for transporting the cell to the substratum, i.e. diffusion (Brownian motion), advection (convection) transport by fluid flow, gas flotation, sedimentation, or active movement due to flagella (**Figure 2.1**). Models or theories of anaerobic granulation that can be grouped into the first step of biofilm formation are selection pressure theory, inert nuclei and methanogen as the nucleation center for granulation.

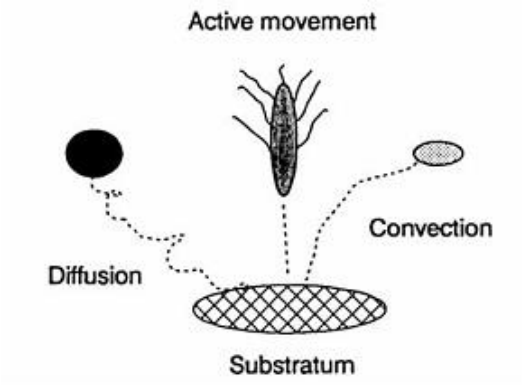


Figure 2.1 The different transport mechanisms for a cell to the substratum [14]

2.3.1.1 Selection pressure theory

Hulshoff Pol et al., (1983) explained that the cause of anaerobic granulation was due to the presence of continuous selection for sludge particles with a high settleability during reactor operation. The selection pressure for particles or granules can be controlled by adjusting the upflow velocity of the reactor (hydraulic loading rate and gas production rate), e.g. UASB reactor. Finely dispersed particle will be washed out from reactor by high hydrodynamics condition inside the reactor. As a result, the heavier particles or nuclei which are resistant for high selection pressure, still remain in the reactor and their size and density will increase. It can also be concluded that under low selection pressure, microbial adhesion and aggregation cannot be advantageous and most of influent will be metabolized by dispersed sludge. These conditions also promote the growth of filamentous form of *Methanosaeta* which cause the formation of bulking type of anaerobic sludge with poor settling characteristics [19].

2.3.1.2 Inert nuclei model

The inert matter content of granules was thought to initiate the granulation process by acting as nuclei for anaerobic microorganisms to attach, and then, form initial embryonic granules [36]. By the formation of initial embryonic granules, subsequent granular growth occurs through biofilm formation around these inert carriers of suspended matter. Hence, the development of a mature granules can be considered as the result of an increase of biofilm thickness [37]. However, several researches reported that granules can be developed without the addition of any inert material and that high concentration of finely suspended matters in the influent are unfavorable to granulation [13, 38].

2.3.1.3 Growth of microbial nuclei

Most methane production (around 70%) is generated from acetic acid degradation, which is mostly degraded by two microorganisms, namely *Methanosarcina* and *Methanosaeta*. Several models or theories considering the growth of acetoclastic methanogens have been proposed which conclude that *Methanosarcina* and *Methanosaeta* species form nuclei for starting the granulation process.

The spaghetti theory for the granulation process stated that filamentous *Methanosaeta* through a multidirectional branched-growth process formed spaghetti-like structured aggregates that entrapped other microorganism and initiated nuclei formation or precursors for the overall granulation process [13]. According to this theory, granules formation is divided into two main phases, 1) formulation of precursors or nuclei (first phase)

and 2) actual growth of the granules from precursors (second phase). The first phase is critical step for the formation of granules. *Methanosaeta* forms small aggregates to finely dispersed matter by the turbulence from gas production. The selection of aggregates is conducted by increasing the upflow velocity. Once the precursors have formed, the granulation process is inevitable by the growth of individual microorganisms and the entrapment of other microorganisms that result in the increasing of the precursor size. At this step, granular formation is still as filamentous appearance, like loose-structured ball of spaghetti with long *Methanosaeta* filaments.

Chen and Lun (1993) also postulated a similar theory about granule formation that was divided into two main steps, i.e. nucleus formation and a nucleus growing into a granule. They concluded that both *Methanosaeta* which has good adhering capacities and *Methanosarcina* which has capacity of growing into clumps by excreting extracellular polymeric substances EPS are the important microorganism for nucleus formation. During the growth of nuclei, various microorganism which syntrophically grow with methanogen play important role on complex substrate degradation [39]. Methanogen is not dominant microorganism in the surface of mature granule, but other mixed microorganism.

2.3.2 Initial reversible adsorption and permanent adhesion

2.3.2.1 Secondary minimum adhesion model

This model assumed that bacterial cells as nothing more than living colloidal particles. Hence, it obeys the laws of physical chemistry and interacts with the surface in the same manner as normal colloidal particles (Van Loosdrecht and Zehnder, 1990). As a result, the adhesion of microorganism is possible to be explained by colloid chemical theories, i.e., the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory which describes the change in Gibbs energy as a function of the distance between particles surfaces. Reversible adhesion occurs in the secondary minimum of the DLVO free energy curve. At separation distance (H) of 5 – 20 nm, the Gibbs energy involved in this reversible process is relatively small because there is no direct contact between two surfaces/cells. Reversible adhesion can transform to irreversible adhesion when a particle or cell prevail over the energy barrier for reaching the primary minimum at small separation distances (H>1 nm). The main disadvantage of this model is that bacterial cells cannot be simply considered as ‘classical colloidal particles’ because they do not have simple geometry or uniform molecular surface composition. The presence of intracellular metabolic reactions probably results on the alteration of surface cell, before and after adhesion.

2.3.2.2 Divalent cation-bridge model

Divalent cations, e.g., calcium (Ca^{2+}), magnesium (Mg^{2+}) and iron (Fe^{2+}), play important roles in the granulation process by neutralizing negative charges of bacterial cell surfaces and functioning as cationic bridges between bacteria [40, 41]. In addition, EPS also tends to bind multivalent metals resulting more stable polymer complexes [40]. However, the additions of Ca^{2+}, Mg^{2+} and Fe^{2+} at high concentration have negative effects on granulation process in certain conditions [14, 19, 40].

2.3.2.3 Extracellular polymeric substances (EPS) bonding model

Schmidt and Ahring (1996) defined EPS as any polysaccharide-containing structure of bacterial origin lying outside the bacterial cell, either as a result of excretion or cell lysis. For more detail, EPS represents the capsular material and peripheral slime, other macromolecules such as proteins, nucleic acids and other polymeric substances in the intercellular space of microbial aggregates (Flemming and Wingerder, 2001; Shin, et.al., 2001).

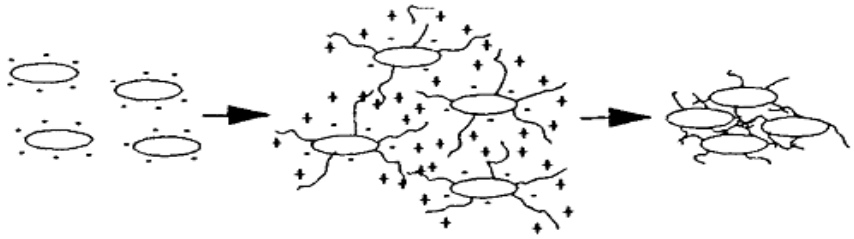


Figure 2.2 The role of surface charge and the production of EPS in granulation [14].

EPS changes the surface negative charge of bacteria resulting in the bonding of bacterial cells and other inert matter to form bacterial aggregates or flocs as shown in **Figure 2.2** [14]. EPS has also been reported as critical factor for maintaining the structural integrity of granular sludge [10, 24]. However, it has also been observed that too high EPS can cause deterioration in granular sludge formation [10, 35]. The amount and type of EPS produced are usually affected by the growth, temperature, carbon source, substrate loading rate and hydrodynamic shear force [10, 14, 26-28, 35, 42, 43].

Cape Town theory was developed by Sam-Soon et al. (1987), who proposed that hydrogen-utilizing methanogen, *Methanobacterium* strain *AZ*, secreted EPS (mainly consist of long-chain polypeptides) under high partial pressure and limited cysteine. The EPS produced then trapped any microorganism leading to the formation of anaerobic pellets which rapidly settled. Wastewaters containing acetate, propionate and butyrate resulted on

inadequate hydrogen partial pressure which cause failure on granulation process based on this theory [44].

Several studies suggested that hydrolytic and acidogenic microorganism have great influence on the EPS production than acetogenic and methanogenic microorganisms, although it was still not clear whether EPS was produced by one specific or various different species [24]. The EPS content of granules and anaerobic sludge fed with acetogenic and methanogenic substrate was significantly lower than that of feeding with acidogenic substrate.

2.3.3 Cell multiplication

2.3.3.1 Syntrophic micro-colony model

The principal of this theory is the mutual relationship among different anaerobic microorganisms inside granules to live synergistically resulting in the efficient transfer of intermediate products among those respective microbial groups. Syntrophic relationship is normally at granule which has the multi-layered structure. The layered structure of granule showed that the granulation process is not a random aggregation of suspended bacteria, but the microbial associations are very high as syntrophic associations among different microorganism for optimum substrate supply and removal of metabolic products [15].

Syntrophic relationships between acetogens and methanogens in granules are important factors for the high specific activity of granules, since those two microbial groups are closely related and the limiting growth microorganism in granule. It has also been investigated that the distance between syntrophic acetogens and methanogens in each layer of granule is very small which allows the interspecies transfer of hydrogen and efficient substrate conversion [15, 17].

The aggregate-forming behaviors of various methanogenic and syntrophic fatty acid-degrading cultures were investigated in order to determine the potential granule-forming of syntrophic associations [45]. The results showed that a butyrate-degrading syntrophic strain produced EPS that could enhance the granulation process. However, their investigation also proved that methanogens *Methanosaeta* and *Methanobacterium formicicum* are an important key to granulation. The good granulation was achieved by syntrophic-enriched nuclei which showed the highest increase on granule diameter compared to the fermentative-enrich nuclei, *Methanosaeta*-enrich nuclei and *Methanosarcina*-enrich nuclei [33]. The granulation started with *Methanosaeta concilii* cells as growth nuclei then syntrophic acetogens attached to the *M. concilii* backbone. In addition,

their study also investigated that the enhancement of granule size could be done by enrichment of propionate-degrading syntrophic consortia [46].

2.3.3.2 H^+ translocation-dehydration theory

This theory was developed by Tay *et al*. (2000) and considers several factors as the main key in the granulation process. Firstly, high negatively charged microbial surfaces facilitate hydrogen bonding with water molecules leading to a strong repulsive hydration interaction between hydration layers of two approaching microorganisms. Secondly, the initiation of bacterial adhesion can be improved when the hydrophobicity of adhering surfaces is increased [47]. According to Tay *et al*., (2000), proton translocation activity across the bacterial membrane causes the dehydration of the cell surfaces by breaking the hydrogen bond between negatively charged groups and water molecules resulting in the increase of surface hydrophobicity. Four stages of granulation process were formulated according to this theory:

a) *Dehydration of bacterial surface*

   Result of proton translocation activity across bacterial membranes.

b) *Embryonic granule formation*

   The relatively hydrophobic bacteria adheres by the action of external hydraulic forces because of the weakened hydration repulsion. Effective metabolite transference promotes the further dehydration of bacterial surfaces resulting in the strong initial aggregate formation. EPS production is also induced.

c) *Granule maturation*

   Intermediate transference determines the distribution of the different bacterial groups leading to well-structured microbial communities in the mature granules.

d) *Post maturation*

   Granule structures are continuously maintained by the mechanism of proton translocation activity. EPS production causes hydration of the surface outside of the granule and prevents the attachment of gas bubbles to the granules.

This model can also explain phenomena happening in the reactor, the enhancement of granulation process by feeding of easily degradable substrates. According to this theory, faster digestion and uptake of easily degradable substrates has led to more rapid activation of proton pumps on the acidogenic cell membranes, which can turn to quick dehydration of the bacterial surface resulting to bacterial adhesion.

For other phenomena, such as sludge washout due to the sudden change of carbon source, it can also be explained by this theory. Sudden change substrate lead to new lag phase in bacterial growth in which new set of enzymes is synthesized (enzymes involved in substrate degradation is substrate specific). Then, new lag phase causes the decrease of proton pumping activity leading to decrease bacterial adhesion and then followed by granule disintegration and washout [38].

However, the bacterial metabolism including proton translocation activity can also be influenced by various internal factors resulting in changes in internal and surface chemical compositions of bacterial cells [21]. According to Hulshoff Pol et al. (2004), bacterial adhesion is a complex process that cannot be explained as a physical chemical process, since bacteria have no sharp surfaces, simple geometries or uniform molecular surface compositions.

2.4 Factors affecting anaerobic granulation

The anaerobic granulation process depends on bacterial growth inside an anaerobic digestion system since the organic and inorganic compounds excreted during bacterial metabolism can promote nuclei formation. Then, the structure of mature granules is influenced by interactions between individual microbial species in anaerobic digestion. Methanogens generally initiated the granular nuclei formation. However, the generation time of this microorganism is longer (3 days at 35^{o}C or 50 days at 50^{o}C) than other microorganism in anaerobic digestion. Hence, it can be assumed that controlling the environmental conditions, as well as creating the optimal growth conditions for slow-growing microorganism (methanogens), is possible to enhance granulation process itself [13].

2.4.1 Wastewater type

Wastewater types are considered to be stronger factors in the granulation process and granule quality e.g. structure of granule, microbial compositions and populations in granules, than reactor design or operational conditions [15, 19, 20]. According to Tay, et.al. (2000), organic substrates can be divided into high-energy and low-energy feeds. High-energy carbohydrate feeding can support acidogens and facilitate the formation of extracellular polymers. The proton pumps is more active along with more readily acidogens take up and metabolize substrate then methanogens obtains the substrate easily. The rapid the growth of

acidogens due to the presence of high-energy substrate in influent facilitate the overall process of granulation in the reactor.

The microbial compositions of anaerobic granule from anaerobic reactors treating different wastewater, i.e., fruit and vegetable cannery, protein-based and brewery, were analyzed at the molecular level [15, 19]. The results found that anaerobic granules from reactor treating fruit and vegetable cannery wastewater had excellent shear strength, settling properties, high cell density and layered-structure composed by methanogen, syntrophs and acidogens. Brewery treated – anaerobic granules also showed good granules quality, i.e., high microbial density and layered-structure. However, anaerobic granules from reactors treating protein – based wastewater showed poor qualities, i.e., poor strength, low settling velocity, low microbial density and non-layered structure. High ratio of polypeptide to carbohydrate in EPS was found at protein – fed anaerobic granule resulting on the lower shear strength compared to cannery treated – anaerobic granule and brewery treated – granule.

Different substrates also provided different surface charges, which are important factors as triggering forces for anaerobic granulation [24]. It has been proven that EPS production related to substrate types and running conditions. DLVO theory was used to explain the microbial adhesion in which microbial adhesion occurred by neutralization of the repulsion forces between similar charged particles. In case of anaerobic granulation, microorganism commonly have negative charges and substrate acts as external electrolytes in which at high substrate concentration, surface charges of substrate tend to increase (positive).

The characteristics of anaerobic granules were also investigated in UASB reactors treating different types of wastewater, i.e., ethanol, carbohydrates and protein-based synthetic wastewater [20]. The results showed that those types of substrates influenced the physicochemical properties of anaerobic granules. Ethanol – fed anaerobic granules were more compact than carbohydrate – fed and protein – fed anaerobic granules. The microbial distributions inside of anaerobic granules were also affected by types of wastewater in which high acidogens colonization was found at carbohydrate – fed and protein – fed anaerobic granules and *Methanobacteriales* were co – dominant in those anaerobic granules. Whereas *Methanosaeta* was reported as dominant methanogens in all types of wastewater.

2.4.2 Seed sludge

Any medium containing proper microbial flora can theoretically be used as seed sludge for anaerobic granulation in a UASB reactor. Potential seed sludge includes manure, fresh water sediments, septic tank sludge, digested sewage sludge and surplus sludge from anaerobic treatment [12]. For reducing the start-up period of reactor and reach high removal efficiency, surplus anaerobic granular sludge is always chosen as initial seed sludge. However, the availability of those granular anaerobic sludge is limited by its high purchase and transportation cost [21]. Therefore, digested sewage sludge, which is abundant amount, is normally used as seed material to substitute granular sludge in start-up period of reactor [21, 23, 26, 32]. The optimum amount of seed sludge used in reactor must also be determined for maintaining the effective contacts between sludge and substrate or influent. Lettinga et.al., (1995) reported that the optimum amount of thick sludge should be between 12 and 15 kg $VSS.m^{-3}$ while the concentration of thinner sludge should be 6 kg $VSS.m^{-3}$ for mesophilic reactor.

To improve the methanogenic activity and granulation process of digested sewage sludge, additional UASB granules are normally supplemented to newly digested sludge during the start-up period. The addition of small amount of crushed granular sludge (8-9% of the volatile suspended solid content) positively influenced the sludge bed granulation [13]. Furthermore, the addition of crushed granular sludge at concentration of 8 – 15% of VSS content had more or same enhancement effect on granulation (higher) than result by addition of only 2% crushed granular sludge. The improvement of granulation process by addition of crushed granules was probably caused by the consequence of supplying specific inoculums which is responsible for granulation. As a result, it is possible that anaerobic granulation can be accelerated by manipulating the microbial composition of the seed sludge.

The consensus is to conclude that knowledge of anaerobic seed sludge contributes the most to anaerobic granulation has not yet been achieved. However, several studies considered that acetoclastic methanogens *Methanosaeta* spp. was the key in granulation. Although, several methanogens, i.e., *Methanobacterium formicicum* and *Methanosarcina mazei* have also been shown to contribute in granule formation [13, 46].

2.4.3 Macro-and micronutrients

Besides carbon and energy sources, anaerobic bacteria also have nutritional requirements for macro-and micronutrients. The important macronutrients for microbial growth and optimal granulation are nitrogen and phosphorus. While, iron and nickel are

obligatory micronutrient needed to optimize the function of methanogenic enzyme systems. Other micronutrients which can contribute to methanogenesis and granulation are calcium, magnesium, molybdenum, tungsten, selenium, potassium, barium, manganese, zinc, sulfur, copper and aluminium [5].

In anaerobic digestion, the macronutrient requirements are determined in terms of COD: N: P ratio of reactor. Gerardi (2003) stated that a minimum COD: N: P ratio of 100:3-4:0.5-1 is needed to provide adequate nitrogen and phosphorus levels. Several studies have reported that the addition of nutrients could be postponed after granule formation without changes in process performance. Yeast extract is a good source of amino acids, minerals and B vitamins, e.g., biotin and folic acid that can be used to improve reactor performance. The absence of nickel, cobalt and iron were reported not affect on the COD removal rate in UASB reactors as long as yeast extract was in the influent. Although the trace minerals in yeast extract was lower than other chemicals used in reactor, the reactor performances significantly decreased along with the decrease of yeast extract supplementation. Yeast extract was reported to enhance the ability of anaerobic bacteria to collect essential trace elements from other sources.

2.4.4 Upflow liquid velocity and hydraulic retention time

Anaerobic granulation can proceed well at relatively high liquid upflow velocity but it does occur under conditions of low hydrodynamic shear force [10]. The combination of high upflow liquid velocity and short hydraulic retention time (HRT) was reported as a favorable condition for anaerobic granulation. The effect of upflow liquid velocity for anaerobic granulation are usually described by selection pressure theory [13]. Long HRT combination with low upflow liquid velocity promote the growth of dispersed sludge and are less favorable for anaerobic granulation process. On the other hand, a short HRT combination with high upflow liquid velocity leads to washout for dispersed sludge (non-granular sludge) and then promote anaerobic granulation.

Flocculent anaerobic sludge can be converted to active anaerobic sludge by manipulating the hydraulic stress and the settleability of anaerobic granules. The effect of upflow liquid velocity on the specific washout rate of the smaller particles is insignificant. While its effect on the mean granule size is positively significant.

2.4.5 Organic loading rate (OLR) and sludge retention time (SRT)

As previously described, the wastewater type applied determines the bulk characteristics or granular sludge than do loading rates [19]. The different sludge

characteristics were observed for the same wastewater and inoculums under different OLRs and SLRs [26]. OLR expressed as kg $COD.m^{-3}.d^{-1}$ is one of important operating parameter which represents the capacity of a reactor to convert organic substrate per unit volume. While, SLR (kg $COD.kg^{-1}VSS.d^{-1}$) represents the capacity of anaerobic population presenting in reactor for converting organic substrate per unit mass. The development of granular sludge bed with good characteristics can be promoted by keeping the OLR in range of 2.0 – 4.5 kg $COD.m^{-3}.d^{-1}$ and SLR in range of 0.1 – 0.25 kg $COD.kg^{-1}VSS.d^{-1}$ [26].

The effects of overloading on granulation time and methanogenic population activity by comparing three reactors, namely A, B and C, under different conditions were investigated. The results showed that OLR, EPS content and granulation were closely related each other. Low loading rate of below 1 kg $COD.m^{-3}.d^{-1}$ for the first 40 days was applied at reactor A in which the start of granulation (white appearance, flocculent nuclei-type materials) was observed for the first time on the 48^{th} day of operation at OLR of 2 kg $COD.m^{-3}.d^{-1}$. Reactor B was operated under slight overloading and start of granulation was observed for the first time on the 7^{th} day at OLR of 2.5 kg $COD.m^{-3}.d^{-1}$ and mature granules were observed at day 39 at OLR of 30 kg $COD.m^{-3}.d^{-1}$. The activity of acetate-consuming methanogens improved slightly during operation but not the same extent as in reactor A. While, reactor C was operated under extreme overloading condition at OLR of 15 kg $COD.m^{-3}.d^{-1}$ on day 14. Granulation process was very poor under these conditions since sludge washout and acidification occurred. EPS content was observed in all reactors and EPS production tend to increase followed by slight EPS decrease as the OLR was fixed at a certain level for period of time which indicated bacterial consumption of EPS part. OLR increased more often in reactor B than that of in reactor A, it can be concluded that the conditions for reactor B could enhance the granulation process. In addition, anaerobic granulation was poorly observed at low OLR [32].

2.4.6 Temperature

Similar with other microbial activity, methanogenic growth and activity are also strongly influenced by environmental temperature. The temperature range for mesophilic methanogens is reported at a range between 30 to 35^{o}C. Temperature fluctuations can affect methanogenic activity significantly and it should be kept at minimum fluctuation around 2-3^{o}C per day for mesophilic methanogens [5].

Operational temperature also strongly influences the population composition of granules. The efficiency of anaerobic digestion process involves more than one microbial

population. Therefore, temperature fluctuations could be advantageous for some microbial groups, but not for some other microbial groups, e.g., acidogens and hydrolytic microorganism are normally resistant to temperature fluctuation represented by rapid VFA production, while methanogen activity is very low at low temperature or temperature fluctuations. Hence, careful monitoring of the VFA to alkalinity ratio is important if there is temperature fluctuation in the reactor [5, 48].

2.4.7 pH

Anaerobic granules consist of microbial groups that are syntrophically related to each other for utilizing complex substrates [5, 17]. According to degradation steps in anaerobic digestion, microbial species involved can be classified into three categories, namely hydrolytic microorganism, acidogenic microorganism and methanogenic microorganism. Those microbial groups have their optimum pH for their growth and activity. Acidogenic or acid-producing microorganism can tolerate low pH and have optimum pH of 5.0 – 6.0. in other side, methanogens or methane-producing microorganism have narrow optimum pH of 6.7 – 7.4 [49]. Hence, methanogens is more sensitive to pH than other microbial groups in UASB reactors. pH fluctuation to 6.0 or 8.0 cause reduction of methanogens activity resulting the serious operational problem leading to reactor failure. Under normal conditions, the decrease of pH resulted from acid production is neutralized or buffered by bicarbonate produced by methanogen microorganisms.

2.5 Formation of multilayered granules

The theory of multi-layered granule formation was first proposed by McLeod, et.al. (1990). The hypothesis of this theory was based on the observation of mature granule morphology by a scanning electron microscope. The center or inner of granule consisted of *Methanosaeta* aggregates which were believed as initial nuclei for granule development. Acetate producing microorganism (H_2 producing acetogens) was then found as second layer after *Methanosaeta* aggregates. Acetate producers provided substrate, i.e., acetic acid, for *Methanosaeta* which was located at inner of granules. As the outer layer, fermentative bacteria attached forming exterior layer in which this microorganism were in direct contact with their substrate in bulk solution. The formation of multi-layered granules consisted of two main phase, namely nuclei formation (nucleation) and nuclei maturation

2.5.1 Nuclei formation (Nucleation)

Nuclei are defined as microbial aggregates that have sizes between 100 – 500 µm [46]. Therefore, nucleation is the growth process of microbial aggregates to the average diameter size of 100 µm. Nucleation phase is important as starting phase to form multi-layered anaerobic granules. The dominant microorganism that involved in nucleation phase is varied. *Methanosaeta spp.* had been reported as dominant microorganism for nucleation. McLeod, et.al. (1990) observed that the inner of anaerobic granule consisted of *Methanothrix* or *Methanosaeta* which was then proposed as initial nuclei that initiated granule development. The granulation process became faster by using *Methanosaeta* which were enriched at beginning of reactor start up [50]. *Methanosaeta* were also reported having hydrophobic characteristics and nearly uncharged at neutral pH in which those conditions are for *Methanosaeta* aggregation forming fiber-like bundles [51]. Morgan et. al., (1991) found that the addition of pure culture of *Methanosaeta* cells could enhance granulation of dispersed digester sludge. In addition, Zheng et. al., (2006) confirmed that filamentous microorganism *Methanosaeta concilii* was found as major cell components of small aggregates in early stage of UASB operation (by fluorescence in situ hybridization analysis). The results indicated that these filamentous microorganism acted as nuclei for the development of microbial granules.

Several researchers have also found that *Methanosaeta* was not the only one of microorganism that could initiate the nuclei formation. Nuclei could be initiate by fermenting microorganism, *Methanosarcina sp.* and syntrophic microorganism [33, 51-53]. El-Mamouni et al. (1997) showed that nuclei containing ethanol-degrading syntrophic consortia could proceed granulation faster than other nuclei containing *Methanosaeta sp., Methanosarcina sp.,* or fermentative microorganisms. From those previous studies, the dominant microorganism in nucleation can be varied and the most important is how the nuclei can retain in reactors and then conglomerates with other microorganism to form mature granules.

2.5.2 Nuclei maturation

Wu et al. (2009) defined mature granules as microbial aggregates that have diameters of more than 500 µm. From this definition, nuclei maturation is the next phase of nucleation to form mature granule. Maturation phase involves the attachment of more microorganism to nuclei surface and forms bigger and denser nuclei (mature granule). McLeod et. al., (1990) observed that acetate producers (H_2 producers acetogens) and fermentative microorganism

were “the coating microorganism” which attached at *Methanosaeta* nuclei as middle and outer layers, respectively. The presence of acetogens and fermentative microorganism could provide substrates for microorganism in the inner layers. The role of “coating microorganism” in the maturation of nuclei were also investigated by other researchers [15, 46, 51]. Zheng et.al. (2006) investigated the syntrophic consortia which assisted the nuclei maturation as coating microorganism. The syntrophic consortia, *Syntrophobacter* was found higher in granules than in bulk biomass which indicated that *Syntrophobacter* selectively attached to nuclei (high settling ability). However, the other syntroph microorganism, such as *Desulfobulbus spp.*, did not seem to assist the nuclei maturation since this microorganism did not attach at nuclei (showed by similar amount of *Desulfobulbus sp.* in granule and bulk biomass). The maturation phase of nuclei also involved the life-cycle of granules in which the coating microorganism attached at the breaking part of large granules and formed new and stable mature granules

2.6 Enhancement of anaerobic granulation

One main disadvantage of a granular reactor, i.e. upflow anaerobic sludge blanket (UASB) reactor, expanded granular sludge bed (EGSB) reactor and internal circulation anaerobic (ICA) reactor, is a long start-up period which is related to the granules formation inside reactor. Various strategies have been applied to enhance the granule formation or shorten the start-up period of reactor operation. Those strategies include the following:

2.6.1 Addition of synthetic and natural polymers

Synthetic and natural polymers are normally used as supporting agents for enhancing particle coagulation and flocculation processes. Many studies investigated the effects of synthetic and natural polymers on anaerobic granulation [7, 22, 23, 25, 30, 54-56].

Chitosan or poly (β-(1-4)-N-acetyl-D-glucosamine) is a natural biopolymer that is produced by the alkaline deacetylation of chitin [57]. It was widely used for removal of humic substances from drinking water, treatment of wastewater from distilleries, removal of oil from wastewaters, treatment of food processing wastes, lignin removal and other application. The chemical composition of chitosan is described by the molar fraction of GlcNAc (2-acetamido-2-deoxy-β-D-glucopyranose) or F_A. This unit is important factor affecting chitosan characteristics such as solubility and charge density [58]. The positive charge of chitosan is resulted from the protonization of amino groups of GlcN (2-amino-2-

deoxy-β-D-glucopyranose). This characteristic was very attractive for microbial flocculation and aggregation since most of microorganism have negative surface charge. The mechanism of microbial aggregation is closely related to mechanism of microbial adhesion to surfaces. Complex interactions such as van der Waals (VDW), electrostatic, hydrophobic and polymer interactions controlled the microbial attachments or aggregations. Chitosan (a polymer which is similar with polysaccharide structure) supplementation significantly enhanced the granules formation in UASB reactors.

The granulation rate at the chitosan-containing reactor was 2.5 times higher than that of at reactor without chitosan supplementation. Polymers chain enhance granulation by bridging among microbial cell resulting complex and strong structure [25]. Tiwari, et al. (2005) also showed that chitosan as additive agent in granulation successfully increased granule size and percentage of larger aggregates in reactor. Chitosan was considered as cationic polymer which could decrease the negativity of microbial surface charges resulting on microbial aggregation. Moreover, the characteristics of chitosan as water absorbing polymer which caused chitosan swelled in water could provide many attachment sites for microorganism. These double effects of chitosan were considered as the most important factors for microbial granulation.

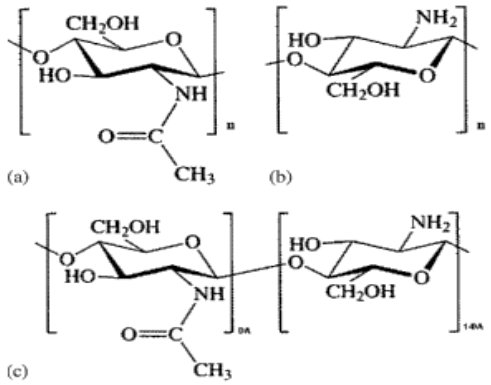


Fig. 2.3 Chemical structure of (a) chitin, (b) chitosan and (c) partially acetylated chitosan [57]

Water absorbing polymers (WAP), such as acrylic and acrylamide, were observed for their effects on microbial granulation. This polymer type was able to swell in water and then provided more a complex network structure for microbial attachments or aggregations. Imai, et al. (1997) investigated the effect of water absorbing polymer (WAP) ST-500D with various concentration for enhancing granulation in UASB reactor fed by glucose and mixed

VFA. Although the rates of granulation were different for glucose and mixed VFA feeding, the granulation time was successfully shortened by this WAP addition. No granulation was observed in reactor without WAP addition. Based on the results, the role of WAP for granulation was assumed based on three formation phases,

- Phase 1: WAP particles absorbed anaerobic sludge resulting in the decrease of biomass washout
- Phase 2: microorganisms attached on WAP particles grew rapidly and formed initial granules in which WAP acted as a nuclei or bio-carrier
- Phase 3: granule bed developed rapidly. Some of WAP particles were decomposed by anaerobic microorganism resulting several fragments of initial granules which then developed as mature granules

The effect of a cationic polymer, AA 184 H polymer or commercial coagulation agent, has been investigated on a reactor start-up and granule development in 6 reactors treated as controls and polymers with various concentrations [23]. Reactors with polymer addition showed better reactor performance, e.g., short start-up time, the highest OLR and high COD removal efficiency, and granule characteristics, e.g., granules size, granule strength and specific activity, throughout the operation than the control reactor. The cationic polymer enhancement in reactor with 80 mg/L concentration showed the most significant effects on biogranulation and reactor start-up due to the optimum concentration of polymer [23].

The addition of bioflocculant (MBF21 bioflocculant) has been conducted to enhance anaerobic granulation in the UASB reactor treating low-strength synthetic wastewater and compared with synthetic polymers (acrylamide-chitosan graft polymer/ACGC and cationic polyacrylamide/PAM) [30]. The results showed that bioflocculant-containing reactor was the most efficient compared to other reactors (polymers addition and control). The granulation rates in bioflocculant-, ACGC-, PAM-containing reactors were enhanced by 50%, 87%, 75% compared to control. Although bioflocculant addition was not the most effective way to develop large-size granules, it showed as the least inhibitory in enhancing microorganism multiplication and improving microbial metabolic activity. The ratio of volatile suspended solids to suspended solid and sludge methanogenic activity of granular samples from bioflocculant-containing reactor were also higher than other three reactors. Granulation was achieved in all four reactors, but granules from polymers-containing reactors appeared earlier and larger than those from control reactor.

In addition, the UASB operation with the addition of cationic polymers (polyacrylamide containing DADMAC) at OLR more than 1.0 kg $COD.m^{-3}.d^{-1}$ and inoculum having SS greater than 110 $g.l^{-1}$ and VSS/SS ratio less than 0.3 could enhance biomass granulation and COD removal efficiency. However, polymer addition at OLR less than 1.0 kg $COD.m^{-3}.d^{-1}$ and thick inoculum sludge did significantly enhance biomass and tend to deteriorate the reactor performance (reduction of COD removal efficiency) [22].

Resmanto, et al. (2010) investigated two cationic synthetic polymers, namely polyacrylamide 3150 and polyacrylate dynafloc 8265 with both optimum doses as 2 $mg.g^{-1}$SS. Dynafloc 8265 showed as the most suitable polymer for coagulation for all specific substrate-adapted microorganism. Moreover, this type of synthetic cationic polymers did not show any inhibitory effect to microbial activities. After 2 months operation, the granule of all substrate-adapted microbial groups can reach the size of 2.0 mm. From the results of FISH-CLSM and microbial activities, the addition of dynafloc 8265 showed positive effects on microbial granulation from specific substrate-adapted microorganisms.

2.6.2 Addition of divalent ions

The presence of divalent ions, such as calcium (Ca^{2+}), magnesium (Mg^{2+}) and iron (Fe^{2+}), could enhance microbial granulation by binding negatively charged cells to promote microbial nuclei formation [27, 40, 41]. Yu et. al., (2001b) investigated the addition of calcium (Ca^{2+}) at various concentrations from 150 – 300 mg/l in order to enhance the biomass accumulation and granulation process. From those concentration range, microbial granulation could be enhanced. However, the results also showed that specific activity of granules decreased along with the increase of influent calcium concentration since higher concentration of calcium tend to lead the precipitation of calcium in the granules which could block the intra-granular pores, severe mass transfer limitation and high ash content in granules [40]. The addition of calcium to UASB reactor appeared to enhance three steps of microbial granulation, i.e., adsorption, adhesion and multiplication but it did alter the predominant microorganism in the granules. Al^{3+} was also reported to have positive effect on sludge granulation. Yu et.al., (2001a) found that aluminium prompted granule formation by allowing aggregates to form earlier and to achieve a larger diameter size.

The addition of iron (Fe^{2+}) to enhance granulation has also been investigated [41]. The addition of ferrous iron at dose of 0.01 g Fe^{2+} per g COD feed allowed COD removal of more than 98% at loading rate of 9 g COD/l per day and high granules diameter which were higher 24% and 56% than that of reactor control (without ferrous iron addition), respectively.

The formation of inorganic precipitate of ferrous sulfide initiated the inert nuclei formation causing the biomass attached onto it.

2.6.3 Application of influent pulsation

The application of external pulsation on influents was aimed to generate hydrodynamic stress during the UASB reactors starting up [59]. Early phase of UASB reactor start up was commonly operated under low OLR which resulted improper hydraulic mixing and mass transfer inside reactor. These conditions lead to poor degassing of sludge which cause biomass washout and long start-up period. Applying external pulsation was able to release gaseous metabolites as well as improved mass transfer (by preventing sludge bed channeling). Pulsation of influent in treatment reactor had higher COD removal and specific methanogenic activity (SMA). Although anaerobic granules produced from the pulsed reactor was smaller, it had well defined shape with presence of channels on its surfaces. These channels indicated that the granules had higher porosity causing the increase of biogas release and nutrient transport from outside to inner core of granules [60]. However, these studies were conducted on a small lab scale of 0.8 l UASB reactors which implied that a deeper investigation is needed in terms of extra cost obstacle for its application on large industrial scale.

2.6.4 Enhancement of extracellular polymeric substances (EPS) production

Several operational conditions of a reactor can be controlled to enhance the EPS production. Extracellular polymers are called bioglue which facilitate cell-to-cell interaction and strengthen microbial structure through formation of complex polymeric matrix. Cell surface characteristics such as cell surface hydrophobicity, surface charge density, binding site and surface morphology may be affected since EPS accumulates at the cell surface. Several previous studies have been conducted in order to increase the EPS production then leading to the enhancement of microbial granulation [10, 24, 32, 43].

Zhou et.al. (2006) investigated the effects of EPS production and electrostatic properties of the substrates on the process of granulation in UASB reactors using three kinds of substrates: glucose, skim milk and mixed VFA. This study proved the effectiveness of slight overloading for stimulating EPS production which could shorten the period of granulation. The results of this research concluded that DLVO theory built up a connection among substrates, EPS and granulation in which bacteria as negative particles, substrate as external electrolytes and EPS as high molecular flocculants which resulted on the electrical neutralization and EPS bonding as important triggering forces for anaerobic granulation.

The sludge granulation under weak, strong and violent shear conditions in an internal circulation anaerobic reactor was investigated by Wu et al. (2009). The granulation consists of two steps namely nucleation and maturation of nuclei. According to the results, nucleation under strong shear conditions, shear rate of 8.28 s^{-1} corresponding to superficial liquid of 2.66 $m.h^{-1}$ and gas velocity of 0.2 $m.h^{-1}$, developed fastest compared to weak shear (0.04 s^{-1}) and violent shear (12.42 s^{-1}) conditions. The average augmentation rate of average sludge diameters were 0.40, 0.57 and 0.41 μm per day for weak, strong and violent shear conditions, respectively. High shear force seemed to accelerate the secretion of extracellular protein. However, over-produced extracellular protein also deteriorate granulation. The maximum concentration of extracellular protein for enhancing granulation was 80.5 mg. g VSS^{-1}. Over that concentration, nucleation was weaken and inhibited which could lead to disruption and washout of nuclei from reactor.

2.6 Extracellular polymeric substances (EPS)

2.7.1 Definition of EPS

The general abbreviation of EPS was aimed to represent different classes or macromolecules, such as polysaccharides, proteins, nucleic acids, lipids and other polymeric compounds, presented in the interior of various microbial aggregates [27]. This broad definition of EPS results on unpredictable and controversial studies related to EPS. Another researchers concluded that EPS are mainly the high molecular weight secretion from microorganisms and the products of cell lysis [61]. EPS are sticky materials secreted by microbial cells in unfavorable or stressful conditions in order to survive in those conditions. EPS are located at both outside of the cells and in the interior of microbial aggregates. The outer EPS was divided into bound EPS (sheaths, capsular polymers, condensed gels, loosely bound polymers and attached organic materials) and soluble EPS (soluble macromolecules, colloid and slimes). These two types of EPS, bound EPS (closely bound with cells) and soluble EPS (weakly bound with cells or dissolved into medium) can be separated by centrifugation resulting on supernatant (soluble EPS) and microbial pellets (consist of bound EPS) [62]. Studies related to EPS are specified for bound EPS, while soluble EPS has very weak interaction with cells (but it affects on the microbial activity and surface characteristics of sludge). EPS are believed having important role on adhesion, matrix structure formation and stabilization of microbial granules [35, 63]. **Figure 2.3** shows two layers model of the

structure of bound EPS, namely the tightly bound EPS (TB-EPS) as inner layer which has certain shape and is tightly bound and stable with cells, and the loosely bound EPS (LB-EPS) which is loose and dispersible slime layer without clear edge [64].

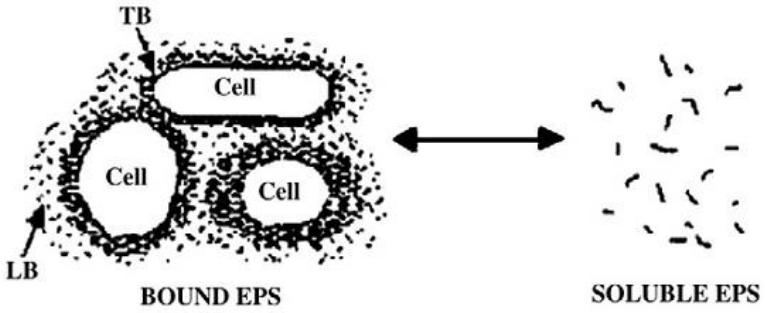


Figure 2.4 Sketch of EPS structure [64]

2.7.2 Composition of EPS

The EPS from granules contains various proportions of protein, polysaccharides, nuclei acids, lipids, humic-like substance and heteropolymers [65]. Only polysaccharides that is synthesized extracellularly, while protein, lipids and nucleic acids presents in extracellular matrix due to excretion of intracellular polymer or as a results of cell lysis [66]. Humic substances may also be a key component of EPS in sludge, approximately 20% of the total amount. The variation of fractions in EPS strongly depend on the extraction method and the origin of sludge. One main consideration related to EPS composition is the ratio of polysaccharides to protein which varied depend on microbial species, growth phase, the type of limiting substrate (C, N and P), oxygen limitation, ionic strength, culture temperature, shear force, etc. [63, 64].

2.7.3 The characteristics of EPS

2.7.3.1 Hydrophobicity and hydrophilicity

The EPS has many charged groups, such as carboxyl, phosphoric, sulfhydryl, phenolic and hydroxyl groups, as well as apolar groups, such as aromatics, aliphatics in protein and hydrophobic regions in carbohydrates. The hydrophobicity areas in EPS is beneficial for the adsorption of organic pollutant. Beside of hydrophobicity, EPS also has hydrophilicity characteristic causing EPS has amphoteric characteristic. EPS could be separated to their hydrophilic and hydrophobic fractions by using XAD resin resulting 7% of hydrophobic which consisted protein, while the hydrophilic mainly consisted of

carbohydrates [67]. The hydrophobicity and hydrophilicity of EPS influence the formation of microbial aggregates and also the sorption sites for organic pollutants [68, 69]

2.7.3.2 Adsorption characteristics

The EPS has several adsorption sites, such as aromatics, aliphatics in proteins and hydrophobic regions in carbohydrates, which can be potential sites for heavy metal adsorption prior to transporting it to the environment. High binding capacity of EPS was based on its estimated numbers of the available carboxyl and hydroxyl groups [68]. In anaerobic granulation, the binding between EPS and divalent ions, Ca^{2+} and Mg^{2+}, is the important interactions to maintain the structure of microbial aggregates [70]. Organic pollutants such as phenanthere, benzene, humic acid and dye, can be adsorbed by EPS [27, 61].

2.7.3 Factors affecting EPS production

2.7.4.1 Carbon source

Glucose, as main the carbon source in the operation of anaerobic sequencing batch biofilm reactor (ASBBR), gave the highest yield of EPS as 23.6 mg EPS/g carbon source compared to other substrates, such as mixed VFA and meat extract, which yields 9.0 and 1.4 mg EPS/g carbon source, respectively [71]. Simple sugars like glucose, fructose and sucrose had been reported as an easy degradable substrate and main precursor for EPS production. The building block of gluconacetan for many EPS should be energy-rich form of monosaccharides. The formation of repeating unit of polysaccharide is formed by addition of activated sugar nucleotides to a lipid carrier. Then, the transportation of the repeating units occurs and crosses the cell membrane to the outer layer and polymerization to form the EPS matrix as the last step.

Lipid rich wastewater commonly causes many severe problems because of the nature of the wastewater, such as reduction in the liquid-phase mass transfer rate, sedimentation hindrance due to the development of filamentous microorganism, development and flotation of sludge with poor activity, clogging and the emergence of unpleasant odors.

2.7.4.2 Microaerophilic condition

Miqueleto, et.al., (2010) found that EPS production could not be detected in ASBBR feeding with glucose in strict or obligate anaerobic conditions (fluxing with nitrogen gas from bottom of reactor), while EPS could be produced from ASBBR without nitrogen fluxing. The results confirmed that EPS was mainly produced by microaerophilic or facultative microorganism when oxygen with very low concentration was available.

Microaerophilic condition is possible to be achieved by recirculation of liquid phase inside reactor and exposing some part of biomass during long fill phase in ASBBR. ASBBR fed with glucose with high carbon to nitrogen (C/N) ratio produced high EPS and showed high oxygen reduction potential (ORP).

a). C/N ratio

The composition of the growth medium affected the substrate conversion to form polymers. Polysaccharide production generally occurs in a medium containing high ratio between carbon and the limiting nutrient, such as nitrogen. Miqueleto, et al., (2010) found that EPS production decreased as the C/N ratio decreased. At high C/N ratio, microorganism may alter the cellular growth pathway to EPS production due to deficiency of nitrogen for protein synthesis. It was also confirmed by decreasing of daily growth of microorganism (yield TVS/day) as the C/N ratio of the substrate increased. Moreover, deficiency in an essential nutrient, such as nitrogen, can limit cellular growth and the energy from carbon excess is used for polysaccharide biosynthesis (which occurs due to increased ATP production when there is an excess of carbon source).

b). Microbial groups

The EPS production was investigated from four different enriched microbial groups, namely the enriched glucose (Glu)-, butyrate (HBu)-, propionate (HPr)- and acetate (HAc)-degrading microorganism [72]. The EPS yields per gram specific substrates were 1.07 mg-EPS_p (proteinaceous EPS) and 0.46 mg-EPS_c (carbohydrate EPS) for HAc-degrading microorganism, 1.52 mg-EPS_p and 0.54 EPS_c for HPr degrading microorganism, 2.00 mg-EPS_p and 0.59 EPS_c for HBu degrading microorganism and 2.39 mg-EPS_p and 0.99 EPS_c for Glucose-degrading microorganism. The results showed that acidogens could produce more EPS than acetogens and methanogens. Acetogens and methanogens were present in both carbohydrate-degrading and short chain fatty acids degrading granules, whereas acidogens were present only in carbohydrate degrading granules. The higher EPS produced by acidogens lead to the formation of mature granules with superior characteristics.

2.7.4 Effect of EPS on granulation

2.7.5.1 EPS effect on settleability of microbial aggregates

The sludge volume index (SVI) represents the settleability of microbial aggregates in which low SVI value indicates good settleability. The negative charge of an EPS could increase the negative charge of a microorganism, resulting in repulsive forces between cells. Repulsive forces between cells decreased the settleability of microbial

aggregates [73]. More LB-EPS also decreased settleability of microbial aggregates due to more bound water in microbial aggregates resulting high porous flocs which had a low density [74]. Too high EPS content in the system must be considered as its effect for increasing SVI value.

2.7.5.2 EPS effect on stability of microbial aggregates

The stability of microbial aggregates is expected to be affected by the presence of an EPS. Stability of microbial aggregates is defined as the ability of microbial aggregates to resist hydrodynamic and mechanical shear stress inside reactors [75]. Those stresses are able to erode particles, bacteria and EPS from the surface of microbial aggregates. A higher EPS content lead to greater sludge stability. The complex interactions caused by EPS such as polymeric entanglement, ion bridging, electrostatic interaction and hydrogen bonds, contribute to the stability of microbial aggregates [61, 76]. Sheng and Yu (2007) proposed a multi-layer structure of microbial aggregates with two main regions, outer and inner regions (**Figure 2.4).** The outer region consisted of a dispersible part and the inner one was a stable part. During exposure to shear stress, the outer region dispersed while the inner part remained stable.

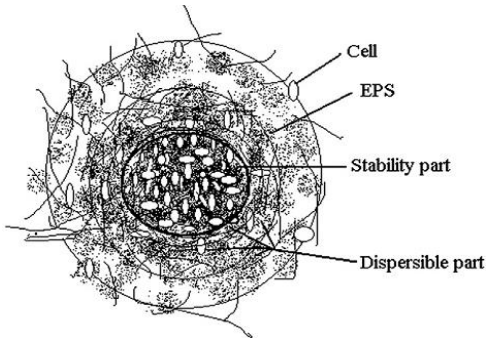


Figure 2.5 Model of multi-layer microbial aggregates based on EPS [76]

2.7.5.3 EPS effect on mass transfer in microbial aggregates

An EPS surrounds the surface and fills the inner part of a microbial aggregate. The presence of EPS on these positions cause substrate must pass through EPS layers in order to be transferred into the cells. The pores in granular sludge which are useful for substrate diffusion and gas releasing can be clogged by the EPS. Thus, it results on decreasing of the mass transfer efficiency of substrates and washout of microbial aggregates. Diffusion coefficients of EPS component are lower than those of water affecting the import of nutrients to the cells and export of metabolites to environment [77]. Too high level of EPS

component in anaerobic reactors need to be carefully considered for its negative effect on mass transfer in microbial aggregates.

2.7.5.4 EPS effect on surface charge of microbial aggregates

There are many charged functional groups in an EPS that influence the surface charge of microbial aggregates. EPS has positive effect to decrease the negative charge of sludge which then results on sludge aggregation. The total EPS content and individual components both were investigated having positive effect on the negative charge of sludge and the effect of protein and humic acid substances were the most significant [78]. Protein is known for its unique charge characteristics in which amino groups are positive and can decrease the negativity of carboxyl and phosphate groups and then decrease the negative surface charges of microorganism.

2.7.5.5 EPS effect in flocculation ability of microbial aggregates

Flocculation ability for microbial aggregates is an important factor to reach low turbidity and a high quality effluent. EPS affects flocculation by two mechanism, namely double layer compression and ion bridging. The other EPS effect on flocculation ability is its protein content. Removal of surface protein by addition of small amount of protein-hydrolyzing enzyme causes deflocculation of microbial aggregates [74, 79]. Wilen et. al., (1990) found that increase of protein or decrease of the humic substance could increase the flocculation ability of sludge. However, those results also concluded that there were complex interactions between individual EPS components and the flocculation of microbial aggregates. The ratio of the main EPS components such as ratio of protein to polysaccharides content seemed more affecting on the microbial flocculation [80]. Loose bound EPS (LB-EPS) and tight bound EPS (TB-EPS) also affect on the microbial flocculation. The excessive amount of LB-EPS in the system had negative effect on sludge flocculation because of weak cell attachment in the system [79].

CHAPTER 3
METHODOLOGY

This research was conducted in two main phases. The first phase was aimed to shorten the formation of initial granule nuclei with high syntroph activities. This initial nuclei was designed as a starting point for multilayered anaerobic granules. The second phase of this research was early maturation phase in which the structure of nuclei or microbial aggregates was strengthened by EPS. Second phase was considered as early maturation phase since the whole maturation phase involves many external natural factors which were difficult to be controlled in this study.

3.1 Phases of initial granule formation

3.1.1 Nuclei formation (nucleation) phase

This phase focused on the time of nuclei formation by the addition of synthetic and natural polymers, dynafloc 8265 and chitosan, respectively. The nucleation of anaerobic granules was concomitantly conducted with adaptation of microorganism to syntroph specific substrate for producing syntroph enriched-nuclei. The nucleation phase was achieved when one of treated reactors showed the nuclei to total aggregates ratio as approximately more than 50% and the average diameter size more than 100 µm.

3.1.2 Early maturation phase

After the nucleation phase, the substrate was then switched from a syntroph specific substrate to glucose as the main carbon source for the microorganisms inside a reactor. The glucose feeding to reactor was aimed for enhancing EPS production which was important for strengthening the nuclei structure as well as entrapped other microorganism to form multilayered anaerobic granules. This early maturation phase was indicated by increase of granule to total aggregates ratio and decrease of small microbial aggregates. The stability of physicochemical and microbial characteristics of anaerobic granule were observed during nucleation and maturation phase for determining the accurate time for the production of multilayered anaerobic granules.

3.2 Seed sludge

Anaerobic seed sludge used as the initial microbial seed was obtained from an anaerobic pond of a wastewater treatment plant (WWTP) in the tapioca starch industry. The initial seed sludge was sieved with 100 µm sieving instrument for removing large particles and mature granules since this research studied the anaerobic granulation which was started from the digested seed sludge that commonly had small diameter sizes. The screening of initial seed sludge quality included the analyze of its volatile suspended solids (VSS), suspended solids (SS), total solids (TS), sludge volume index (SVI), size distribution and specific microbial activities. The characteristics of inoculum seed sludge from wastewater treatment plant are shown in **Table 3.1**

Table 3.1 The characteristics of seed sludge for reactor inoculation

The characteristics of seed sludge (stock)	
Volatile suspended solids (VSS)	9.88 $g.l^{-1}$
Suspended solids (SS)	10.84 $g.l^{-1}$
Total solids (TS)	11.06 $g.l^{-1}$
VSS/SS ratio	0.91
Average particle size	47 µm
Sludge volume index (SVI)	88.56 $ml.g^{-1}$ SS
Extracellular polymers composition	
- Polysaccharides	20.75 $mg.g^{-1}$SS
- Protein	72.06 $mg.g^{-1}$SS
The specific activities of seed sludge	
- Specific methanogenic activity	0.13 g COD. g^{-1} VSS. day^{-1}
- Propionic acid utilization activity	0.22 g COD. g^{-1} VSS. day^{-1}
- Butyric acid utilization activity	0.29 g COD. g^{-1} VSS. day^{-1}
- Glucose utilization activity	2.28 g COD. g^{-1} VSS. day^{-1}

3.3 Cationic polymers

3.3.1 Synthetic cationic polymer (dynafloc 8265)

Dynafloc 8265 was obtained from Dyna Fluid Co. Ltd., Thailand. This synthetic polymer is commonly used for flocculating or sludge dewatering in liquid – solid separation processes. The characteristics of this polymer are as follows,

- Cationic polyacrylate
- White granular powder
- Density 850 $g.cm^{-3}$
- Wide effective pH range (1 – 14)

For stock solutions, dynafloc 8265 was dissolved in deionized water with a final concentration 0.1 – 0.5% prior to use for research.

3.3.2 Natural cationic polymer (Chitosan)

The chitosan was provided by Taming Enterprises Co. Ltd., Thailand. The characteristics of this chitosan are as follows:

- Degree of deacetylation 94.2%
- Molecular weight 3.5×10^5 Dalton
- Moisture content 8%
- Ash content 0.91%

A chitosan solution was prepared by diluting 1 g chitosan in 100 ml of 1% acetic acid. The solution was stirred at 200 rpm overnight.

3.4 Reactors

Three upflow anaerobic sludge blanket (UASB) reactors were used in this research. The reactors were made from flexi glass with an internal diameter of 86 mm, effective height of 860 mm and working volume 4.5 of liters. Recirculation line from upper port to sludge bed was provided in each reactor to enhance granulation and maintain efficient substrate degradation. Reactor 1 (R1), 2 (R2) and 3 (R3) were operated as control reactor (no polymer addition), reactor with synthetic polymer (dynafloc 8265) addition and reactor with natural polymer (chitosan) addition, respectively. The configuration of UASB reactors is as seen in **Figure 3.1.**

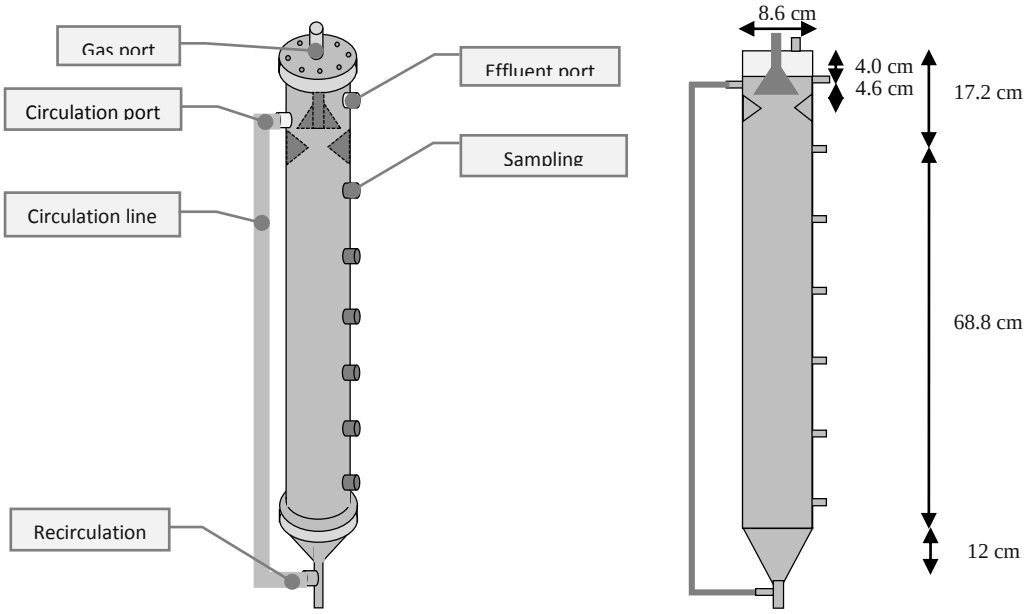


Figure 3.1 The configuration of UASB reactors

3.5 Wastewater

The macro and micro compositions of synthetic wastewater used in this research are listed in **Table 3.2.** The carbon sources used in this research were separated into two main substrates (depending on the phase of the reactors operation). The first phase (nucleation) used syntroph specific substrates that consisted of mixed volatile fatty acids (VFAs), i.e. acetic, propionic and butyric acids with ratio 2:1:1 (based on gram COD) as the main substrate. For second phase (early maturation), main substrate was switched from mixed VFA to glucose for enhancing the production. The concentrations of syntroph specific substrate and glucose were varied depend on the organic loading rate applied during reactor operations.

Table 3.2 Composition of synthetic wastewater [81]

Formulation of synthetic wastewater					
$(NH_4)_2SO_4$	132	$mg.l^{-1}$	*Nutrient solution**		
$NaH_2PO_4.H_2O$	75.5	$mg.l^{-1}$	$FeCl_3.6H_2O$	1.5	$g.l^{-1}$
$CaCl_2.2H_2O$	50	$mg.l^{-1}$	H_3BO_3	0.15	$g.l^{-1}$
$MgSO_4.7H_2O$	90	$mg.l^{-1}$	$CuSO_4.5H_2O$	0.13	$g.l^{-1}$
Yeast Extract	10	$mg.l^{-1}$	KI	0.18	$g.l^{-1}$
*Nutrient solution**	0.3	ml	$MnCl_2.4H_2O$	0.12	$g.l^{-1}$
			$Na_2MO_4.2H_2O$	0.06	$g.l^{-1}$
			$ZnSO_4.7H_2O$	0.12	$g.l^{-1}$
			$CaCl_2.6H_2O$	0.12	$g.l^{-1}$
			EDTA	10	$g.l^{-1}$

3.6 Anaerobic granulation study

3.6.1 Nucleation phase by synthetic and cationic polymer additions (phase I)

3.6.1.1 Optimum doses of synthetic and natural polymers

Synthetic and natural polymers were used to shorten the nucleation phase in this research. The polymers used in this research were dynafloc 8265 and chitosan as synthetic and natural polymers, respectively. A jar test was conducted to observe the optimum dose of polymer addition [25]. Based on jar test as preliminary research, dynafloc 8265 and chitosan were optimally added at concentration of 2 $mg.g^{-1}$ SS and of 13 $mg.g^{-1}$ SS, respectively. The main objective of polymers addition was to reduce the negative charge on microbial surface (reduction the repulsion force between two neighboring microbial cells) resulting on sludge coagulation to form aggregates. The optimum dose of polymer was determined as minimum dose which performed the lowest turbidity of supernatant after sludge coagulation or aggregates formation and its lowest toxicity effects on microorganism. In this study, the additions of polymers were conducted at every week during reactor operation for nucleation phase.

3.6.1.2 Operational conditions for nucleation phase

The research overview for the operational conditions of the nucleation phase can be seen in **Figure 3.2.** The first additions of polymers were conducted concomitantly with the inoculation of seed sludge in UASB reactor. Thereafter, polymers were routinely added

every week during operation time. Reactors were operated with different organic loading rate started at 0.5 to 1.5 kg $COD.m^{-3}.d^{-1}$. Increase of OLR was conducted by increase of substrate concentration (mixed VFA). The circulation velocity was kept as 1 $m.h^{-1}$ since the shear force at this velocity showed significant effect on granulation process [10, 13]. Initial nuclei can be categorized based on its size which is between 100 – 600 µm [46]. Time for nuclei formation was investigated as its relationship with the increase of nuclei size. The nuclei phase was terminated until nuclei ratio and the average diameter size were approximately 50% of total sludge and more than 100 µm, respectively.

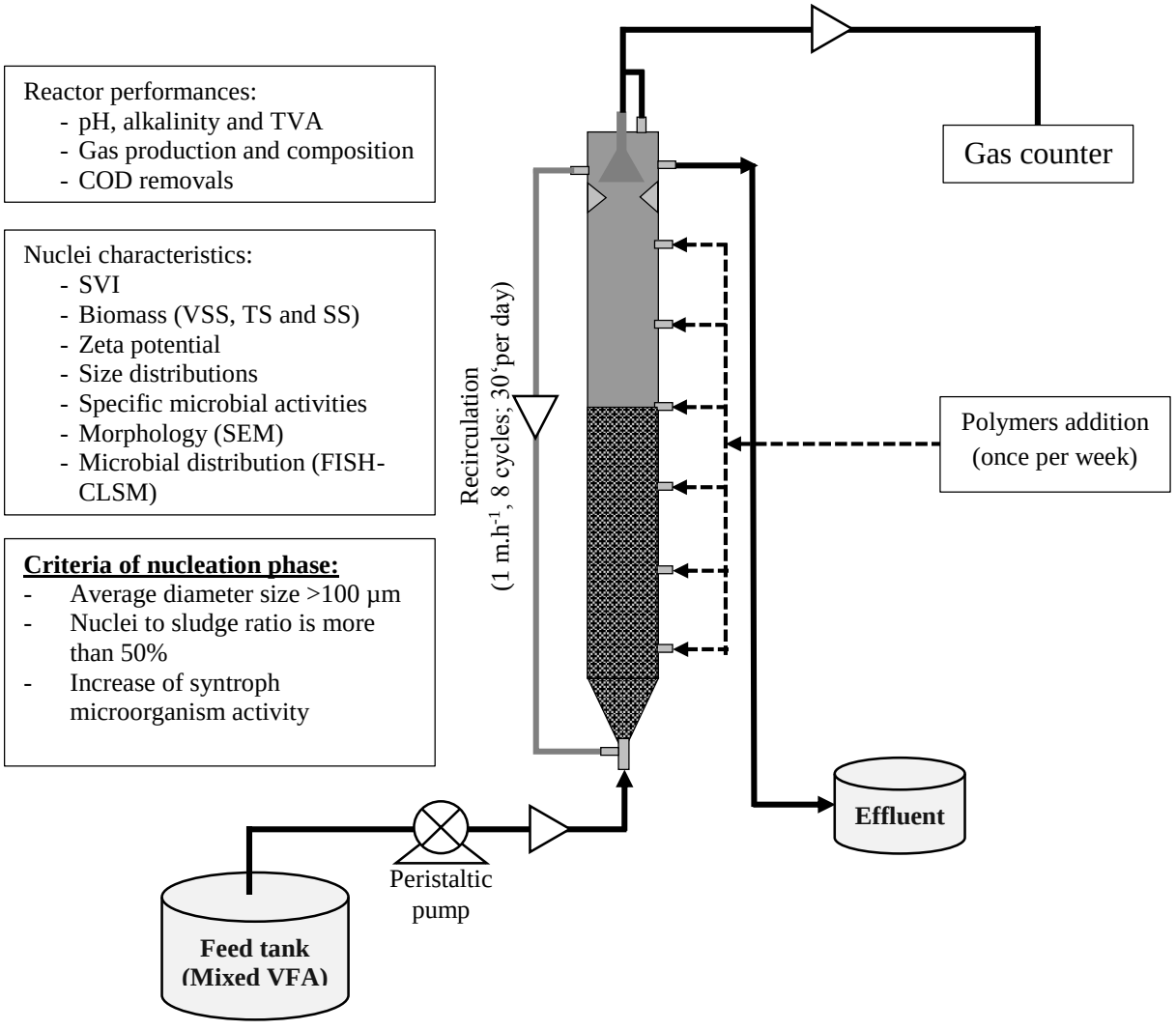


Figure 3.2 Schematic diagram of adaptation and nucleation phase

3.6.2 Early maturation phase (phase II)

The early maturation phase was conducted by stimulating EPS production by feeding reactors with glucose as the main carbon source. EPS production from glucose as substrate is higher than that of from volatile fatty acids and protein [24]. EPS enhance anaerobic granulation by trapping other microorganism and strengthen the granules structure [27]. The substrate was switched from mixed VFA to glucose when nuclei to total sludge ratio reached more than 50% (one reactors). Granule was defined as microbial aggregates with diameter size more than 600 µm [10]. The stability of granule, i.e., granule ratio, specific microbial activities and morphology, was used to determine the period of the early maturation phase.

The OLR of the reactors in the nuclei maturation phase was stepwisely increased from 0.5 kg $COD.m^{-3}.d^{-1}$. There was no polymer additions in UASB reactors during this phase. Therefore, EPS was considered as the main factor affecting granulation in early maturation phase. The scheme of early maturation phase can be seen in **Figure 3.3**. The parameters which were analyzed for this phase is similar with the analysis for the nuclei formation phase which included reactor performances, physicochemical and microbial characteristics of granules.

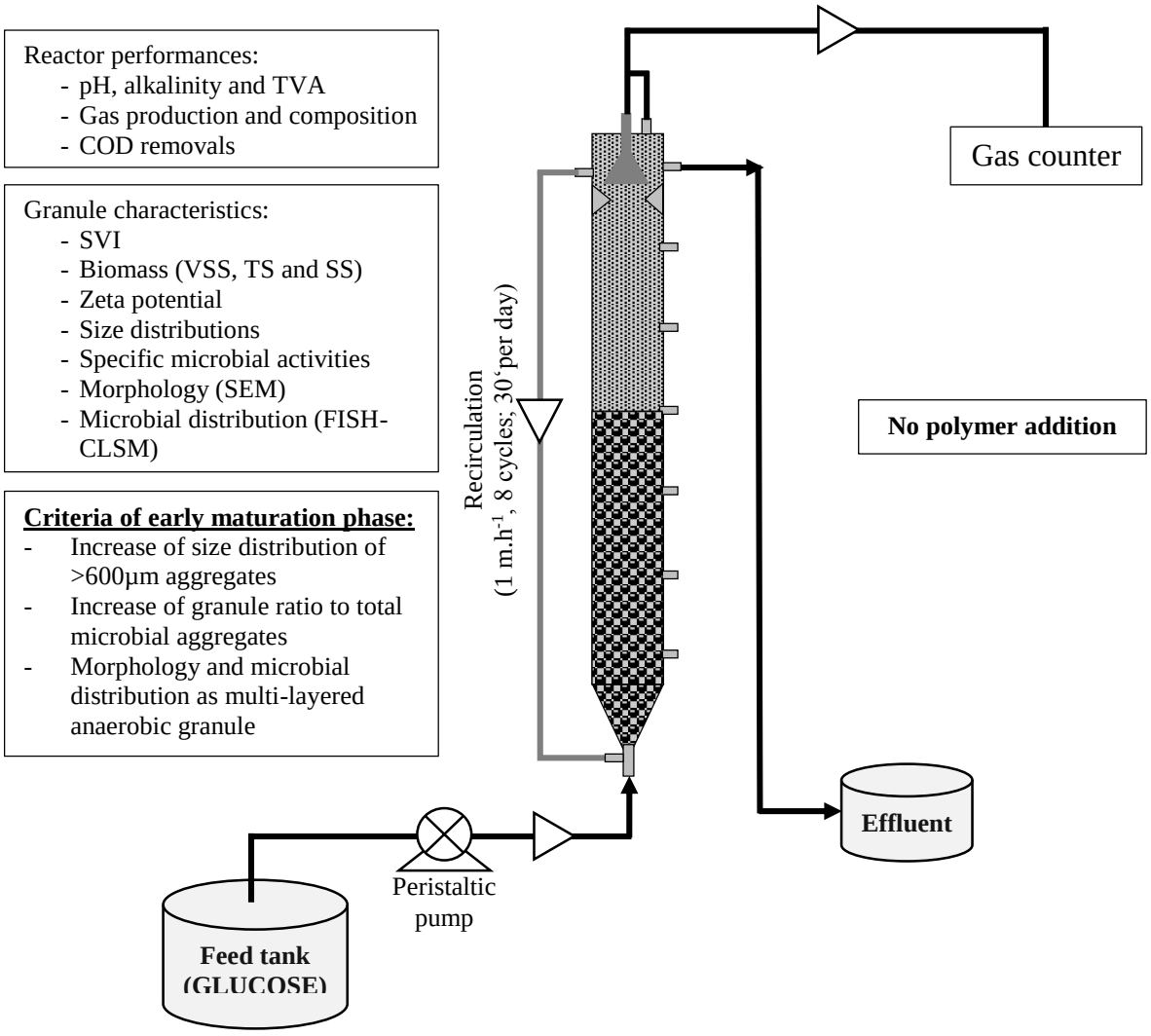


Figure 3.3 Schematic diagram of early maturation phase

3.7 Monitoring reactor performances

The performance and stability of the UASB reactors during nucleation and early maturation were monitored through the analysis of COD, VSS, SS, pH and alkalinity based on the APHA standard method [82] as shown in **Table 3.3**. Biogas production and composition were also monitored by water replacement method and gas chromatography (GC) analysis. Reactor performances were daily analyzed until steady state condition.

Table 3.3 Methods for analyzing the reactor performance

Parameter	Analysis method
pH	pH meter
Alkalinity	Titration method
TVA	Titration method
COD	Close reflux [82]
TS, VSS and SS	Standard method [82]
Gas production	Water replacement and gas counter
Gas composition	GC analysis

3.8 Microbial aggregate characteristics

3.8.1 Physiochemical characteristics

Physiochemical characteristics of microbial aggregates were represented by the value of SVI, size distribution, zeta potential and compositions.

3.8.1.1 Sludge volume index (SVI)

SVI represented the settling ability of microbial aggregates [82]. The SVI value was defined as the volume in ml occupied by 1 g sludge after settling for 30 minutes. The value of SVI can be calculated by the formulation below.

$$\text{SVI} = \frac{\text{settled sludge volume (ml/l)x1000}}{\text{suspended solids (mg/l)}}$$

3.8.1.2 Size distribution

Microbial aggregates can be considered as solid particles based on a physical definition. Therefore, the particle size and its distribution were determined by a laser particle analyzer using Mastersizer 2000 (Malvern, UK). This instrument can analyze the particle having size range between 0.02 to 2000 μm. The size distribution of microbial aggregates was represented as percentage of certain group size to total particles analyzed. The average diameter size was calculated based on De Brouckere or volume mean moment D(4,3) method which was integrated in laser particle analyzer, Mastersizer. The analysis of the size distribution was conducted in a laboratory of The Department of Chemical Engineering, King Mongkut's University of Technology Thonburi (KMUTT), Thailand.

3.8.1.3 Zeta potential

Zeta potential analysis is also considered to be a solid particle. The value of the zeta potential indicates charge behavior of microbial aggregates within solution (wastewater). The microbial aggregation or coagulation is commonly at zeta potential value between -30 to +30 mV. Therefore, Particle with zeta potential more positive than +30 mV or more negative -30 mV are normally considered stable or no flocculation. At isoelectric point or zeta potential near zero mV, the coagulation is the most expressive. Zeta potential of microbial aggregates was analyzed by Zetasizer (Malvern, UK). The analysis of zeta potential was conducted in MTEC Lab, National Science and Technology Development Agency (NSTDA), Thailand.

3.8.1.4 EPS compositions

The EPS was composed from various components, such as protein, polysaccharides, humic substances, nucleic acid, lipids and others. However, many researchers considered that protein and polysaccharide were the main composition of EPS affecting on microbial granulation [27, 61, 75]. EPS extraction from microbial aggregates in this research was based on modified chemical extractions in cold condition [64, 83]. The microbial aggregates with wastewater was ultrasounded for 3 minutes with 40 W. Then, the dispersed aggregates was added with formaldehyde 60 µl and incubated for one hour. The cold extraction was then applied by addition of NaOH 1 M and incubation for 3 hours in cold condition (ice). Centrifugation at 10,000 rpm for 20 minutes at 4^{o}C was applied to separate supernatant (contained total EPS) and sediment (sludge). The compositions of polysaccharides and protein in clear supernatant were analyzed with phenol sulfuric and Bradford methods, respectively [84-86].

3.8.2 Microbial characteristics

The microbial characteristics of anaerobic granules were represented by specific microbial activities, morphology of granules and microbial distribution in granules.

3.8.2.1 Specific microbial activities

The specific microbial activities included the activities of glucose degrading microorganisms (acidogens), propionate – and butyric degrading microorganisms (syntrophs) and acetate – degrading microorganisms (acetoclastic methanogens). The value of specific microbial activity was defined as the maximum gram substrate (COD) which was degraded by one gram microorganism (VSS) per day (g COD.g^{-1} VSS.d^{-1}). 5 ml of seed sludge was inoculated into a 50 ml vial contained various substrates (depend on specific

microorganism) in anaerobic conditions. Glucose was used as main substrate for determining the activity of glucose degrading microorganism (GDM) which represented acidogens microorganism. Propionate and butyrate were used as main substrates for determining the activity of propionate – and butyrate degrading microorganism (PDM and BDM), respectively. While acetate was main substrate for determining the activity of acetoclastic methanogens (ACM). The daily depletion of substrate (glucose, propionate and butyrate) during the time was analyzed for the activities of GDM, PDM and BDM. For the activities of ACM, the methane production was monitored and recorded. The glucose concentration was analyzed by dinitrosalycylic (DNS) acid method [87]. The propionate and butyrate concentrations were analyzed by GC with flame ionized detector (FID). The condition of GC-FID were:

- Injector: temperature 250^{o}C, split mode
- Column: stabiwax – DA with length 30 m, inner diameter 0.25 mm and film thickness 0.25 µm, temperature 60^{o}C
- Detector: Flame Ionization Detector (FID). Temperature 250^{o}C

While gas production and composition were analyzed by the water replacement method and GC with thermal conductivity detector (TCD), respectively.

3.8.2.2 Morphology of microbial aggregates

Scanning electron microscopy (SEM) was used to analyze the morphology of microbial aggregates. The sample preparations for an SEM involved fixation in 2% paraformaldehyde, 2% glutaraldehyde and cacodylate buffer 0.05 M overnight at 4^{o}C [88] . Fixed granules were gold coated and then viewed in a scanning electron microscope, JEOL model JSM-5410LV Japan.

3.8.2.3 Microbial distribution inside anaerobic granule

Fluorescence in situ hybridization (FISH) combined with confocal laser scanning microscopy (CLSM) method was used to determine the microbial distribution inside anaerobic granules. The *Eubacteria* and *Archaea* groups were analyzed to represent the bacteria and methanogen as general. FISH procedure was conducted based on Raskin, et.al., (1994) [89]. The microbial aggregates are dehydrated in alcohol series of 50, 80 and 100% for 3 minutes each. Hybridization of dehydrated microbial aggregates was conducted with hybridization buffer and probes for 3 hours at 46^{o}C. The probes for *Eubacteria* and *Archaea* characteristics are shown at **Table 3.4**. After hybridization, the microbial aggregates were then washed by washing buffer twice at 48^{o}C for 20 minutes each. The microbial aggregates

was then placed into concave object glass. Antifade solution was added above microbial aggregates prior to be closed with cover glass. The FISH samples are analyzed and monitored by CLSM with specific color spectrum for green (FITC) and red (cy3).

Table 3.4 Oligonucleotide probes for *Eubacteria* and *Archaea* [90]

Probe name	**Target group**	**Probe sequence (5'-3')**	**Formamide (%)**	**label**
ARC915	*Archaea* domain	GTGCTCCCCCGCCAATTCCT	15	Cy 3 (red)
EUB338	*Bacteria* domain	GCTGCCTCCCGTAGGAGT	15	FITC (green)

CHAPTER 4
RESULTS AND DISCUSSION

The main objectives of this research were to build syntroph adapted microbial nuclei (nucleation phase) and to form mature granules from nuclei (early maturation phase). In the nucleation phase, the operation of UASB reactors were conducted based on different cationic polymer additions. Reactor 1 (R1), reactor 2 (R2) and reactor 3 (R3) were operated during nucleation phase as control reactor (no cationic polymer addition), synthetic cationic polymer (dynafloc 8265) and natural cationic polymer (chitosan), respectively. The additions of cationic polymer were aimed to shorten nucleation time of microbial aggregates in R2 and R3. Nucleation phase was focused on the development of microbial nuclei (based on physical characteristics), i.e., nuclei ratio and average diameter size, and the increase of specific microbial group activities. Nucleation time was achieved when nuclei ratio and average diameter size of microbial aggregates in one of treated reactor were >0.5 and >100 µm, respectively. The adaptation of seed sludge under syntroph specific substrate (mixed VFA with COD ratio of acetate: propionate: butyrate = 2:1:1) was concomitantly conducted for enhancing specific microbial activity, especially acetogens and methanogens. As a result, nucleation phase was expected to produce good microbial nuclei with high specific microbial activities.

After the nucleation phase, the early maturation phase was started in all reactors by switching the main carbon source from mixed VFA to glucose. There were no cationic polymer additions during early maturation phase. Glucose as main carbon source in this phase was used to enhance EPS production which could help the maturation of nuclei. EPS had several characteristics than cationic polymers such as its complex polymeric characteristics that were able to maintain and strengthen the structure of microbial aggregate. Early maturation phase was focused on the development of microbial granule characteristics such as granule ratio to total microbial aggregates, structure of granule and microbial distribution (FISH-CLSM) in granules. The early maturation phase was achieved when granule ratio was stable and structure and microbial distribution in granule were similar to mature granule.

4.1 Reactor performances and their stabilities

During the nucleation and early maturation phases, the performance and stabilities of UASB reactors, such as pH, TVA/Alkalinity ratio, COD removal and methane yield, were routinely observed. The operation conditions of UASB reactors can be seen in **Table 4.1**. The OLR was stepwisely increased as COD removal was stable (higher than 70-80%). During nucleation phase, syntroph specific substrate, i.e., mixed VFA with COD ratio of acetate: propionate: butyrate = 2:1:1, was used as main substrate. The addition of cationic polymers, dynafloc 8265 and chitosan, into R2 and R3, respectively, were conducted every weeks resulting on 9 times additions at the end of nucleation phase, day 58. Main substrate was then switched from mixed VFA to glucose at day 59 which indicated the start of early maturation phase. The nucleation phase was conducted until day 58 which was then followed by early maturation phase until day 118.

Table 4.1 The operating conditions of UASB reactors during nucleation and early maturation phase

Phase	Feeding	OLR (kg COD.m^{-3}.d^{-1})	HRT (day)	Operation day
Nucleation	Syntroph specific substrate (mixed VFA)	0.5	1	0 – 8
		0.7	1	9 – 17
		1.0	1	18 – 33
		1.5	2	33 – 58
Early maturation	Glucose	0.5	2	59 – 74
		0.7	2	75 – 88
		1.0	2	88 – 118

pH and TVA/alkalinity were observed during the nucleation and early maturation phases to investigate the stability of the reactors (**Figure 4.1**). During nucleation phase, pH of all reactors were in range 7.1 -7.7. Mixed VFA as main substrate in this phase contributed on this pH range. Mixed VFA, i.e., acetate, propionate and butyrate, was shortly degraded by anaerobic microbial groups in reactors to acetate and then methane as final product. pH range during nucleation phase was optimum for methane producing microorganism, methanogen, which was considered as the most sensitive microorganism in anaerobic

digestion system [1, 5]. Stable pH conditions related to the TVA/alkalinity ratio that represented the ability of alkalinity as a buffering agent for pH fluctuation due to VFA accumulation. The alkalinity was added into reactors to maintain alkalinity concentration inside reactors in range of 2000 – 2500 mg $CaCO_3.l^{-1}$. Highest TVA/alkalinity were observed in all reactors at early operation (OLR 0.5 kg $COD.m^{-3}.d^{-1}$) as approximately 0.4 which were probably caused by first encounter of microorganism to mixed VFA feeding. However, TVA/alkalinity ratio of all reactors were in range 0.2 – 0.3 indicating reactors were in good condition. TVA/alkalinity ratio in range 0.1 – 0.35 indicated healthy anaerobic reactor in which the alkalinity inside reactor was capable to accommodate pH fluctuation [91].

During the early maturation phase, the pH in all reactors were lower compared to that during the nucleation phase. Substrate switching from mixed VFA to glucose may contribute to this pH decrease. Glucose was more complex than mixed VFA in which the degradation of glucose resulted more intermediate products (organic acids) that caused pH decrease in all reactors [92]. pH values in R1 and R2 were lower than that in R3 which were followed by higher TVA/alkalinity ratio. From day 80, pH values of R1 and R2 were lower than 7.0 (approximately 6.7 – 6.8) and TVA/alkalinity were in range 0.4 – 0.5. pH value and TVA/alkalinity ratio in R1 were stable in range 7.0 – 7.1 and 0.3 – 0.4 until day 118, respectively. These pH conditions during early maturation phase may affect the activity of specific microbial groups inside reactors, especially methanogens.

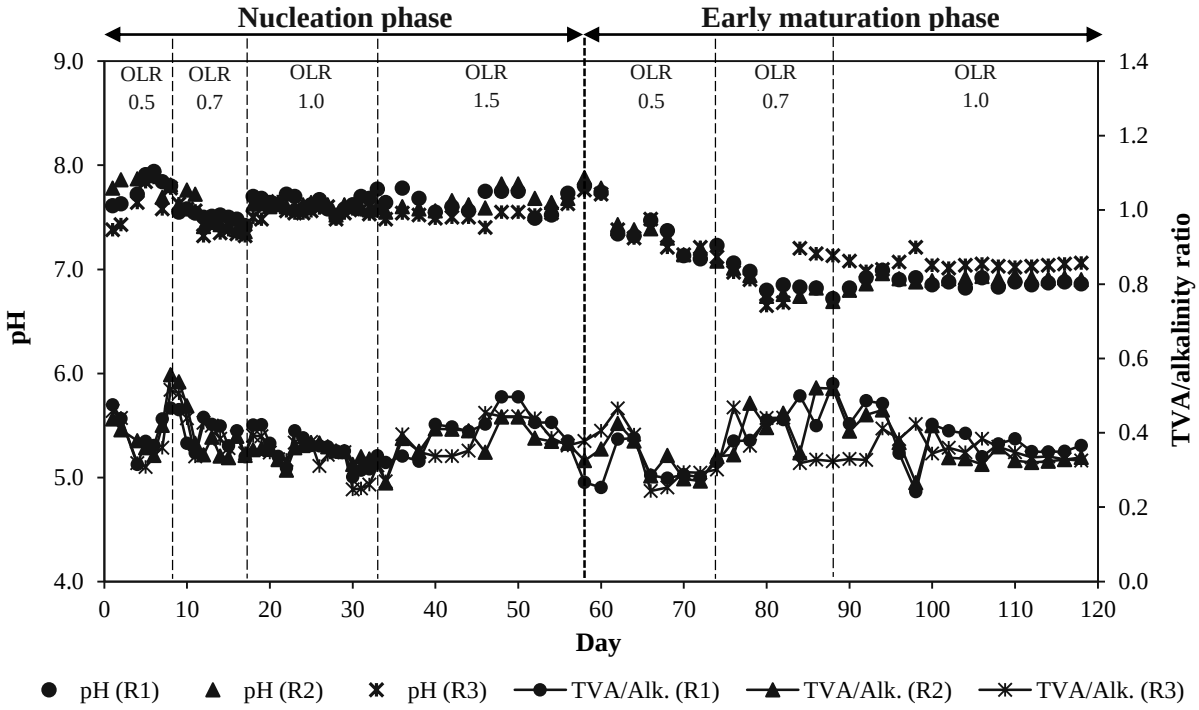


Figure 4.1 pH and TVA/alkalinity ratio during the nucleation and early maturation phases

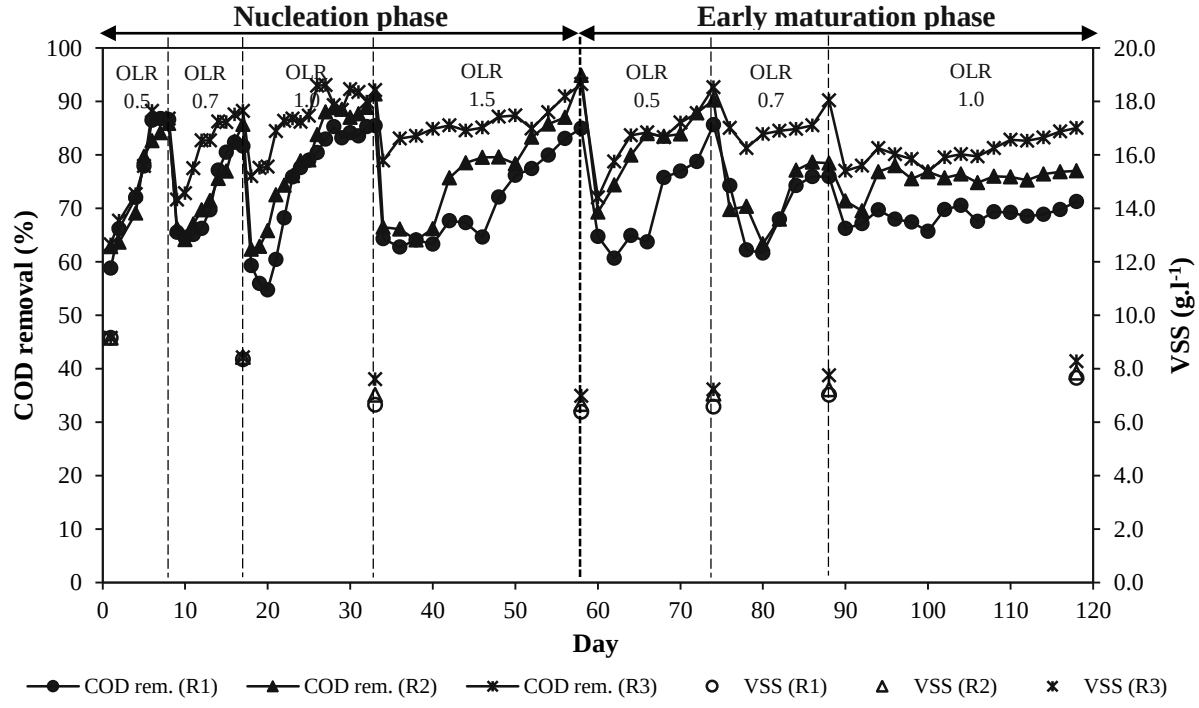


Figure 4.2 COD removal and VSS concentration during the nucleation and early maturation phase

Random variations of COD removal during nucleation and early maturation phases were observed (**Figure 4.2**). COD removal represented the ability of microorganism to

degrade organic matter inside reactors. The possibility of negative effect from cationic polymer additions inside reactors could be determined by observing COD removal. R1 as control reactor showed the lowest of COD removal (approximately 60 – 70%) from day 0 until day 58 compared to the treated reactors with cationic polymer additions, R2 and R3. COD removal efficiency in R2 and R3 was in the range of 70 – 90%. In R1 and R2, COD removals were found low (approximately 60%) at several days after increase of OLR (day 0-2, 9-12 and 34-42). The reason for these low COD removal was due to the first encounter of microorganism in R1 and R2 to higher OLR. These tendencies were not found in R3, chitosan addition, in which the COD removals in R3 were approximately 80% at several day after increase of OLR. Although R1 and R2 showed lower COD removal for several days after increase OLR, those COD removals were constantly high at day 58 as 80 – 90%. During early maturation phase (day 59 – 118), COD removals in all reactors showed similar tendencies in which low COD removal was found after increase OLR at day 59 – 62 (OLR 0.5 kg $COD.m^{-3}.d^{-1}$) , 76 – 80 (OLR 0.7 kg $COD.m^{-3}.d^{-1}$) and 90 – 94 (OLR 1.0 kg $COD.m^{-3}.d^{-1}$). However, COD removals in R1 and R2 were still lower until day 118 as approximately 60% and 70% than that in R3, respectively.

Low COD removal after increasing OLR during the nucleation phase is probably related to the biomass concentration inside the reactors i.e. volatile suspended solids (VSS). During nucleation, VSS concentration in all reactors tend to decrease until day 58 which were caused by biomass washout, slow growth microorganism and sampling. R1 as reactor with no polymer addition showed the lowest VSS concentration as a result of high biomass washout during nucleation phase as 9.2, 8.4, 6.7 and 6.4 $g.l^{-1}$ at day 0, 17, 33 and 58, respectively. Low COD removals in R1 may also related to food to microorganism (F/M) ratio, in which F/M ratio in R1 was found higher than that in R2 and R3. While, in reactors with cationic polymer additions, R2 and R3, VSS concentrations were higher than in R1 resulting on lower F/M ratio which caused the optimum degradation of organic matter (mixed VFA). Although VSS concentration tend to increase during early maturation phase, COD removals in R1 were still low as approximately 60% until day 118. These maybe caused by adverse effect of high F/M ratio during nucleation phase to the activity of specific microbial groups inside reactor, especially methanogens.

Higher COD removals in R2 and R3 than in the control reactor (R1) during the nucleation and early maturation phases indicated that there were no issues of toxicity and limitations of substrate diffusion, which were caused by the addition of cationic polymers

addition. The addition of dynafloc 8265 and chitosan as cationic polymer to shorten nucleation time did not adversely affect on COD removal efficiencies in R2 and R3, respectively. Wang, et al., (2005) stated that the utilization of cationic polymers should be carefully selected for granulation study because of the possibility of its toxicity and poor contact between microorganisms to substrate. Microbial granules cannot be judged in term of "large" only, but also in term of their activity. Acrylamide chitosan graft copolymer (ACGC) resulted on large microbial aggregates but low VSS/SS ratio and microbial activity was found in this microbial aggregates due to monomer toxicity of ACGC [30]. The addition of cationic polymers could result on limitation of substrate diffusion or poor contact between microorganism and substrate [22, 29]. The addition of cationic polymers on seed sludge could form barrier layers surrounding a bacterial cells resulting on the resistance of substrate diffusion into each microbial cell. Cationic polymers also enhanced the thickness of sludge bed inside reactor resulting poor contact between microorganism and substrate. Bhunia and Ghangrekar, (2008) used thick inoculum concentration as more than 110 g $SS.l^{-1}$ which resulted on low COD removal in reactor with cationic polymer additions. However, the inoculum used in this present study was approximately 10.7 g $SS.l^{-1}$ which was far less thick than in previous studies. Higher COD removals in R2 and R3 confirmed that dynafloc 8265 and chitosan were suitable cationic polymers for granulation considered for toxicity and poor contact between microorganism and substrate issues. However, natural cationic polymers, e.g., chitosan, was more preferred than synthetic cationic polymers for the reason of environmental safety.

This study was not mainly focused on methane production and yield. However, those parameters were still important to consider in an anaerobic digestion system. As a final result of anaerobic digestion, methane production was main factor for determining the successful operation of anaerobic reactors. In this study, methane productions in all reactors increased along increase of OLR during both nucleation and early maturation phases (**Figure 4.3)**. The methane production among R1, R2 and R3 were not significant different during nucleation phase at low OLR of 0.5 - 1.0 kg $COD.m^{-3}.d^{-1}$ and early maturation at OLR 0.5 kg $COD.m^{-3}.d^{-1}$. However, methane productions in R3 were higher than that in R1 and R2 at high OLR of 1.0 – 1.5 kg $COD.m^{-3}.d^{-1}$ during nucleation phase and OLR of 1.0 kg $COD.m^{-3}.d^{-1}$ during maturation phase.

The highest methane production was found during the nucleation phase as approximately 2000 $ml.d^{-1}$ at OLR 1.5 kg $COD.m^{-3}.d^{-1}$. Higher biomass washouts in R1 and

R2 during the nucleation phase may cause these lower methane productions than that in R3 (**Figure 4.3**). Methane productions during nucleation phase in all reactors were higher compared to early maturation phase. At similar OLR of 1.0 kg $COD.m^{-3}.d^{-1}$, methane productions in R3 were approximately 1500 $ml.d^{-1}$ and 900 $ml.d^{-1}$ during nucleation and early maturation phases, respectively. Mixed VFA feeding during nucleation phase resulted higher percentage of methane and less carbon dioxide compared to glucose feeding during early maturation phase (**Table 4.2**). In this study, biogas productions at same OLR 1.0 kg $COD.m^{-3}.d^{-1}$ were not significant different as approximately 1500 and 1600 $ml.d^{-1}$ during both nucleation and early maturation phases, respectively. However, methane and carbon dioxide compositions in R3 at OLR 1.0 kg $COD.m^{-3}.d^{-1}$ during nucleation phase were in range 75 – 80% and 6 – 8%, respectively. At same OLR condition of OLR 1.0 kg $COD.m^{-3}.d^{-1}$, methane compositions in R3 were lower as approximately 63 – 66% which was followed by higher carbon dioxide composition as 23 – 25%. Mixed VFA was efficiently degraded into acetate and final products, methane and carbon dioxide through acetogenesis and methanogenesis. While glucose was degraded through acidogenesis, acetogenesis and methanogenis in which during acidogenesis and acetogenesis, the degradation of substrate could possibly produce more carbon dioxide (**Table 4.2**).

Table 4.2 The anaerobic degradation of glucose and volatile fatty acids [5, 93]

Phase	Reactions
Acidogenesis	• $C_6H_{12}O_6 \rightarrow 3CH_3COOH$ (glucose) (acetate) • $C_6H_{12}O_6 \rightarrow 2C_3H_6O_3$ (glucose) (lactate) • $C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2O$ (glucose) (butyrate) • $C_6H_{12}O_6 + 2H_2 \rightarrow 2\ CH_3CH_2COOH + 2H_2O$ (glucose) (propionate) • $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3CH_2OH + 2CO_2 + 2H_2$ (glucose) (ethanol)
Acetogenesis	• $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$ (glucose) (acetate) • $CH_3CH_2OH + 2H_2O \rightarrow CH_3COOH + 2H_2$ (ethanol) (acetate) • $CH_3CH_2COOH + 3H_2O \rightarrow CH_3COOH + CO_2 + H_2 + 3H_2O$ (propionate) (acetate) • $CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2O$ (butyrate) (acetate)
Methanogenesis	• $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$ (carbon dioxide) + (hydrogen) → methane • $2CH_3CH_2OH + CO_2 \rightarrow CH_4 + 2CH_3COOH$ (ethanol) (methane) + (acetate) • $CH_3COOH \rightarrow CH_4 + CO_2$ (acetate) (methane) + (carbon dioxide)

Methane yield was defined as the amount of methane produced for a given quantity of organic matter that has been removed and which can be used to characterize the metabolic activity of the methanogenic ecosystem. It was also observed to represent the balance between substrate degradation and methane production in anaerobic digestion system. Methane production and yield increased along with increase of OLR in all reactors during

nucleation and early maturation phase which were probably caused by gradual adaptation of microorganism inside reactors to higher load of substrate. **Figure 4.3** shows that there were no significant different of methane yields in all reactors during nucleation phase at OLR 0.5 – 1.0 kg $COD.m^{-3}.d^{-1}$ and early maturation phase at OLR 0.5 kg $COD.m^{-3}.d^{-1}$. However, at higher OLR of 1.5 kg $COD.m^{-3}.d^{-1}$ during nucleation phase and OLR 1.0 kg $COD.m^{-3}.d^{-1}$ during early maturation phase, methane yields in R3 were significantly higher than that in R1 and R2. Methane production and yield in R3 during nucleation phase at OLR 1.5 kg $COD.m^{-3}.d^{-1}$ were observed as approximately 0.3 $m^{-3}CH_4.kg^{-1}$ $COD_{removed}$ which were higher than in R1 and R2. R3 also showed higher methane yield than that in other reactor during early maturation phase at OLR 1.0 kg $COD.m^{-3}.d^{-1}$ as approximately 0.24 m^{-3} $CH_4.kg^{-1}$ $COD_{removed}$. The result of methane yield closely related with methane production in each reactor. The methane yield in all reactors during early maturation phase was lower than that during nucleation phase. Theoretically, both methane yields of carbohydrates and mixed VFA are 0.35 l CH_4/g COD [94]. However, the different substrate used in each phase may result on different methane yield in same OLR in which glucose degradation contributed on higher carbon dioxide production which could decrease the methane yield during early maturation phase as shown at **Table 4.2**. Glucose was degraded by anaerobic microorganism from acidogenesis, acetogenesis and methanogenesis phase releasing more carbon dioxide, thus decreased the methane yield. However, mixed VFA was only degraded through acetogenesis and methanogenesis causing more efficient methane production.

Based on the results of the methane production and yield, the control reactor (R1) had the lowest methane production and yield as compared to reactors with cationic polymer addition (R2 and R3). Higher biomass washout in R1 as a result from the absence of cationic polymer addition, probably caused these low methane production and yield. Methane productions and yield in R2 and R3 were found higher than control reactors. These results indicated that those reactors with cationic polymer addition could retain more microorganism inside reactor due to microbial aggregation which prevented biomass washout from reactors. Moreover, the negative effects such as toxicity of cationic polymers and limitation of substrate diffusion were not observed in R1 and R2 considering their high methane production and yields.

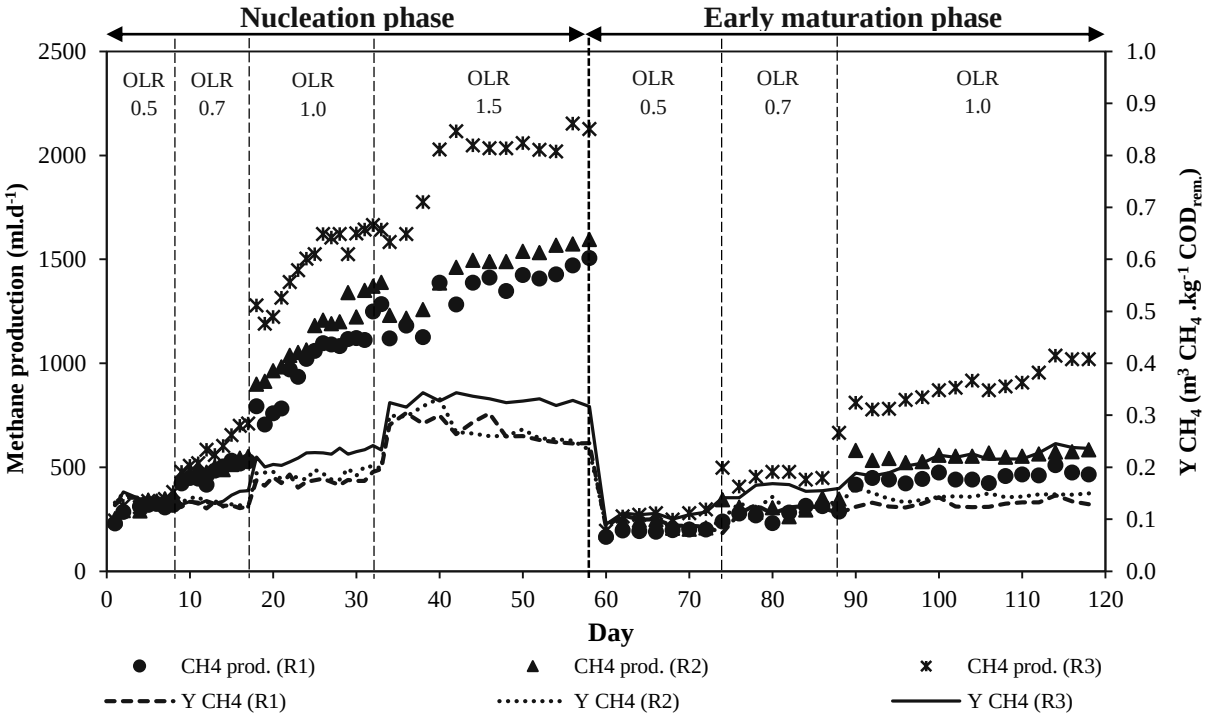


Figure 4.3 Methane production and yield during nucleation and early maturation phases

4.2 Size distribution

4.2.1 Size distribution of microbial aggregates during UASB operation

Beside the characteristics of microbial activity and distribution, good granule nuclei or mature granules, which is a tiny microbial ecosystem, can be determined based on its physical characteristics, such as size distribution and average diameter size. Based on the physical characteristics, the nuclei and granules were defined as microbial aggregates with diameter 100 – 600 µm and over than 600 µm, respectively [10, 46]. In this study, size distributions of microbial aggregates were divided into three main groups as 0 – 100 µm (representing the initial aggregates), 100 – 600 µm (nuclei) and >600 µm (granule). The size distribution of microbial aggregates in this study was analyzed by laser particle analyzer (wet method) which was able to analyze the microbial aggregates with size range of 0.02 – 2000 µm.

The results of size distribution analysis showed that initial microbial aggregates with size ranges of 0-100, 100-600 and >600 µm were approximately 99.4, 0.6 and 0%, respectively (**Figure 4.4**). Microbial aggregates with size range less than 100 µm was dominant than other size range which resulted on the initial average diameter size as 47 µm.

As mentioned previously, the initial seed sludge was firstly screened by 100 µm sieving instrument prior to inoculate into reactors. However, nuclei (size range 100-600 µm) was also found in initial seed sludge as 0.6% from total microbial aggregates. The presence of small amount of nuclei in initial seed sludge was probably caused by the self-aggregation of some microorganisms during storage or before inoculation into reactors.

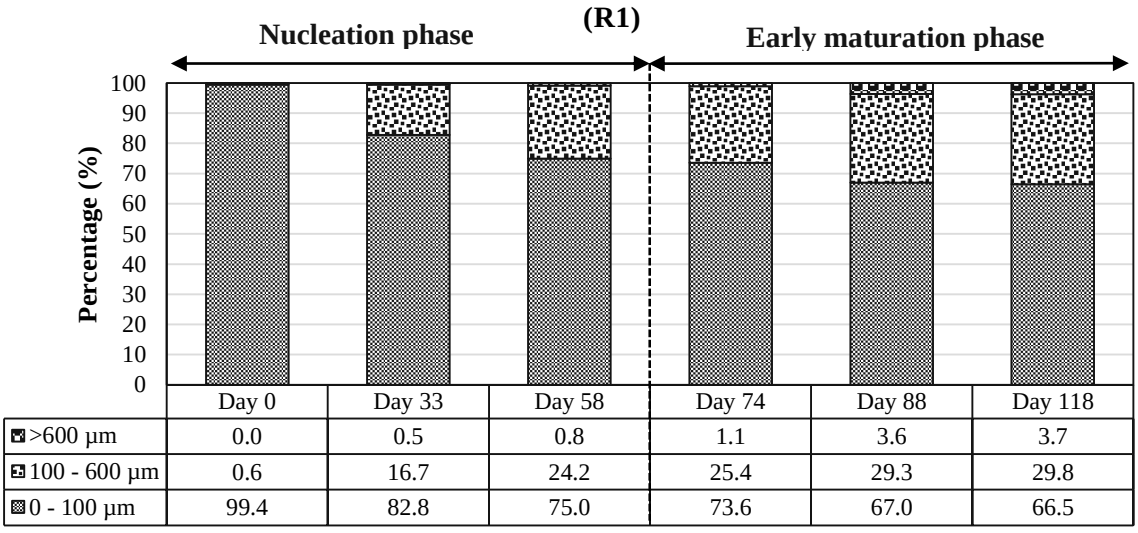
The developments of the sizes of the microbial aggregates size during the nucleation phase were caused by microbial self-aggregation or the addition of cationic polymers. The criteria for nucleation phase in this study was based on average diameter size (>100 µm) and nuclei ratio (>0.5 or 50%). Size distribution of microbial aggregates in R3 showed significant development from day 0 until day 58 (end of OLR 1.5 kg $COD.m^{-3}.d^{-1}$). **Figure 4.4** showed that size distribution of nuclei (100-600 µm) in R3 significantly increased as 0.6, 24.6 and 55.1% of total microbial aggregates at day 0, 33 and 58, respectively. Those increases of nuclei were followed by the decreases of microbial aggregates with size range less than 100 µm as 99.4, 73.6 and 36.8% of total microbial aggregates at day 0, 33 and 58, respectively. Size distribution of granule also slightly increased as 0, 1.8 and 8.2% of total microbial aggregates at day 0, 33 and 58, respectively. According to the size distribution of nuclei in R3, which reached 55.1% of total microbial aggregates, the nucleation time was considered to complete at day 58. The addition of chitosan in R3 successfully increased the size of microbial aggregates. Chitosan triggered on microbial aggregation in R3 resulting stable and large microbial aggregates.

The development of microbial aggregates was also observed in R1 and R2 during the nucleation phase. However, the increases of nuclei in R1 and R2 were not significantly high as in R3. The size distributions of nuclei in R2 (dynafloc 8265) were slightly higher than that in R1 (control reactor) as 0.6, 19.6 and 25.9% of total microbial aggregates at day 0, 33 and 58. While size distributions of nuclei in R1 at day 0, 33 and 58 were 0.6, 16.7 and 24.2% of total microbial aggregates. Granules were also found in R1 and R2 with low percentages in those both reactors as less than 3% of total microbial aggregates. The result of size distribution which was showed by nuclei in R2 (slightly different with size distribution of microbial nuclei in R1) were unexpected since the addition of dynafloc 8265 could trigger on microbial aggregation (preliminary jar test analysis). On jar test analysis, dynafloc 8265 was able to coagulate seed sludge resulting on clear supernatant and thick seed sludge aggregation. However, the ability of dynafloc 8265 to retain microbial aggregates as large aggregates was different compared with chitosan. Dynafloc 8265 was able to coagulate

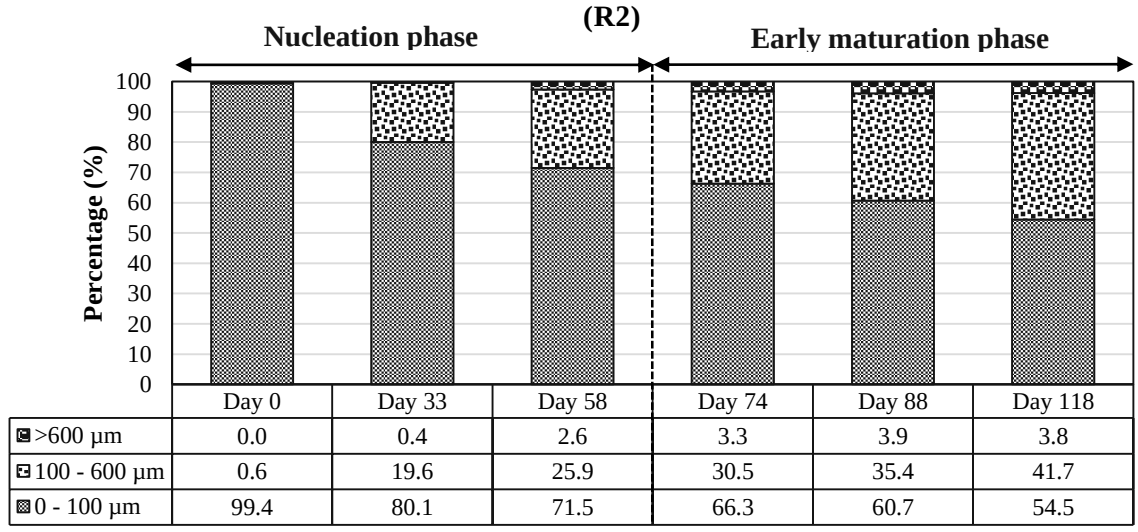
microbial seed sludge on the first 24 hours and then those large microbial aggregates tend to break during reactor operation due to weak aggregation. This tendencies resulted on small and slow microbial aggregation in R2. Moreover, although both dynafloc 8265 and chitosan were cationic polymers, the characteristics of dynafloc 8265 were very different with chitosan in which chitosan had characteristics similar with exopolymeric substances (EPS) [7, 25, 30].

Size distributions of microbial aggregates in all reactors were also observed during the early maturation phase, which was conducted without the addition of cationic polymers. Therefore, the development of microbial aggregates during was mainly affected by EPS which was enhanced during this phase by substrate switching to glucose. The main criteria for determining early maturation phase was the formation and development of granule (size range >600 µm). Early maturation phase was terminated when the size distribution of granules was stable or showed good stability of physicochemical and microbial characteristics. At the end of nucleation phase (day 58), granules in R1, R2 and R3 were approximately 0.8, 2.6 and 8.2% of total microbial aggregates, respectively. Although chitosan could trigger on the formation of microbial granule as 8.2% of total aggregates at day 58, it was still considered that chitosan was not strong enough for the formation of large microbial aggregates or granules. For that reason, EPS production was then enhanced by glucose feeding during early maturation phase. EPS was known as its role to strengthen the structure of microbial granule and bridge the neighboring nuclei or aggregates resulting on larger and stronger granules [27, 95].

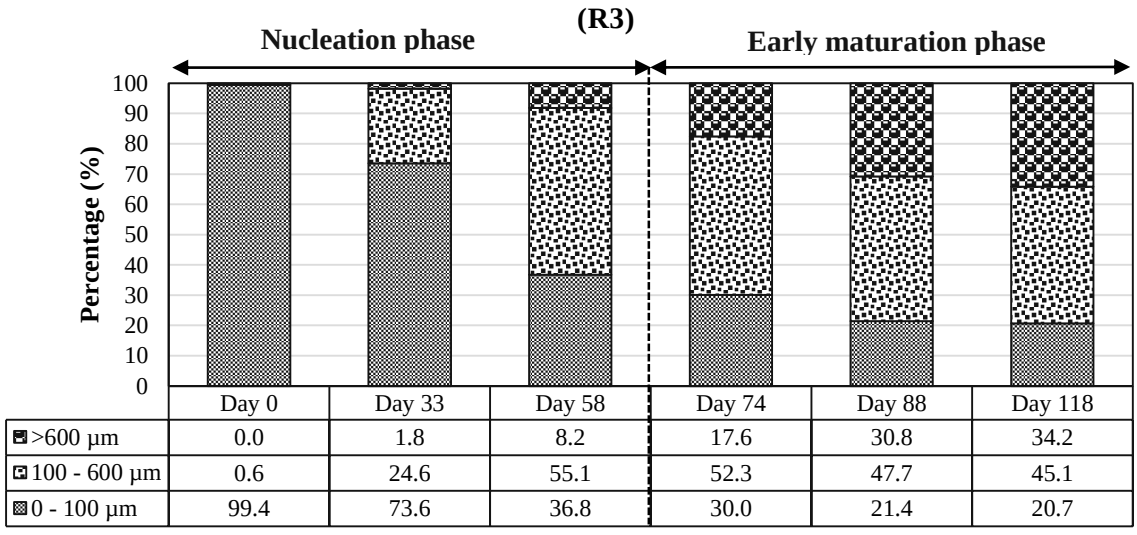
Figure 4.4 shows that the highest developments of granule were observed in R3. The size distributions of microbial granule in R3 at day 74, 88 and 118 were approximately 17.6, 30.8 and 34.2% of total microbial aggregates. The increase of size distributions of granule were followed by the decrease of microbial aggregates with diameter size less than 100 µm as 30, 21.4 and 20.7% of total microbial aggregates at day 74, 88 and 118, respectively. However, size distribution of nuclei in R3 tend to decrease during early maturation phase as 52.3, 47.7 and 45.1% of total microbial aggregates at day 74, 88 and 118, respectively. It seemed that the aggregation in R3 during early maturation phase did not only involve microbial aggregates with size less than 100 µm, but also nuclei. Nuclei with other nuclei or smaller microbial aggregates was probably associated by EPS producing larger microbial aggregates or granules.



	Day 0	Day 33	Day 58	Day 74	Day 88	Day 118
>600 µm	0.0	0.5	0.8	1.1	3.6	3.7
100 - 600 µm	0.6	16.7	24.2	25.4	29.3	29.8
0 - 100 µm	99.4	82.8	75.0	73.6	67.0	66.5



	Day 0	Day 33	Day 58	Day 74	Day 88	Day 118
>600 µm	0.0	0.4	2.6	3.3	3.9	3.8
100 - 600 µm	0.6	19.6	25.9	30.5	35.4	41.7
0 - 100 µm	99.4	80.1	71.5	66.3	60.7	54.5



	Day 0	Day 33	Day 58	Day 74	Day 88	Day 118
>600 µm	0.0	1.8	8.2	17.6	30.8	34.2
100 - 600 µm	0.6	24.6	55.1	52.3	47.7	45.1
0 - 100 µm	99.4	73.6	36.8	30.0	21.4	20.7

Figure 4.4 Size distributions of microbial aggregates in all reactors

Accumulation and adhesion of small similar microbial aggregates was also found in early formed microbial granules in UASB reactor fed by glucose as main substrate [24]. This indicated that agglomeration or aggregation of nuclei or small microbial aggregates could be the main key for understanding granulation process under enhanced EPS productions. Size distributions of granule were also observed in R1 and R2. However, the development of granules in R1 and R2 were slower compared to R3. The size distributions of nuclei in R1 at day 74, 88 and 118 were 25.4, 29.3 and 29.8% of total microbial aggregates, respectively, which were lower compared to size distribution of nuclei in R2 as 30.5, 35.4 and 41.7% of total microbial aggregates at day 74, 88 and 118, respectively. The increases in the size distributions of the nuclei in R1 and R2 were followed by the decrease of microbial aggregates with sizes less than 100 µm. From these results, it was assumed that the agglomeration or aggregation of small microbial aggregates was probably triggered in each reactor due to the effect of the EPS. The early maturation phase was completed at day 118 which considered on slow development of size distribution of microbial granules in R3 from day 88 to 118 as 30.8% to 34.2% of total microbial aggregates. At the end of early maturation phase, size distribution of microbial granules in R1, R2 and R3 were approximately 3.7, 3.8 and 34.2% of total microbial aggregates, respectively.

4.2.2 Average diameter size

Average diameter size is one of the most important properties of microbial aggregates. In general approach of granule characteristics, larger granules lead to higher settling ability. The increase of average sludge diameter can be used as a factor to determine the nucleation time. Nucleation time was defined as the augmentation process of microbial aggregates to diameter size >100 µm. Therefore, nucleation time in this study was terminated when average diameter size of microbial aggregates in one of reactors reached >100 µm. Average diameter sizes of microbial aggregates in all reactors were calculated by De Brouckere or volume mean moment D (4,3) method which was integrated in laser particle analyzer, Mastersizer.

The average diameter size of microbial aggregates in all reactors tends to increase during the nucleation and early maturation phases. The tendencies of increase of average diameter size were in lined with the increase of size distribution of microbial aggregates. Average diameter size of microbial aggregates in R3 was the highest among other reactors during nucleation phase **(Figure 4.5**). At day 0, 33 and 58, the average diameter sizes of microbial aggregates in R3 were approximately 47, 93 and 115 µm, respectively. While the

average diameter size of microbial aggregates in R2 were 47, 78, and 89 µm at day 0, 33 and 58, respectively. The lowest average diameter size was observed in microbial aggregates in R3 as 47, 71 and 76 µm at day 0, 33 and 58, respectively. From these results, nucleation time was firstly achieved in R3 at day 58 which showed average diameter size of microbial aggregates as 115 µm, higher than other reactors. The increase of average diameter size of microbial aggregates in R2 and R3 were affected by the addition of cationic polymers, dynafloc 8265 and chitosan. While, the increase of average diameter size of microbial aggregates in R1 was probably due to microbial self-aggregation since no addition of cationic polymer was conducted in this reactor. During the nucleation phase, other factors that contributed to microbial aggregation, such as EPS, are limited by mixed VFA feeding. Therefore, it could be concluded that the effect of EPS on microbial aggregation during nucleation phase was minimized. Based on the type of cationic polymers used during nucleation phase, chitosan could enhance the average diameter size of microbial aggregate better than dynafloc 8265.

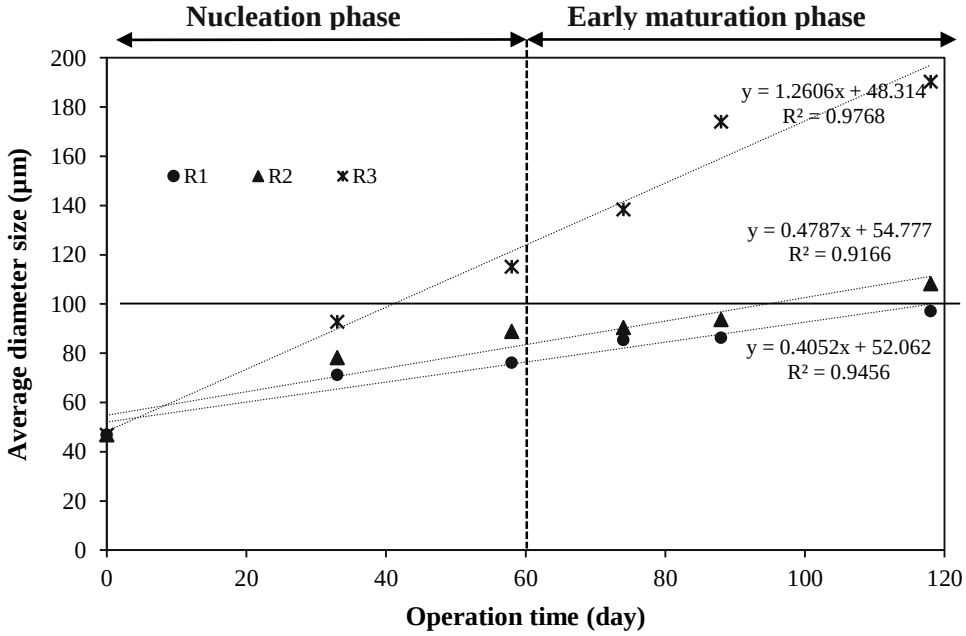


Figure 4.5 The average diameter size of microbial aggregates in all reactors

The average diameter of microbial aggregates in each reactor was also observed during the early maturation phase. The absence of cationic polymer addition in this phase indicated that the increase of average diameter size of microbial aggregates in each reactor was affected by EPS. **Figure 4.5** shows that the average diameter size of microbial aggregates increased until the end of the early maturation phase (day 118). The early

maturation phase was terminated because there was no significant increase of average diameter size of microbial aggregates in R3 from day 88 to 118. Moreover, size distribution of microbial granule also showed no significant increase of this parameter (**Figure 4.4**). During early maturation phase, the average diameter sizes of microbial aggregates in R3 were still higher than that in R1 and R2. The average diameter sizes of microbial aggregates in R3 were approximately 139, 174 and 190 µm at day 74, 88 and 118, respectively. While the average diameter sizes of microbial aggregates in R2 at day 74, 88 and 118 were approximately 90, 94 and 108 µm, respectively. The lowest of average diameter sizes of microbial aggregates during early maturation phase were observed in R1 as 85, 86 and 97 µm at day 74, 88 and 118, respectively. According to average diameter size, microbial aggregates in R2 reached nucleation time at day 88 as its average diameter size was approximately 108 µm (although size distribution of microbial nuclei in R2 was still approximately 42% of total microbial aggregates). The increases of average diameter size of microbial aggregates in all reactors were probably caused by EPS production during early maturation phase. Microbial nuclei resulted from nucleation phase affected as the starting point for the increase of microbial aggregates, especially in R3.

Linear relationships between the average diameter size and the operational time for microbial aggregates in all reactors during the nucleation and early maturation phases are also shown in **Figure 4.5**. The increases of average diameter size of each reactor were closely related with duration of reactor operation time as linear coefficient (R^2) of R1, R2 and R3 were approximately 0.98, 0.92 and 0.98 (more than 0.9), respectively. The average augmentation size rates of microbial aggregates in R1, R2 and R3 were approximately 1.3, 0.5 and 0.41 $\mu m.d^{-1}$, respectively.

4.2.3 Nuclei ratio

As mentioned before, the nuclei was physically defined as the microbial aggregates with diameter 100 – 600 µm [46]. Therefore, nucleation time can be stated as the development process of small microbial aggregates to aggregates with diameter size 100 – 600 µm. Nuclei ratio (%) was defined as the ratio between nuclei to total microbial aggregates inside reactor. Wu, et.al. (2009) investigated that the nuclei ratio was approximately 47% of total microbial aggregates in successful operation for the formation of nuclei in internal circulation anaerobic reactor. Therefore, in this study, it was proposed that the nucleation phase was achieved if one of reactors showed a nuclei ratio of more than 50%.

The main objective for the nucleation phase was to build a syntroph adapted nuclei and also to shorten the formation time of nuclei by the addition of cationic polymers. the nuclei ratio of microbial aggregates in all reactors tend to increase during nucleation phase (**Figure 4.6**). The highest nuclei ratio was observed in microbial aggregates in R3. At day 0, 33 and 58, the nuclei ratio in R3 significantly increased as 0.59, 24.6 and 55.1%, respectively. the nuclei ratio in R2 (dynafloc 8265 addition) were 0.59, 19.6 and 25.9% at day 0, 33 and 58, respectively. While the lowest nuclei ratio was found in R1 (control reactors) as 0.59, 16.7 and 24.2%. As described earlier, the addition of chitosan significantly increased microbial nuclei in R3 resulting higher nuclei ratio to total microbial aggregates than that in other reactors. The nuclei ratio in R2 were slightly higher compared to that in R1 during nucleation phase. Although dynafloc 8265 as cationic polymer was routinely added into R2, this cationic polymer was not efficient enough for triggering microbial aggregation. Dynafloc 8265 was good for settling or coagulating seed sludge for a short-time operation, such as water purification, but dynafloc 8265 could not maintain the structure of microbial nuclei, which was shown by strong microbial aggregation in the first 24 hours. The large microbial aggregates tend to disaggregate into smaller particles during the UASB operation.

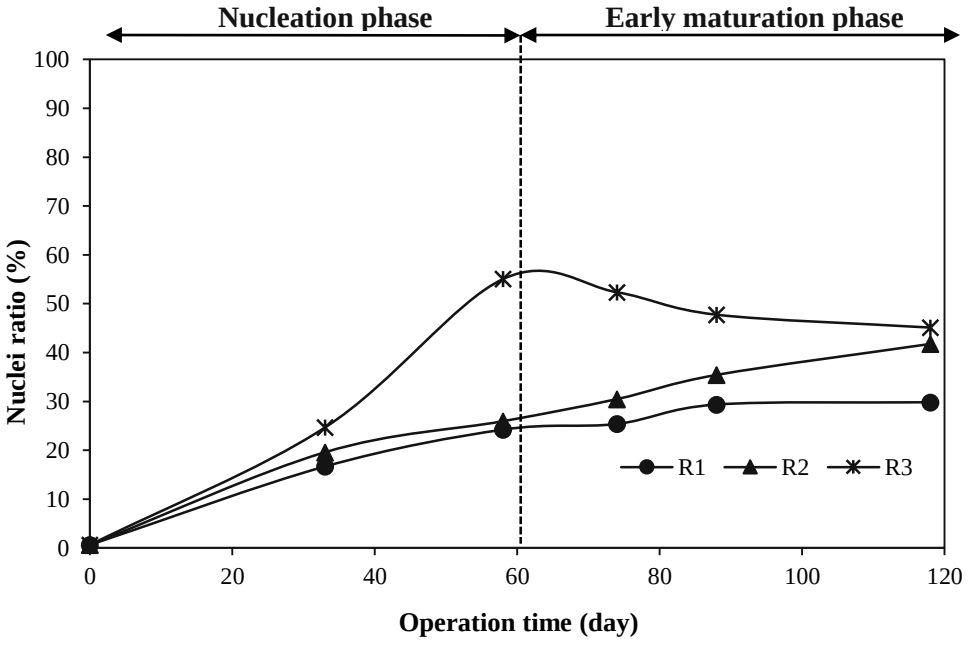


Figure 4.6 Nuclei ratio in all reactors during nucleation and early maturation phase

The development of nuclei ratio in R3 during the early maturation phase tended to decrease, which was different than during the nucleation phase. The developments of the

nuclei ratios in R1 and R2 during the early maturation phase still increased until day 118 which were similar to that during the nucleation phase. According to nuclei ratio, nucleation time had not achieved since the nuclei ratio in R1 and R2 at day 118 were approximately 29.8 and 41.7%, respectively. Nuclei ratio in R3 during early maturation phase were 52.4, 47.7 and 45.1% at day 74, 88 and 118, respectively. In R3, size distributions of nuclei and small aggregates tend to decrease during early maturation phase which were followed by increase of microbial granule. It seemed that small microbial aggregates and nuclei aggregated each other to form microbial granule or larger microbial aggregates.

4.2.4 Granule ratio

The formation of granules was the main objective for the early maturation phase. The granule ratio (%) represents the ratio between granule (microbial aggregates >600 µm) to total aggregates inside reactors. Although the formation of granule was main objective during early maturation phase, granule ratio was observed in each reactor since the first day of nucleation phase. **Figure 4.7** showed the development of granule ratio during nucleation and early maturation phase. Granule ratio in each reactor during nucleation phase was low. The highest granule ratio during nucleation phase were observed in R3 as 0, 1.8 and 8.2% at day 0, 33 and 58, respectively. Granule ratios in R1 and R2 were very low, less than 2.6%, during nucleation phase. At the initial day, no microbial granule was observed in each reactors. The effect of microbial self-aggregation (R1) and cationic polymer additions (R2 and R3) also affected the formation of microbial granules. However, it seemed that the effect of cationic polymers addition were not strong enough to maintain the structure of microbial granule resulting on disaggregation to be smaller microbial aggregates and nuclei. The addition of cationic polymer, dynafloc 8265 or chitosan in R2 and R3, respectively, was not too effective on the formation of granule since granule ratio in both reactors were very low. It was supposed that the characteristics of cationic polymers, especially chitosan, was effective only the formation of microbial nuclei (microbial aggregates with diameter size less than 600 µm).

Granule ratios were then observed during the early maturation phase as the formation of the microbial granules was the main objective in the early maturation phase. The characteristics of nuclei produced in each reactor during nucleation phase determined the formation of granule during early maturation phase. Granule ratio in R3 significantly increased during early maturation phase as 17.6, 30.8 and 34.2% at day 74, 88 and 118,

respectively. Nuclei ratio in R3 at day 58 which was approximately 55.1% could act as good starting point for the increase of granule ratio during early maturation phase.

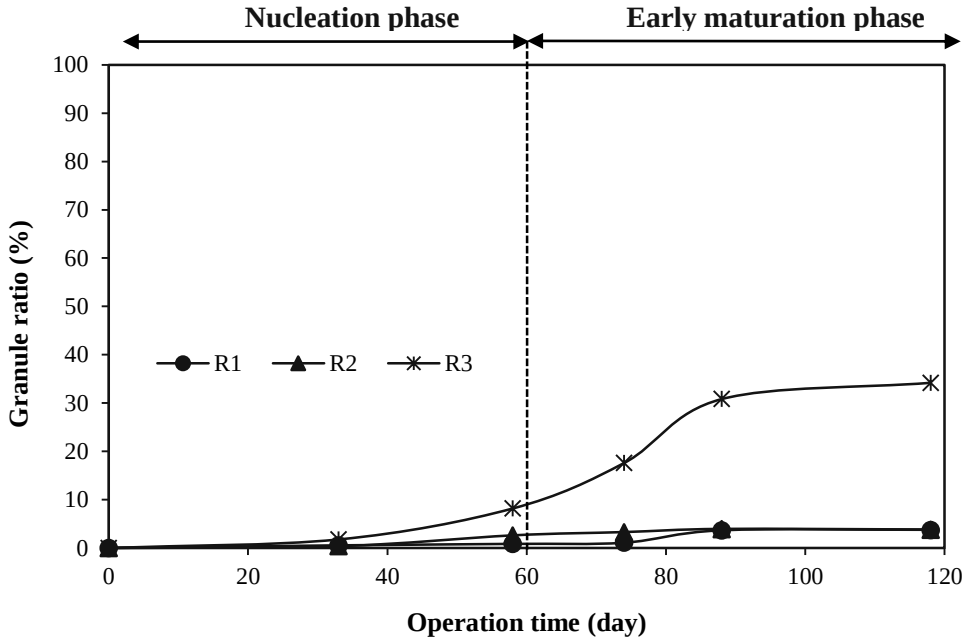


Figure 4.7 Granule ratios in all reactors during the nucleation and early maturation phase

It was supposed that the formation of granule was caused by nuclei aggregation with another nuclei and smaller microbial aggregates. These granule structure was then strengthened by EPS which was enhanced during early maturation phase. Granule nuclei in R1 and R2 tend to slightly increase (approximately <4%) during the early maturation phase. In this phase, only nuclei ratio in R1 and R2 significantly increased until day 118. The size distribution of microbial aggregates at the end of nucleation phase (day 58) determined the development of microbial granules during early nucleation phase. Higher nuclei ratio in R3 (55.1%) at day 58 lead to significant increase of granule ratio due to the aggregation of nuclei with other nuclei which was then strengthened by EPS. EPS as polymeric matrix which located around nuclei could trap other microorganism or small microbial aggregates to form larger microbial aggregates. This aggregation tendencies in R3 was supported by the developments of nuclei ratio in R3 which tend to decrease during early maturation phase. However, nuclei ratio in R1 and R2 was lower at day 58 as 24.2 and 25.9%, respectively, which were followed by size distribution of microbial aggregates <100 µm as 75 and 71.5%, respectively. These size distributions of microbial aggregates <100 µm in R1 and R2 significantly decrease during early maturation phase. It can be concluded that those

microbial aggregates (<100 µm) aggregated each other and formed microbial nuclei instead of microbial granule during early maturation phase.

4.3 Zeta potential

The zeta potential is a physical characteristic of suspension, which is exhibited by any particle inside of it. In a granulation study, microorganisms in a reactor can be considered to be physical particles in emulsion or suspension. It is supposed that if all the particles in emulsion have high negative or positive zeta potential, they tend to resist each other and no flocculation happened. However, the particles are able to come together and flocculate each other when the particles have low zeta potential value (near zero or isoelectric point). Particle with zeta potential more positive than +30 mV or more negative -30 mV are normally considered stable or no flocculation. The characteristic of microbial surface charges during granulation study under the addition of cationic polymers was important factor to consider. Zeta potential analysis could be used to observe the microbial surface charges in each reactor. The additions of cationic polymer, dynafloc 8265 and chitosan, were aimed to provide positive charges into reactors which were able to decrease the negativity of microbial surface charges. By decreasing the negativity of microbial surface charges, the microbial flocculation or aggregation could be stimulated.

The zeta potential values in each reactor tended to increase during the nucleation phase (**Figure 4.8**). Initial zeta potential value for seed sludge was approximately -26.4 mV. Chitosan addition significantly affected on the increase of zeta potential value in R3 as -26.4, -19.7 and -10.5 mV at day 0, 33 and 58, respectively. While zeta potential value in R2, dynafloc 8265 addition, slightly increased as -26.4, -23.2 and -22.15 mV at day 0, 33 and 58. The lowest value of zeta potential were observed in R1, control reactor, as -26.4, -24.4 and -24.5 mV at day 0, 33 and 58, respectively. From these results, chitosan addition was able to decrease the negativity of microbial surface charges than dynafloc 8265.

The development of zeta potential values in each reactor during the early maturation phase tended to slightly increase (**Figure 4.8**). The absences of cationic polymer addition during early maturation phase probably caused slight increase of zeta potential values, especially in R2 and R3. Therefore, the enhancement of EPS production during early maturation phase may affect on zeta potential values in each reactors. Zeta potential values in R3 were -8.2, -7.1 and -6.6 mV at day 74, 88 and 118. While zeta potential values in R2

at day 74, 88 and 118 were -20.5, -19.2 and -19.1 mV, respectively. Zeta potential values in R1 were the lowest as -23.3, -21.4 and -20.3 mV, respectively. Zeta potential values in R3 were higher than that in R1 and R2, which stimulated microbial aggregation

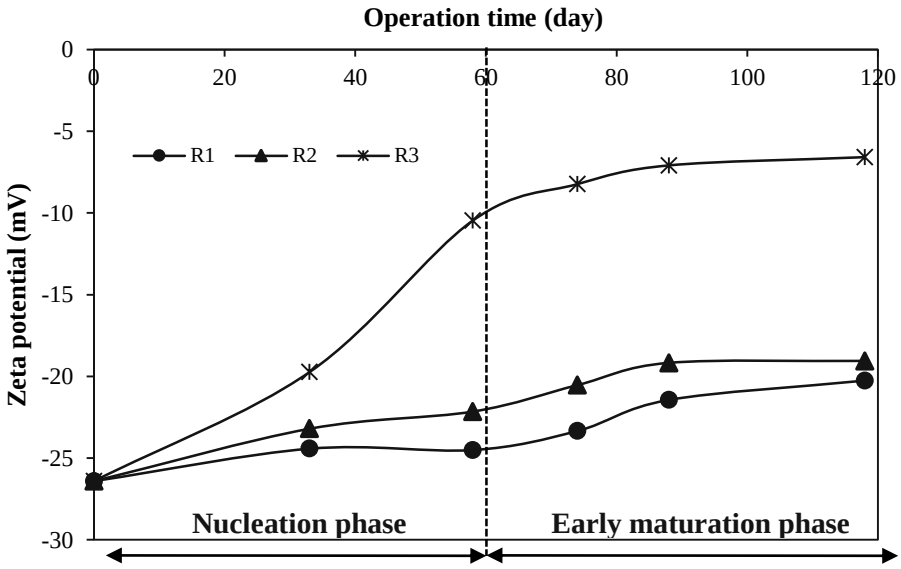


Figure 4.8 Zeta potential of microbial aggregates in all reactors during the nucleation and early maturation phases

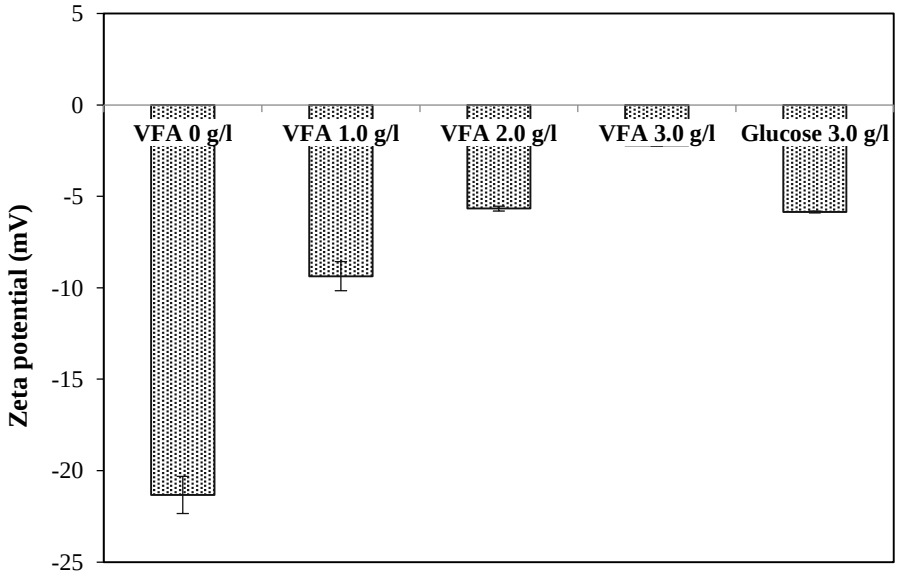


Figure 4.9 Zeta potential values of mixed VFA and glucose.

The zeta potential values of substrates i.e. mixed VFA and glucose, during the nucleation and early maturation phase were observed in order to know the possibility of bias effects of cations from substrates fed into each reactor. During the nucleation phase, the increases of mixed VFA concentration were followed by the increase of zeta potential values (**Figure 4.9**). Zeta potential values of mixed VFA with concentration 0, 1.0, 2.0 and 3.0 $g.l^{-1}$ were approximately -21.3, -9.4, -5.7 and -2.21 mV, respectively. While zeta potential value of glucose 3.0 $g.l^{-1}$ was approximately -5.9 mV. High concentration of those substrate as main substrate during nucleation and early maturation phases showed less negative zeta potential values than microbial negative charge. Theoretically, those zeta potential characteristics of substrates contributed on the decrease of microbial negative charge during nucleation and early maturation phase. However, the results showed that high increase of zeta potential was only happened in R3 while R1 and R2 showed similar tendencies for their low increase of zeta potentials. From the results, it can be concluded that there was no significant effect of substrate concentrations on the increase of zeta potential of microbial aggregates in each reactor. The lack of substrate concentration effect on zeta potentials of microbial aggregates in all reactors was probably because those substrates had been degraded into its simple products, such as methane and carbon dioxide.

As previously described, the addition of cationic polymers was able to decrease the negativity of microbial surface charges. Zeta potential values related to the microbial surface charges. Linear relationship was plotted between zeta potential values and large microbial aggregates (more than 100 µm) ratio to total aggregates during nucleation and early maturation phases. Large microbial aggregates (>100 µm) represented microbial nuclei (diameter size 100 – 600 µm) and microbial granule (diameter size >600 µm). **Figure 4.10** shows that there are close relationships between the increase of zeta potential values (mV) and large microbial aggregates ratio during nucleation and early maturation phases which were indicated by the linear coefficients (R^2) of R1, R2 and R3 as 0.78, 0.95 and 0.98, respectively. The closest relationship between the increase of zeta potential values and the increase of large microbial aggregates ratio (R^2 of R3 was 0.98). Therefore, it can be concluded that the addition of natural cationic polymer, chitosan, into R3 could significantly decrease the negativity of microbial surface charge in R3 leading to the formation of larger microbial aggregates during nucleation and early maturation phases.

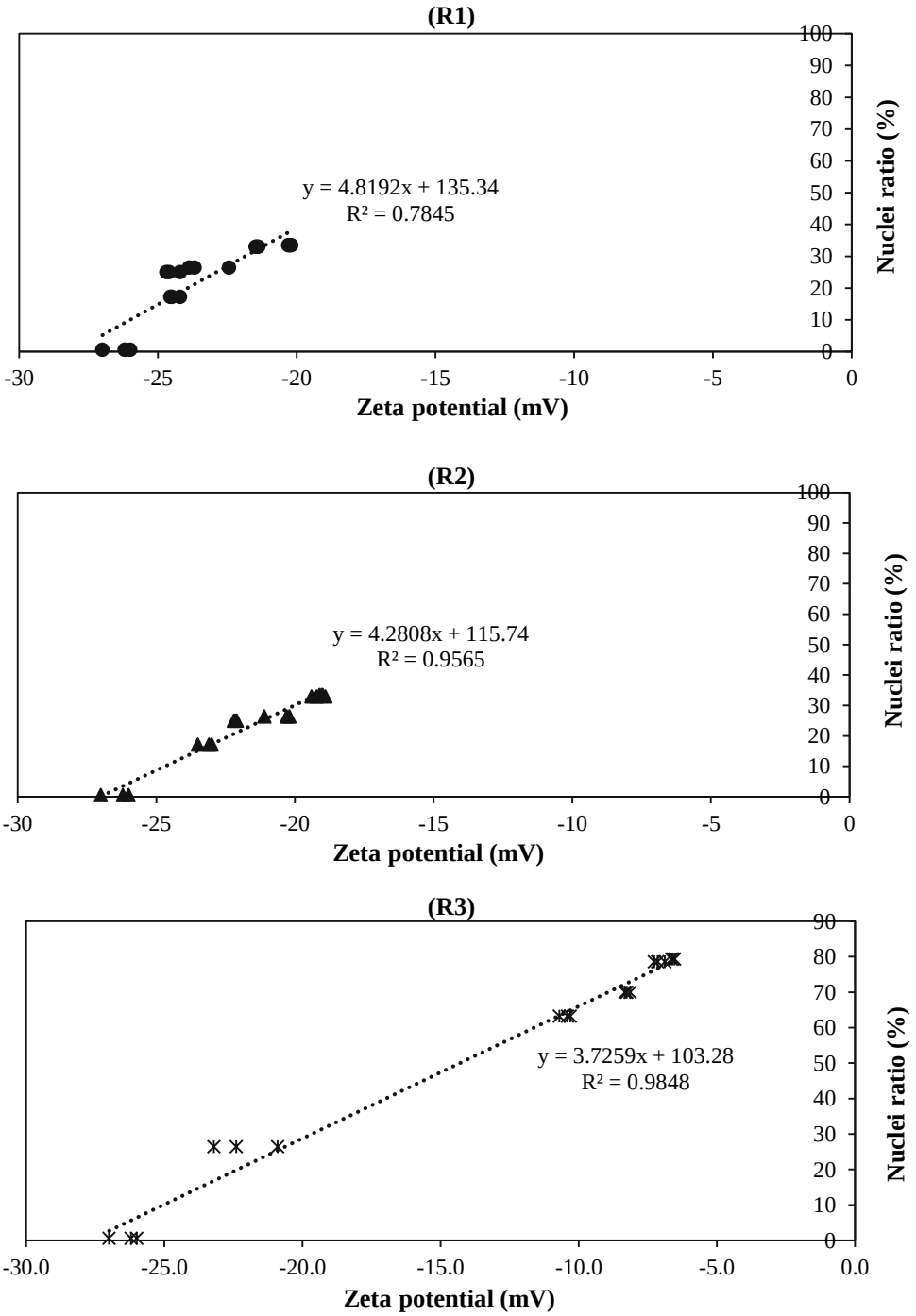


Figure 4.10 Linear relationship between zeta potential and nuclei ratio in all reactors

4.4 Sludge volume index (SVI)

The sludge volume index (SVI) represents the volume of sludge in ml that settles in a certain period of time (normally 30 minutes) with unit of ml settled sludge per gram suspended solids (ml.g^{-1} SS). Low SVI value indicates better setting characteristics of

microbial aggregates or granules. Non-granular sludges with poor settling characteristics commonly have high SVI value. Previous research found that SVI of granules were approximately 10 – 20 ml.g^{-1}SS which were higher than that of flocculent sludge as 20 – 40 ml. g^{-1}SS [26, 36, 96]. In this study, the inoculations of seed sludge into reactors were concomitantly conducted with the addition of cationic polymers, dynafloc 8265 and chitosan, resulting different initial SVI as 92.7, 91.8 and 74.9 ml.g^{-1}SS, for microbial aggregates in R1, R2 and R3, respectively.

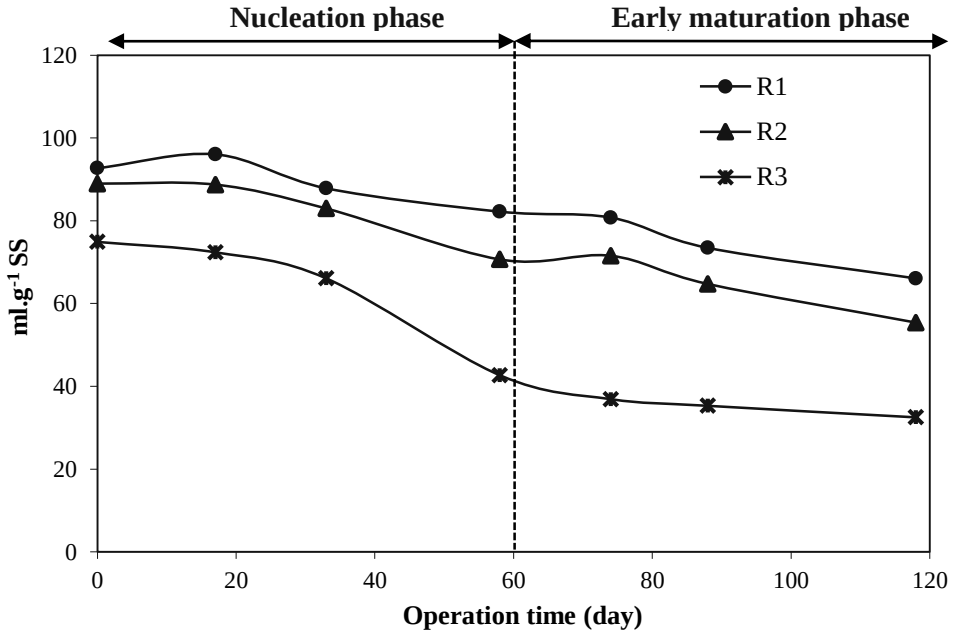


Figure 4.11 SVI of microbial aggregates in all reactors

The SVI values of microbial aggregates in all reactors tended to decrease during the nucleation phase (**Figure 4.11**). The lowest SVI values were observed in microbial aggregates in R3 which were 72, 66 and 43 ml.g^{-1}SS at day 17, 33 and 58, respectively. While microbial aggregates in R2 showed SVI values as 89, 83 and 71 ml.g^{-1}SS at day 17, 33 and 58, respectively. Microbial aggregates in control reactor, R1, showed the highest SVI values as 96, 88 and 82 ml.g^{-1}SS at day 17, 33 and 58, respectively. The addition of dynafloc 8265 in R2 was not effective to decrease SVI values which were slightly higher than that in control reactor R1. However, the addition of chitosan could significantly decrease SVI values during nucleation phase.

The SVI values during the early maturation phase were also observed in each reactor. The SVI values of each reactor continued to slightly decrease during early maturation phase. Microbial aggregates in R3 still showed the lowest SVI values compared to other reactors as

37, 35 and 33 ml.g^{-1}SS at day 74, 88 and 118, respectively. While the highest SVI values were observed in microbial aggregates in control reactor R1 as 81, 73 and 66 ml.g^{-1}SS at day 74, 88 and 118, respectively. SVI values of microbial aggregates in R2 were slightly higher than that in R1 as 72, 65 and 55 ml.g^{-1}SS.

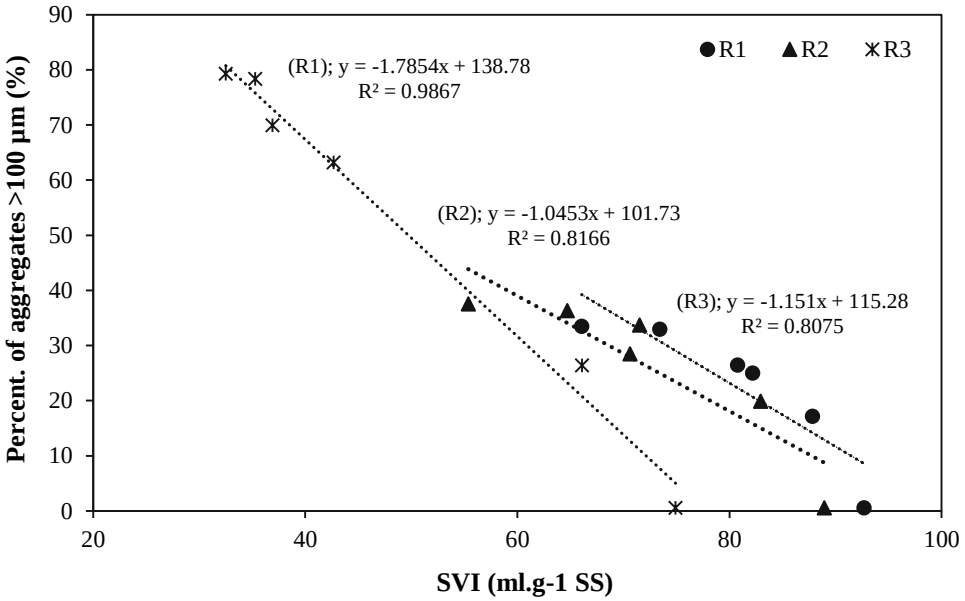


Figure 4.12 The linear relationship between SVI and percentage of aggregates with diameter size >100 µm (nuclei and granule)

The size distribution of the microbial aggregates in each reactor was related to the SVI values. The addition of chitosan in R3 provided cations which could decrease the negativity of microbial surface charges in R3. Less negative of microbial surface charges triggered the microbial aggregation resulting the formation of larger microbial aggregates (nuclei and granules). Large microbial aggregates commonly led on high settling ability of microbial aggregates or low SVI value. Based on size distribution results, R3 showed highest nuclei and granule ratio during nucleation and early maturation phase than other reactors. High nuclei and granule ratio in R3 closely related with low SVI value in R3 during nucleation and early maturation phase. Linear relationships between SVI value and percentage of microbial aggregates with diameter size >100 µm (nuclei and granule) in each reactor were shown at **Figure 4.12**. Linear coefficients (R^2) in R1, R2 and R3 were 0.81, 0.82 and 0.99, respectively, which indicated that higher percentage of microbial aggregates with diameter >100 µm (nuclei and granule) was closely related with SVI value in R3.

4.5 Extracellular polymeric substances (EPS)

EPS play an important role in the process of anaerobic granulation as a bio-glue. The positive effects of EPS are commonly related to EPS characteristics which are able to regulate the hydrophobicity of cell surfaces and provide a large number of electrostatic binding sites [35, 95]. EPS consists of 20-30 fold more binding sites than microbial cell surface. Therefore, it can connect the neighboring bacteria and inert particles as a bridge to form large microbial aggregates [35, 74, 97]. EPS is excreted by microbial cells and exposed at their surfaces under suitable physiological conditions. EPS consists of complex mixtures of polymeric substances excreted by microorganisms, lysis and hydrolysis products, and adsorbed organic matter from wastewater [24, 25, 27, 35].

In this study, EPS was analyzed based on their main components namely polysaccharides and protein. **Figure 4.13** shows the polysaccharides – and protein – EPS ($EPS_{polysaccharides}$ and $EPS_{protein}$) during nucleation and early maturation phases. T-test confirmed that there were no significant different of $EPS_{polysaccharides}$ and $EPS_{protein}$ in each reactor during nucleation phase ($\alpha = 0.05$). Because the EPS productions in each reactor during nucleation phase were not significantly different, the addition of cationic polymers had main role on the formation of microbial nuclei, especially microbial nuclei in R3. $EPS_{polysaccharides}$ tend to decrease during nucleation phase which was probably caused by mixed VFA feeding (produce less EPS). The ratio between $EPS_{protein}$ to $EPS_{polysaccharides}$ increased during nucleation phase. The ratio between $EPS_{protein}$ to $EPS_{polysaccharides}$ in each reactor were also not significantly different for each reactors. Ratio of $EPS_{protein}$ to $EPS_{polysaccharides}$ during early maturation in each reactor was approximately 3.2 – 6.3. The highest ratio of $EPS_{protein}$ to $EPS_{polysaccharides}$ was 6.3 which was observed in R1 at day 58.

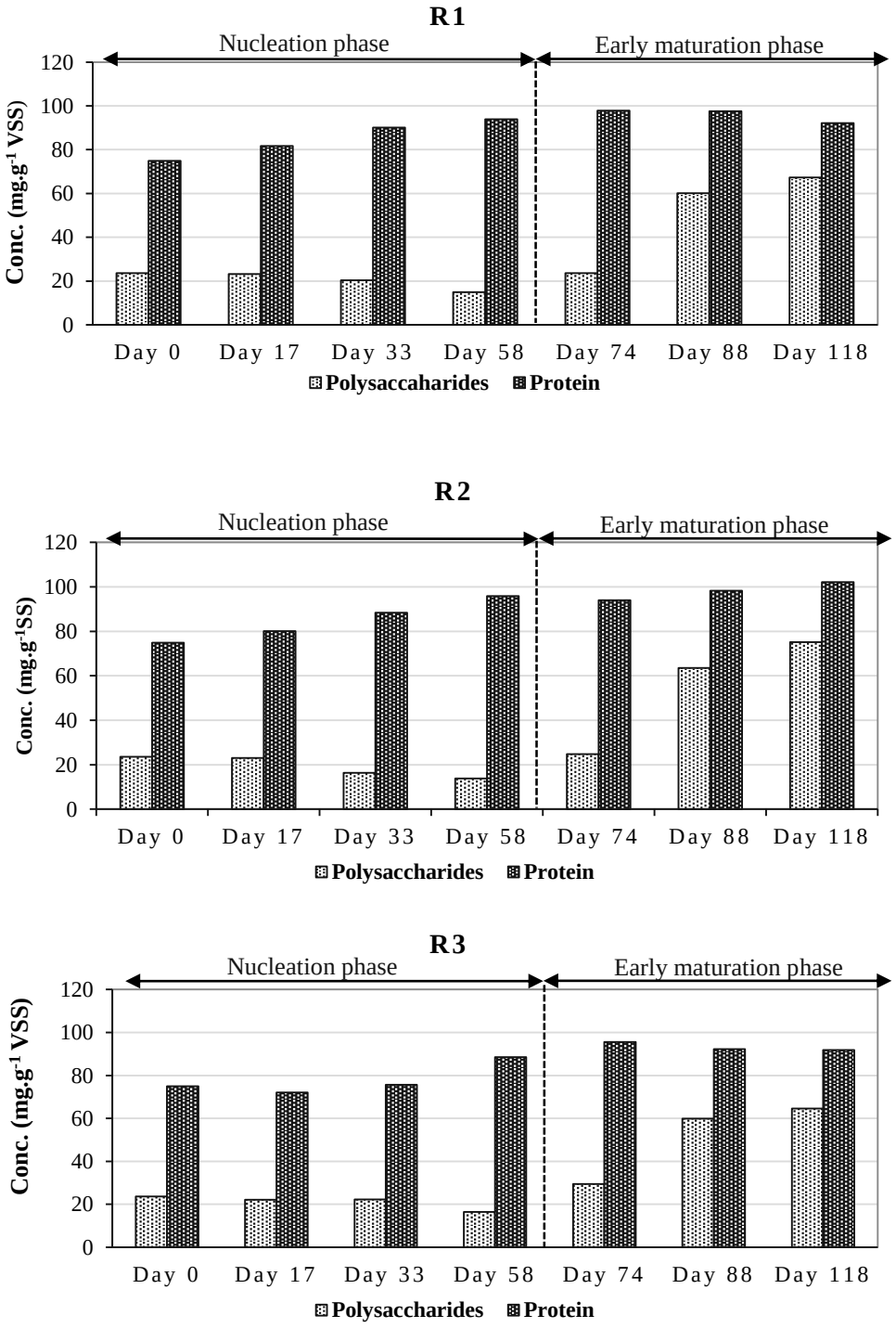


Figure 4.13 EPS production during the nucleation and early maturation phase

Glucose as the main substrate during the early maturation phase was able to enhance the production of EPS $_{polysaccharides}$ in each reactor. EPS $_{polysaccharides}$ significantly increase at day 88 and 118, at OLR 0.7 and 1.0 kg COD.m^{-3}.d^{-1} in each reactor. Among those reactors, the amount of EPS $_{polysaccharides}$ and EPS $_{protein}$ were not significantly different. The increase

of $EPS_{polysaccharides}$ in each reactor was followed by stable concentration of $EPS_{protein}$ until day 118 resulting on lower ratio of $ECP_{protein}$ to $ECP_{polysaccharides}$. $ECP_{protein}$ to ECP - $_{polysaccharides}$ ratio during early maturation phase in each reactor was approximately 1.4 – 4.2 which was lower than during nucleation phase. EPS produced during early maturation phase was important for microbial aggregation since in this phase, there were no cationic polymers addition into R2 and R3. Therefore, the increases of nuclei and granule diameter size were caused by the presence of EPS. EPS may contribute on maturation of granule by bridging small particles and microbial nuclei to form larger microbial aggregates. Based on size distribution results, granule ratio in R3 significantly increased during early maturation phase as 17.6, 31.8 and 34.2% at day 74, 88 and 118. Nuclei and granule ratio in R3 at the end of nucleation phase (day 58) were approximately 55.1 and 8.2%, respectively. Maturation of nuclei in R3 during early maturation phase was faster than that in R1 and R2 which may be affected by the amount of nuclei at the end of nucleation phase. These nuclei, with more EPS produced during early maturation phase, bridged with smaller aggregates or other nuclei and then formed mature granule which were larger than nuclei. Nevertheless, nuclei and granule ratio at R1 and R2 were very low at day 58 as less than 25.9 and 2.6%, respectively, which were followed by high percentage of small microbial aggregates with diameter less than 100 µm (72 – 75% of total microbial aggregates). Therefore, the formation of nuclei still continued in R1 and R2 during early maturation phase in which small microbial aggregates connected each other to form nuclei. It was shown by the results of size distribution which stated that the increases of nuclei in R1 and R2 were significantly higher than the increase of granule (**Figure 4.6**).

$EPS_{polysaccharides}$ are the only EPS components that are synthesized extracellularly for a specific function, while $EPS_{protein}$ and other minor components, such as lipids, humic acid and nucleic acid are the results of cell excretion or cell lysis [27]. Cell lysis contributed on the increase of $EPS_{protein}$ during nucleation phase while $EPS_{polysaccharides}$ was limited due to mixed VFA was used as main substrate in this phase. Different with early maturation phase, glucose could enhance the production of $EPS_{polysaccharides}$ and also microbial growth resulting on lower ratio of $EPS_{protein}$ to $EPS_{polysaccharides}$. The composition of EPS components such as polysaccharides and protein depends on several factors such as microbial species, growth phase, the type of limiting substrate, ionic strength, etc. [27, 64]. However, the characteristics of microbial aggregates were also related to the ratio of $EPS_{protein}$ to $EPS_{polysaccharides}$. Higher protein to polysaccharides ratio in EPS contributed on lower shear

strength and poorer settleability resulting on poor strength and stability of microbial aggregates [19, 98]. The specific gravity and mechanical strength of the granules significantly increased with the decrease of the ratio of proteins to polysaccharides [99]. Wu, et.al., (2009) also confirmed that higher protein to polysaccharide ratio in EPS lead to disruption of microbial aggregates. The addition of cationic polymers, such as dynafloc 8265 and chitosan, were not enough to maintain the stability of large microbial aggregates resulting on low granule ratio during nucleation phase. During early maturation phase, the production of $EPS_{polysaccharides}$ tend to increase while $EPS_{protein}$ tend to stable resulting on low ratio of protein to polysaccharides of EPS. These low ratio of protein to polysaccharides of EPS in early maturation phase contributed on the formation of nuclei and microbial granules in each reactor in the absence of cationic polymer additions.

4.6 Specific microbial activity

Good microbial granules are not only related to good physical characteristics such, as high nuclei ratio, granule ratio and high settling ability, but also to microbial characteristics, such as specific microbial activities inside reactors. This study focused on the formation of multilayered anaerobic granules which had good physicochemical and also microbial characteristics. Good microbial characteristic of microbial granule was designed by the formation of syntroph adapted nuclei during nucleation phase and granule formation during early maturation phase. The formation of nuclei was concomitantly conducted with the adaptation of microbial seed sludge to syntroph specific substrate, i.e., acetate: propionate: butyrate with COD ratio 2: 1: 1. It was important to know the activities of specific microbial groups which represented the ability of special microbial groups to degrade their specific substrates. In this study, the activities of specific microbial groups were categorized based on the utilization on specific substrates such as glucose-degrading microorganism (GDM), butyrate-degrading microorganism (BDM), propionate-degrading microorganism (PDM) and acetoclastic methanogen (ACM). The activities of specific microbial groups of microbial aggregates in each reactor were observed during nucleation and early maturation phases. The increase of syntroph adapted microorganism, such as BDM, PDM and ACM, were main criteria during nucleation phase which represented syntroph relationship in microbial aggregates. While, the balances between methanogen

(ACM) and non-methanogens (GDM, BDM and PDM) was important parameters to observe during the early maturation phase.

4.6.1 Glucose – degrading microorganism (GDM)

The activity of GDM represented the activity of acidogenic microorganisms for its ability to degrade glucose and produce acids. The GDM was an important microorganism for the early maturation phase as main EPS producers that can enhance granule maturation. The activity of GDM of microbial aggregates in each reactor was still monitored during nucleation phase in which mixed VFA was used as main substrate.

The activity of GDM in all reactors tended to decrease during nucleation phase (**Figure 4.14**). The highest activity of GDM was observed in R1 as 1.68, 1.62 and 1.40 g COD.g^{-1} VSS.d^{-1} at day 0, 33 and 58, respectively. While, the lowest of the activity of GDM was found at microbial aggregates in R3 as 1.68, 1.47 and 1.24 g COD.g^{-1} VSS.d^{-1} at day 0, 33 and 58, respectively. The syntroph specific substrate (mixed VFA) used as main carbon source may cause the decrease of GDM activity in all reactors during nucleation phase. However, there is no significant difference ($p < 0.05$) for the decrease of GDM from initial day until day 58 (end of nucleation). The adaptation of microorganism under syntroph specific substrate during nucleation phase could not significantly decrease the activity of GDM. Compared to other microbial groups, acidogens were well known as the most resistant microorganisms to unfavorable conditions [5].

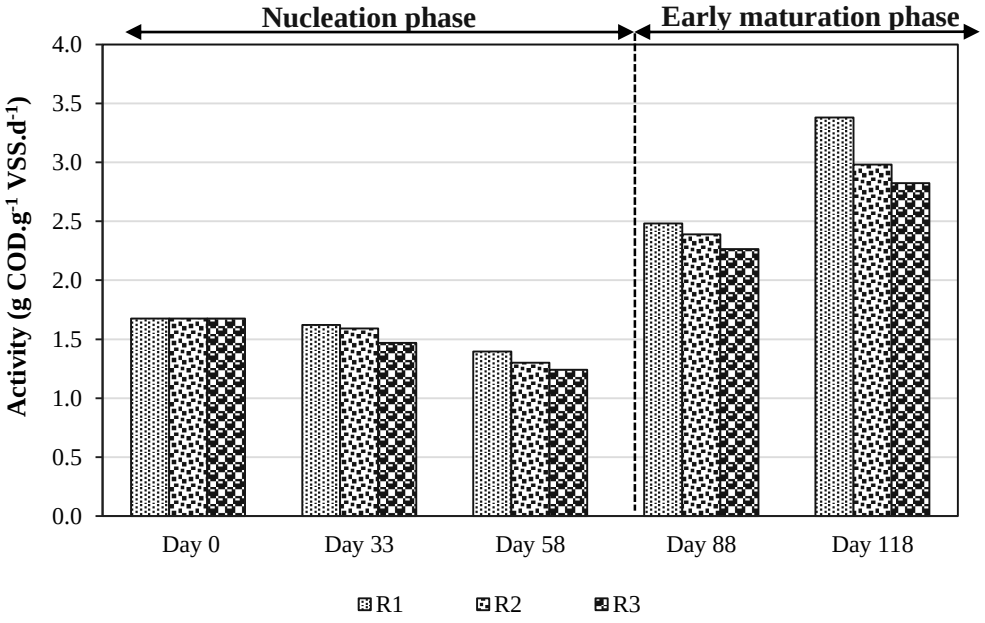


Figure 4.14 The activity of GDM during the nucleation and early maturation phases

The syntroph specific substrate was then switched to glucose as the main carbon source in the early maturation phase, which affected to the fast increase of activities of GDM in the microbial aggregates in all reactors. The highest and lowest activity of GDM was also found in R1 and R3, respectively. The activities of GDM at microbial aggregates in R1 at day 88 and 118 were 2.48 and 3.38 g $COD.g^{-1}$ $VSS.d^{-1}$, respectively. While, the activities of GDM at microbial aggregates in R3 were 2.27 and 2.83 g $COD.g^{-1}$ $VSS.d^{-1}$ at day 88 and 118, respectively. GDM or acidogenic microorganism was considered as the fastest growth microorganism (generation time about 1.4 hours) [100]

4.6.2 Butyrate – degrading microorganism (BDM)

Butyrate and propionate are the most important intermediate products in the syntroph reactions, because of thermodynamics restriction in which their degradations are considered as the rate limiting step in anaerobic digestion. Those intermediate acids can accumulate in anaerobic reactors causing unstable operation [101]. Butyrate was produced from glucose during acidogenesis and then was degraded into acetate in acetogenesis. Butyrate-degrading microorganism (BDM) was considered as one of syntroph microorganism in anaerobic digestion system. BDM had an important role in acetogenesis to degrade butyrate to be two molecules of acetic acids. However, the activity of BDM depend on syntroph relationship with H_2 utilizing microorganism (hydrogenotrophic methanogen) because high H_2 concentration could inhibit the activity of H_2 producing microorganism, such as BDM and PDM. The activity of BDM in microbial aggregates in all reactors were observed during nucleation and early maturation phase (**Figure 4.15**). The activity of BDM of microbial aggregates in all reactors tend to increase during the nucleation and early maturation phase. The highest and lowest activity of BDM was observed at microbial aggregates in R1 and R3, respectively. Microbial aggregates in R1 had the activity of BDM as 0.29, 0.35, 0.45, 0.48 and 0.53 g $COD.g^{-1}$ $VSS.d^{-1}$ at day 0, 33, 58, 88 and 118, respectively, which was higher than the activity of BDM at microbial aggregates in R2 and R3. The lowest activity of BDM was found at microbial aggregates in R3 as 0.29, 0.34, 0.39, 0.43 and 0.46 g $COD.g^{-1}$ $VSS.d^{-1}$ at day 0, 33, 58, 88 and 118, respectively.

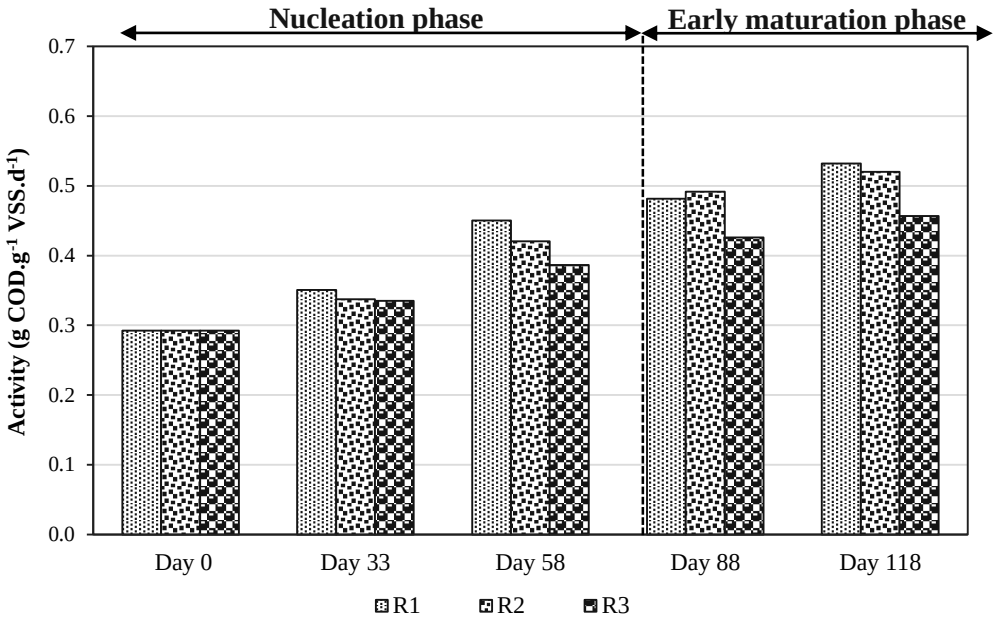


Figure 4.15 The activity of BDM during the nucleation and early maturation phases

During the nucleation phase, all reactors were fed by syntroph specific substrates (mixed VFA), consisting of acetate: propionate: butyrate with ration 2:1:1 (based on g COD). The adaptation of microbial aggregates to syntroph specific substrate successfully increased the activity of BDM during nucleation and early maturation phase. The developments of activities of BDM in all reactors slightly increased during early maturation phase. Glucose was used as main substrate in early maturation phase. Based on glucose fermentation pathway, glucose can be degraded to produce butyrate under slight low pH (6-7) [92]. Therefore, the adaptation process to syntroph specific substrate was still continue which resulted on the increase of the activity of BDM until day 118 (end of early maturation phase).

4.6.3 Propionate – degrading microorganism (PDM)

Propionate – degrading microorganism (PDM) is also one of the syntrophs microorganism in acetogenesis phase of anaerobic digestion system. PDM utilizes intermediate products of anaerobic digestion, i.e., propionate, to produce acetate. The activities of PDM at microbial aggregates in all reactors tend to increase during nucleation phase, while those activities tend to be stable during early maturation phase (**Figure 4.16**). The activities of PDM for microbial aggregates in R1 were 0.17, 0.30 and 0.53 g $COD.g^{-1}$ $VSS.d^{-1}$ at day 0, 33 and 58, respectively. In R2, the activities of PDM were 0.17, 0.28 and 0.51 g $COD.g^{-1}$ $VSS.d^{-1}$ at day 0, 33 and 58, respectively. The activities of PDM for microbial aggregates in R3 were observed as 0.17, 0.24 and 0.52 g $COD.g^{-1}$ $VSS.d^{-1}$ at day 0, 33 and

58, respectively. At the end of nucleation phase (day 58), the activities of GDM at microbial aggregates in R1, R2 and R3 were not significant different ($p < 0.05$) as 0.53, 0.51 and 0.52 g COD.g^{-1} VSS.d^{-1}, respectively. The adaptation of microbial aggregates to syntroph specific substrates during the nucleation phase successfully increased the activity of PDM in all reactors.

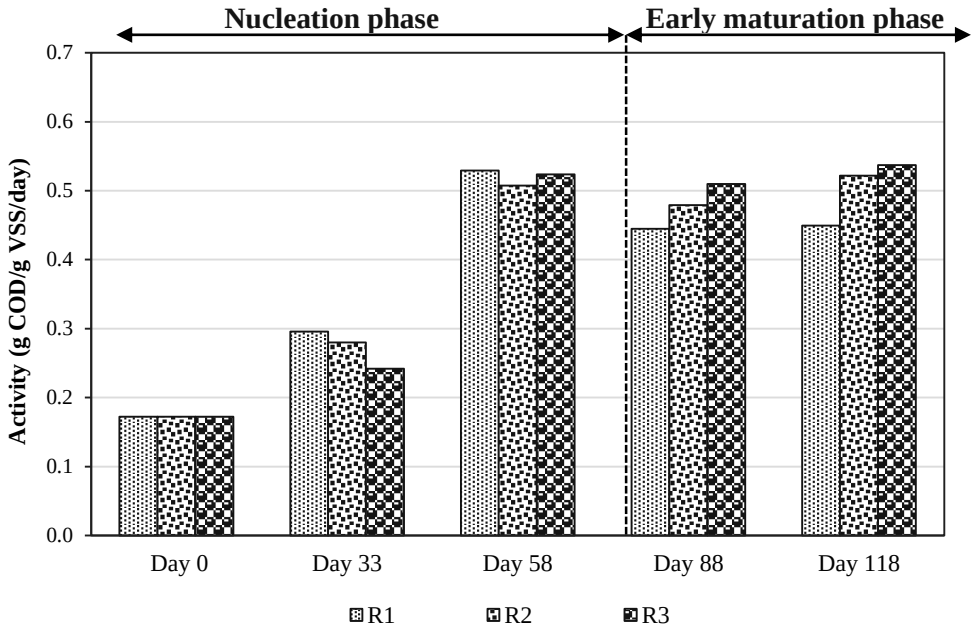


Figure 4.16 The activity of PDM during the nucleation and early maturation phases

The activity of PDM tend to be stable and not significantly different in R2 and R3 during the early maturation phases (**Figure 4.16**). While the activities of PDM in R1 tend to slightly decrease until day 118. During this phase, the activities of PDM in R1 were approximately 0.44 and 0.45 g COD.g^{-1} VSS.d^{-1} at day 88 and 118, respectively. In R2, the activities of PDM were 0.48 and 0.52 g COD.g^{-1} VSS.d^{-1} at day 88 and 118, respectively. While the activities of PDM in R3 at day 88 and 118 were 0.51 and 0.54 g COD.g^{-1} VSS.d^{-1}, respectively. The substrate switching from mixed VFA to glucose could be the reason for these stable activities. The possibility of glucose fermentation pathway can be varied depend on the environmental conditions. Glucose was possible to be degrade into ethanol, lactate, butyrate, propionate and acetate. These variable pathway of glucose degradation caused the propionate production may not sufficient for adaptation of PDM, which was different from nucleation phase (mixed VFA feeding was controlled at a specific ratio).

4.6.4 Acetoclastic methanogen (ACM)

Methane was mainly produced from the utilization of acetate and hydrogen by methane-producing microorganisms in anaerobic reactors. The cleavage of acetate and the reduction of carbon dioxide are considered as major pathways to methane production. Acetoclastic methanogens utilize acetate to produce methane, while hydrogenotrophic methanogens use hydrogen. Acetoclastic methanogens was responsible for 70% of the methane production, although its specific activity is ten times smaller and four times slower in maximum growth rate than hydrogenotrophic methanogens. It can be concluded that the population of acetoclastic methanogens is at least 10 times more important than that of hydrogenotrophic methanogens [102]. Therefore, it was important to investigate the activity of acetoclastic methanogens (ACM) during nucleation and early maturation phases.

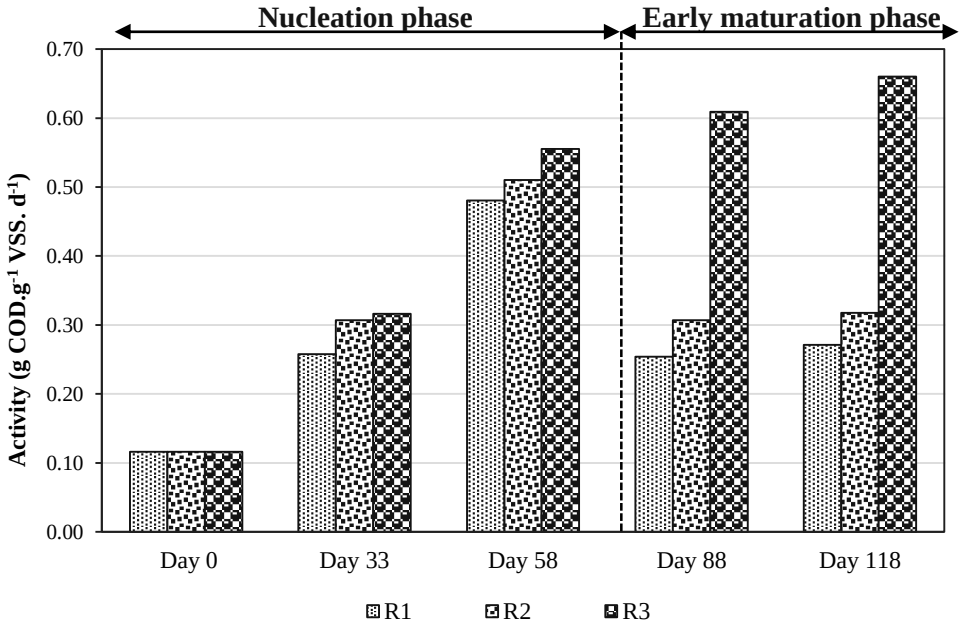


Figure 4.17 The activity of ACM during nucleation and early maturation phase

During the nucleation phase, the activity of ACM by the microbial aggregates in all reactors tended to increase as a result of the adaptation of seed sludge under mixed VFA feeding. At the end of nucleation phase (day 58), the activities of ACM at microbial aggregates in R1, R2 and R3 were significantly higher 0.48, 0.51 and 0.56 g $COD_{CH4}.g^{-1}$ $VSS.d^{-1}$, respectively, compared to initial activity of ACM which was 0.12 g $COD_{CH4}.g^{-1}$ $VSS.d^{-1}$.

The different tendencies of the activity of ACM were observed in microbial aggregates in R1 and R2 during the early maturation phase, which tended to decrease, while

the activity of ACM at microbial aggregates in R3 still increased until the end of the early maturation phase. The activities of ACM of microbial aggregates in R1 at day 88 and 118 were 0.26 and 0.28 g $COD_{CH4}.g^{-1}$ $VSS.d^{-1}$, respectively. While the activities of ACM of microbial aggregates in R2 were observed as 0.31 and 0.32 g $COD_{CH4}.g^{-1}$ $VSS.d^{-1}$ at day 88 and 118, respectively. The activities of ACM of microbial aggregates in R3 continued to increase during early maturation phase as 0.61 and 0.66 g $COD_{CH4}.g^{-1}$ $VSS.d^{-1}$ at day 88 and 118, respectively. The glucose feeding during early maturation phase affected on the decreases of the activity of ACM of microbial aggregates in R1 and R2. The degradation of glucose resulted on intermediate products, i.e., organic acids, which could decrease pH and then affected on the methanogen activities in R1 and R2.

4.6.5 The relationship between diameter size of aggregates and specific microbial activities

There are several advantages of granule over than dispersed granule in anaerobic digestion system, such as high settling ability, toxic resistance and specific microbial activity. The reason for high settling ability were mentioned in previous sub-chapters. Microbial granule performed good toxic resistance because of multilayered structure of granule. In multilayered anaerobic granule, less resistant microorganism (such as methanogen group) was in the inner part of granule while more resistant microorganism (such as acidogenic and acetogenic microorganism) was in the outer and middle layer of granule [15, 17, 103]. The outer layer microorganism was firstly exposed if the environmental condition was unfavorable for microbial growth.

Highly specific microbial activity in anaerobic granule was caused by efficient substrate degradation inside the granules. Multilayered anaerobic granule allows better substrate degradation than dispersed sludge. The outer layer consists of hydrolytic and acidogenic microorganism hydrolyze more complex substrate to be organic acids. Acetogenic microorganism in the middle layer degrade those organic acids to be acetic acid and hydrogen which were then utilized by acetoclastic and hydrogenotrophic methanogen in the inner layer of granule, respectively. The microorganism inside granule work as ‘a team’ to degrade complex substrate. There is syntroph relationship for acetogenic microorganism (acetate and H_2 producers) with methanogen (acetate and H_2 consumers). The activity of those microbial groups must be in balance. High acetate and H_2 accumulation are able to decrease the activity of those syntroph microorganism, acetogenic microorganism and methanogen. Highly active methanogen was preferred because of its roles which utilize H_2

and acetate to produce methane. The activity of methanogen is the main factor to concern than other microbial groups.

In this study, the balance of microbial activities was represented by the activities of GDM (acidogens), PDM and BDM (acetogens) and also ACM (acetoclastic methanogens). **Figure 4.18** shows the summary of the activity of specific microbial groups of microbial aggregates in each reactors during nucleation and early maturation phases. The activities of each microbial group, i.e. GDM, BDM, PDM and ACM, had been discussed at previous sub-chapter. The activity of GDM tend to slightly decreased during nucleation due to mixed VFA feeding into reactors and then their activities significantly increased during early maturation phase since glucose was used as main substrate, replacing mixed VFA. Glucose was easy to degrade by acidogens microorganism in each reactor resulting on the highest activity in term of g $COD.g^{-1}$ $VSS.d^{-1}$ compared to other activities of microbial groups. During nucleation, the activities of PDM and BDM in each reactor significantly increased as a response to mixed VFA feeding. However, the activities of PDM and BDM tend to slightly increased during early maturation phase. The activities of ACM representing methanogens in each reactor significantly increased during nucleation phase (adaptation to mixed VFA feeding). However, the development of the activities of ACM during early maturation phase were different each reactor. The activities of ACM in R1 and R2 decreased at day 88 and 118. This decrease was probably caused by low pH as a result from glucose degradation during early maturation phase. However, the activities of ACM in R3 still increased until day 118. It seemed that the large structure of microbial aggregates in R3 protected methanogens from adverse effect of low pH resulted from glucose degradation.

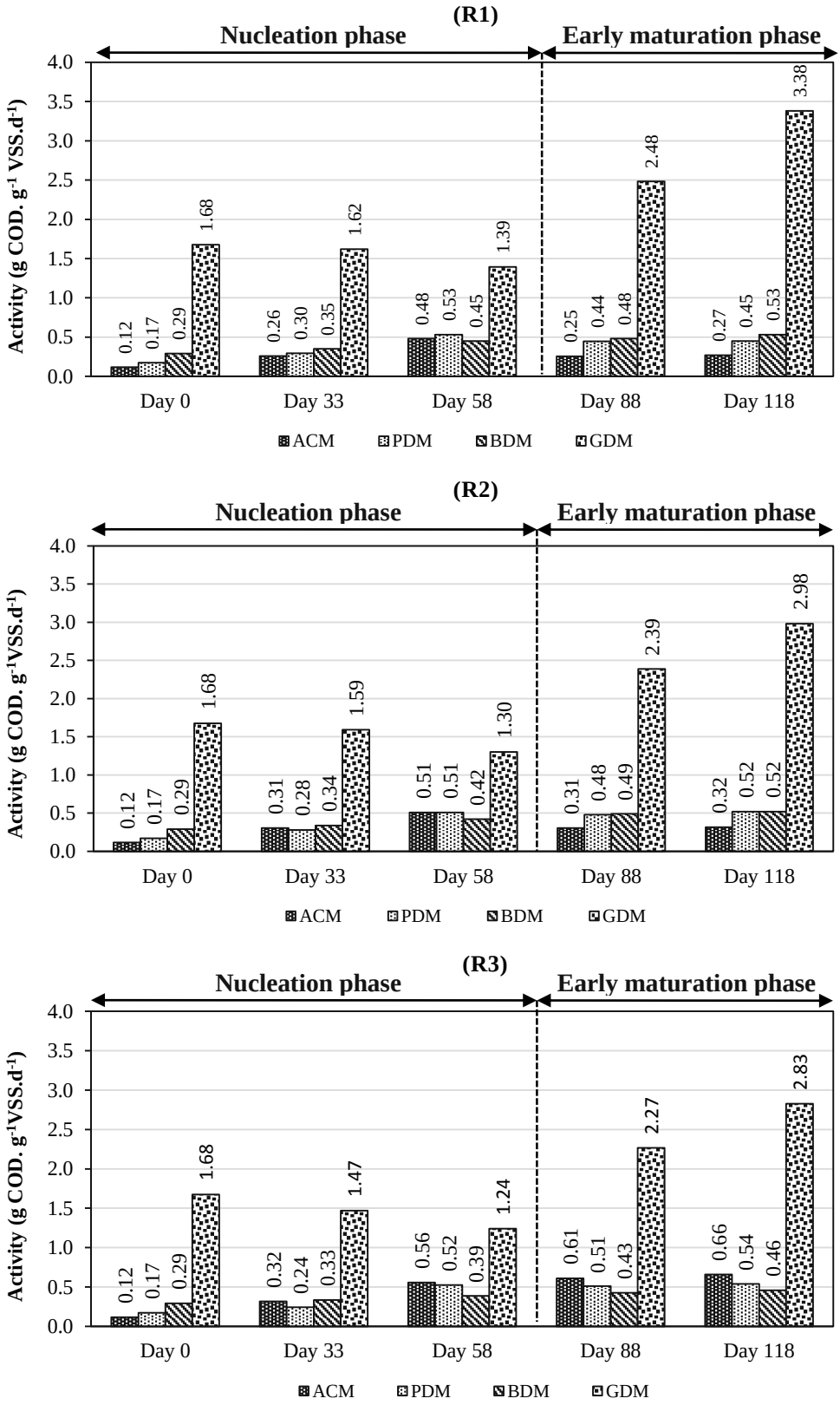
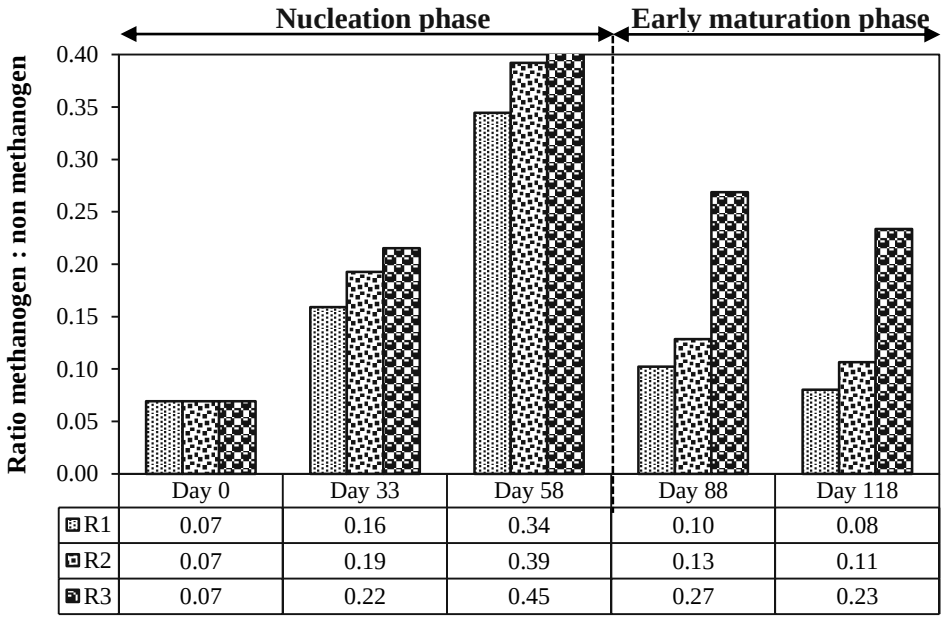


Figure 4.18 The activities of specific microbial groups in each reactor during the nucleation and maturation phases

The balances between the activities of methanogen to non-methanogen were important factors to observe because it represented the balance of microbial activity degrade substrate in anaerobic condition. Unbalanced condition between the activities of methanogen and non-methanogen resulted on failure of the operation of anaerobic reactor. Many studies observed the balances between non methanogen and methanogen activities in anaerobic digestion [18, 52, 104, 105]. However, due to the complexity of microbial relationship in anaerobic digestion system which involved many microorganism, it was difficult to analyze the activity of each individual anaerobic microorganism. An approach to analyze the complexity of the activities of microbial groups in anaerobic digestion was to categorize them based on their main trophic groups, namely hydrolytic, acidogenic, acetogenic-and methanogenic microorganism. Collins, et.al. (2003) categorized that acetogens consisted of ethanol-, butyrate and propionate-utilizing microorganism and methanogens consisted of acetate-and H_2/CO_2 – utilizing microorganism. Meepian, et.al. (2003) investigated the activity of glucose-utilizing microorganism to represent acidogens, butyrate-and propionate-utilizing microorganism for acetogens and acetate-utilizers microorganism for methanogens. MacLeod, et.al. (1993) and El-Mamouni, et.al. (2003) analyzed the activity of glucose-utilizing microorganism for representing acidogens, ethanol- and propionate-utilizing microorganism for acetogens and H_2-, formate- and acetate-utilizing microorganism for methanogens. In this study, the activity of GDM was used to represent the activity of non-methanogens due to the complexity of glucose degradation which could produce more substrates (organic acids, hydrogen, acetate and carbon dioxide) for other microbial groups. The activity of acetogens or H_2 producing microorganism was represented by the activities of PDM and GDM. While the activity of ACM represented the activity of methanogens. For more general approach in this study, the balance of microorganism in anaerobic digestion system could be represented by the balance between methanogens (ACM) to non-methanogens (GDM, PDM and BDM) as shown at **Figure 4.19**.



	Day 0	Day 33	Day 58	Day 88	Day 118
R1	0.07	0.16	0.34	0.10	0.08
R2	0.07	0.19	0.39	0.13	0.11
R3	0.07	0.22	0.45	0.27	0.23

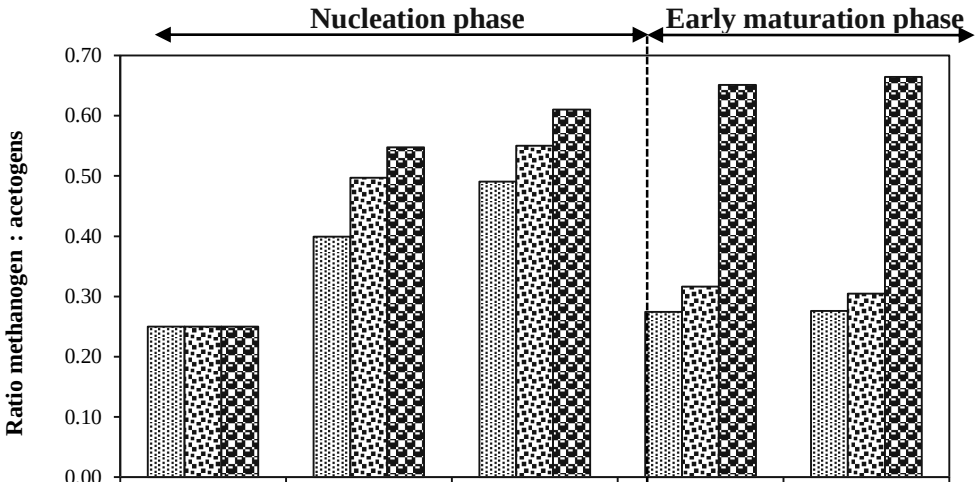
Figure 4.19 Ratio of the activity of methanogens (ACM) to non-methanogens (GDM) during nucleation and early maturation phases

The ratio between methanogen to non-methanogens activities in each reactor increased during the nucleation phase from day 0 until day 58. Initial seed sludge showed the ratio of methanogen to non-methanogen activities as 0.07. While, the ratio of methanogen to non-methanogen activities at day 58 in R1, R2 and R3 were approximately 0.34, 0.39 and 0.45, respectively. The highest of ratio of methanogen to non-methanogen activities during nucleation phase was observed in R3 as 0.45. During early maturation phase, the ratio of methanogen and non-methanogen activities tend to decrease, especially in R1 and R2. This ratio was significantly low in R1 and R2. At day 118, the ratio of methanogen to non-methanogen activities in R1, R2 and R3 were approximately 0.08, 0.11 and 0.23, respectively.

The ratio of methanogen to non-methanogen activities during the nucleation phase was higher than that during early maturation. Mixed VFA used as main substrate in nucleation phase affected on the ratio of methanogen to non-methanogen activities in each reactor. This substrate enhanced the specific activities of methanogens in each reactor and caused on slight decrease of the activities of GDM or acidogens. However, glucose which was fed into reactors during early maturation phase significantly enhanced the activity of GDM as non-methanogen. High activities of GDM resulted on fast glucose degradation

which caused high production of organic acids as intermediates products prior to degrade into methane as final product. It seemed that high intermediates products during the early maturation phase adversely affected the activity of ACM in R1 and R2. However, the activity of ACM in R3 still increased until day 118 in which the methanogens was probably protected by large structure of microbial aggregates in R3. In mature anaerobic granules, the ratio of methanogen to non-methanogens varied depend on the origin of granule, type of substrate and the adaptation of granules to specific substrates. MacLeod, et.al. (1993) also investigated the ratio between methanogen (acetate – utilizing microorganism) to non-methanogen activities (glucose-and propionate – utilizing microorganism) was approximately 0.35 for mature anaerobic granule from UASB reactor treating cheese whey wastewater. In addition, El-Mamouni, et.al. (1995) found that the ratio of methanogen to non-methanogen activities in syntroph enriched microorganism (one year enrichment process) was approximately 0.5 which was later proven as the best initial microorganism for granulation. According to previous references, it was possible to achieve higher ratio of methanogen to non-methanogen activities. The decrease of ratio of methanogen to non-methanogen activities during early maturation phase could possibly be recovered since this decrease was a part of adaptation of microbial aggregates to glucose as main substrate.

Besides the ratio of methanogen to non-methanogen activities, it was important to know the ratio between methanogens (ACM) to acetogen (PDM and BDM) activities that represented the syntroph relationship between acetogens and methanogens in microbial aggregates in each reactor. **Figure 4.20** shows that the ratio of methanogens to acetogens activities significantly increased during the nucleation phase because of microbial adaptation to mixed VFA (acetate, propionate and butyrate), which was used as the main substrate during this phase. The degradation of propionate and butyrate resulted on acetate. Similar substrate feeding in all reactors allowed similar tendencies for the specific microbial activities in each reactor. The highest ratio between methanogen and acetogens activities was observed in microbial aggregates of R3. Microbial aggregates of R1 showed the lowest ratio between methanogen and acetogenic microorganism during nucleation phase. Balanced ratio between methanogen and acetogenic activities affected on the efficient degradation of mixed VFA during nucleation and early maturation phase. These results were confirmed by COD removal efficiencies in all reactors at day 58 were approximately more than 80% (**Figure 4.20**).



	Day 0	Day 33	Day 58	Day 88	Day 118
R1	0.25	0.40	0.49	0.27	0.28
R2	0.25	0.50	0.55	0.32	0.30
R3	0.25	0.55	0.61	0.65	0.66

Figure 4.20 Ratio of methanogen (ACM) to acetogen activities (BDM and PDM) during nucleation and early maturation phases

The ratio of methanogen to acetogenic activities during the early maturation phase showed different tendencies as compared to the nucleation phase (**Figure 4.20**). In microbial aggregates of R1 and R2, the ratio methanogen to acetogens activities tend to decrease while the increase of ratio methanogen to acetogens activities were observed in microbial aggregates of R3. At day 58, the ratio of methanogen to acetogens activities was approximately 0.61 which then continued to increase as 0.65 and 0.66 at day 88 and 118, respectively. Substrate switching from mixed VFA to glucose seemed to adversely affect the activity of ACM in R1 and R2. Glucose degradation produced organic acids resulting on decrease of pH. It seem that the accumulation of organic acids and low pH affected the activity of ACM in R1 and R2 because of direct exposure of those organic acids and low pH to dispersed and small microbial aggregates in R1 and R2 (based on their size distribution characteristics).

Microbial aggregates of R3 had larger diameter sizes that were considered as nuclei and granules. In microbial aggregate of R3, it seemed that multilayered anaerobic granule had been formed in which the most sensitive microorganism (methanogen) was located in the inner part of microbial aggregates while less sensitive microorganism (acidogenic and acetogenic microorganism) was at outer part. Therefore, the activity of ACM in R3 was less affected by the accumulation of organic acids and low pH as results from glucose

degradation. Moreover, the substrate diffusion inside microbial aggregate of R3 was not adversely affected which was indicated by high COD removal efficiency. According to the ratio of methanogen to acetogen activities of microbial aggregates in R3 as 0.66 at day 118, it was supposed that this value was good for indicating the balance of syntroph relationships in R3. Collins, et.al. (2003) investigated that the ratio between methanogens (acetate- and H_2/CO_2-utilizing microorganism) to acetogens (butyrate- and propionate-utilizing microorganism) was approximately 0.62 for anaerobic granule from UASB treating citric acid wastewater. In addition, the ratio of methanogen (acetate- and H_2- utilizing microorganism) to acetogens (ethanol- and propionate- utilizing microorganism) in syntroph enriched nuclei was approximately 0.47. This syntroph enriched nuclei then developed as the best precursor for granulation process [33, 52].

4.7 Microbial morphology by scanning electron microscopy (SEM)

4.7.1 Morphology of initial seed sludge

Initial seed sludge was sieved under 100 µm – sieving instrument prior to inoculation into UASB reactors. The sieving procedure was conducted to remove large microbial aggregates and other impurities. As a result of sieving procedure, the average diameter size of initial seed sludge was 47 µm. The morphologies of initial seed sludge, nuclei and microbial granules were important to observe for investigating the development of microbial aggregates morphology during nucleation and early maturation phases. SEM was used as main instrument to analyze the morphology of microbial aggregates.

Based on the result of SEM analysis, one of the microbial aggregates in initial seed sludge was observed having a diameter size of approximately 50 – 60 µm. According to the structure and diameter size, initial seed sludge was not considered as microbial nuclei. Based on physical characteristic, nuclei can be considered as microbial aggregates with diameter 100 – 600 µm [46]. Sieving procedure as pre-treatment before inoculation could not separate microbial aggregates to individual anaerobic microorganism. Small microbial aggregates with diameter size less than 100 µm were observed as result from sieving procedure (**Figure 4.21**).

Microorganisms in anaerobic digestion naturally aggregated with other microorganisms to form small aggregates. However, these small microbial aggregates were weak and the formation of larger microbial aggregates was difficult to occur in initial seed

sludge. Mixed microorganism, such as cocci-, rod- and filament-shaped microorganism was observed in microbial aggregates of initial seed sludge. The abundant and various amount of microorganism in initial seed sludge was needed to form good multilayered anaerobic granules.

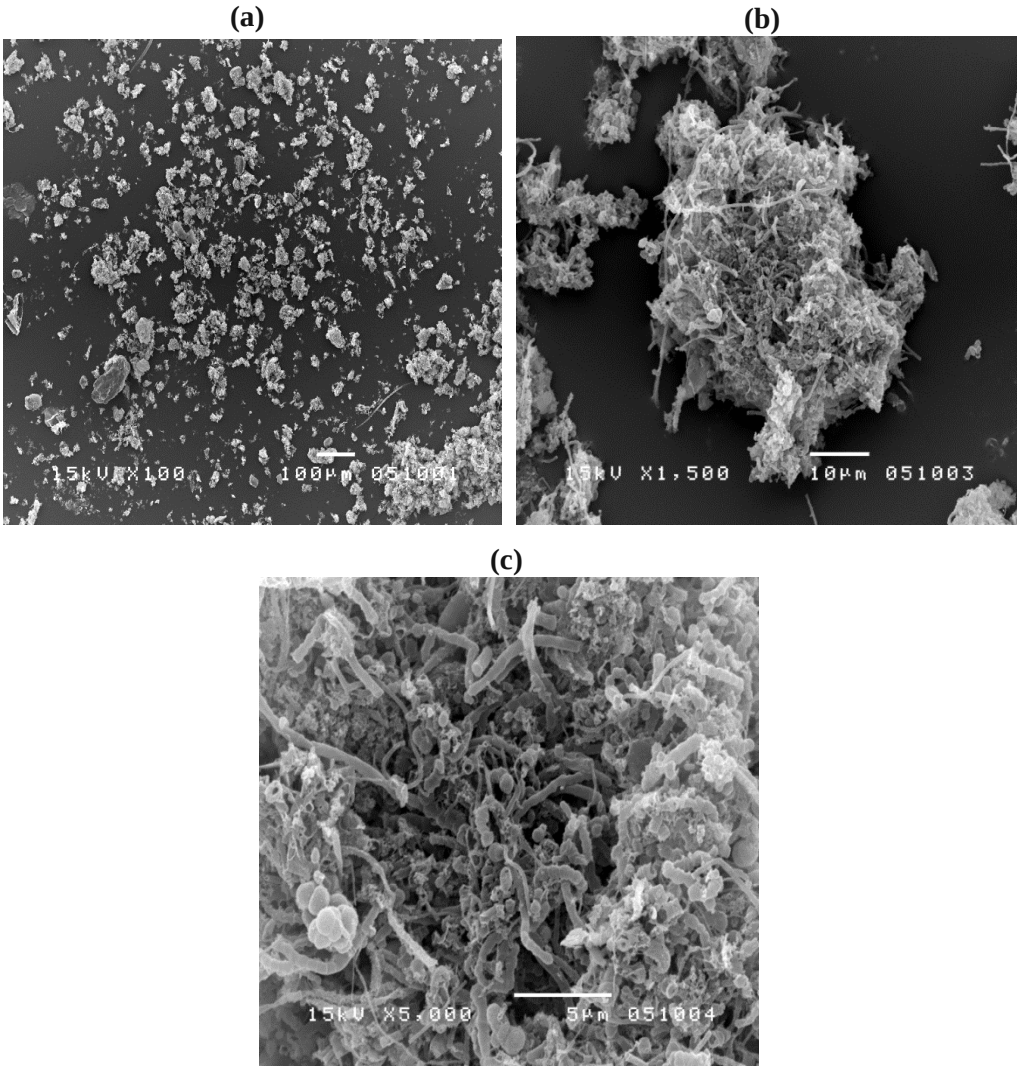


Figure 4.21 Morphology of initial seed sludge with magnifications of (a) 100x, (b) 1500x and (c) 5000x

4.7.2 Morphology of microbial aggregates in the end of nucleation phase

The effect of cationic polymer additions, dynafloc 8265 and chitosan, on the morphology of microbial aggregates at the end of the nucleation phase (day 58) was also observed by SEM analysis. The dominant microorganism at the outer and inner parts of the microbial aggregate were also investigated in microbial aggregates in each reactor.

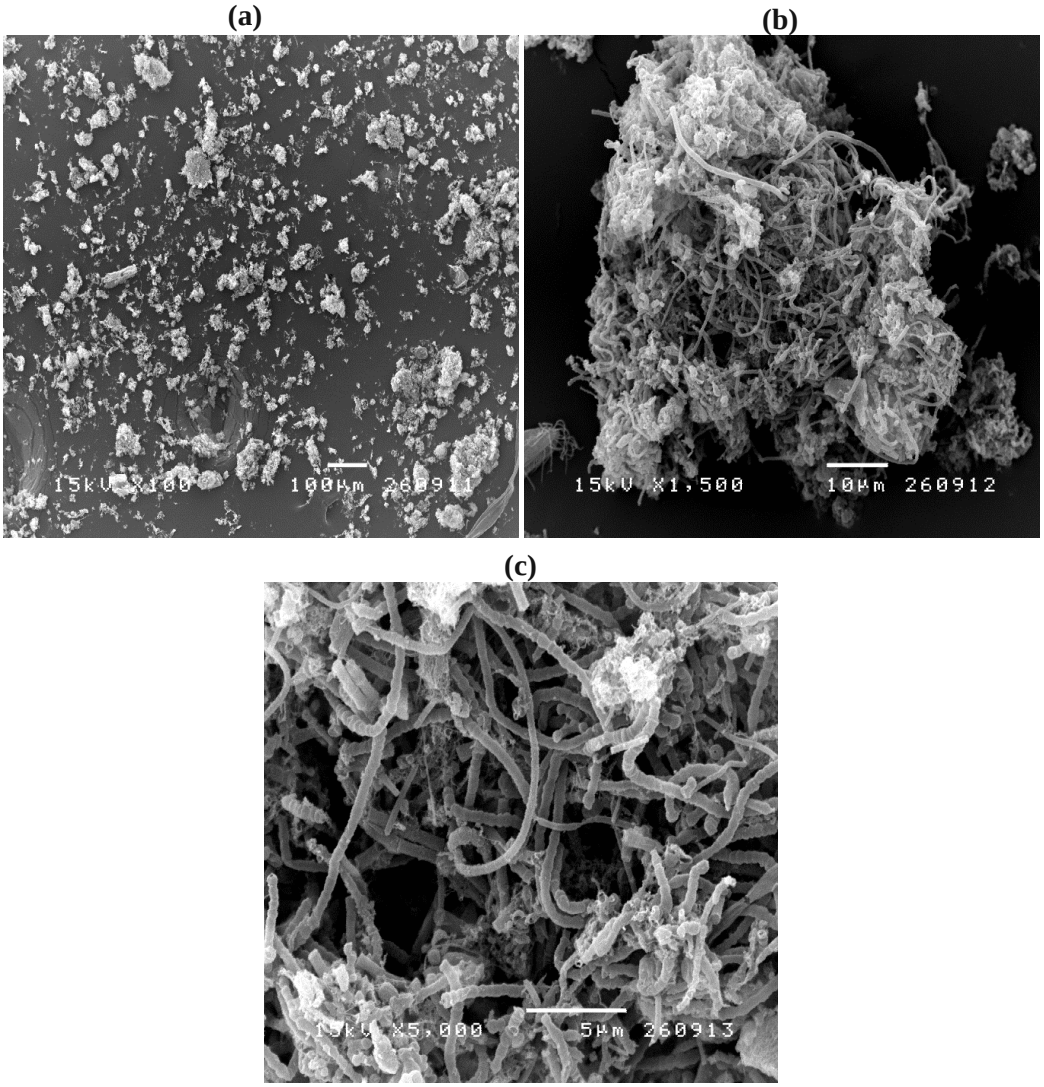


Figure 4.22 Morphology of microbial aggregates in R1 at the end of nucleation phase with magnifications of (a) 100x, (b) 1500x and (c) 5000x

Figure 4.22 shows the morphology of microbial aggregates in the control reactors, R1, at the end of the nucleation phase (day 58). The diameter size of microbial aggregate in R3 was approximately 60 – 70 µm. The morphology of microbial aggregates in R1 seemed fragile and weak. Filamentous microorganism appeared as dominant microorganism inside

microbial aggregates of R1. No polymer addition into the R1 affected filamentous microorganism aggregated each other to form small aggregates. The self-aggregation of filamentous microorganism to form aggregates was correlated with the previous *Spaghetti theory* and multilayered theory [18, 106]. Lower EPS production during nucleation formed weak and fragile structures.

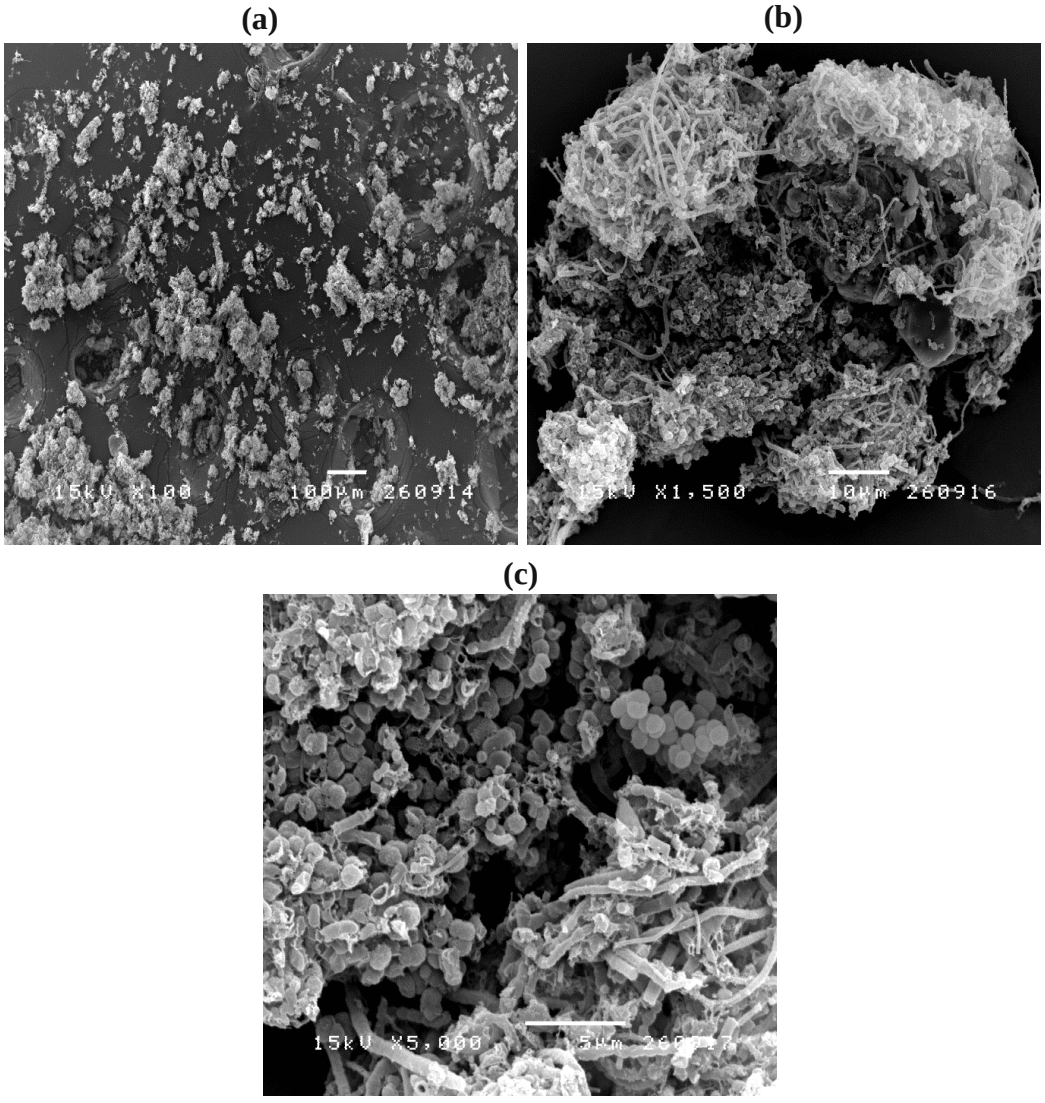


Figure 4.23 Morphology of microbial aggregates in R2 at the end of nucleation phase with magnifications of (a) 100x, (b) 1500x and (c) 5000x

The morphology of microbial aggregates in R2 was observed at various magnifications (100x, 1500x and 5000x), as shown at **Figure 4.23**. The structure of microbial aggregate was weak and fragile, similar to microbial aggregate of R1. However,

the diameter size of microbial aggregate in R2 was slightly larger as 70 – 80 µm than that in R1. This result was in line with the result of size distribution which showed that average diameter size and size distribution of large microbial aggregates (>100 µm) in R2 was higher than that of R1. According to the dominant microorganism observed in microbial aggregate of R2, the result was different with R1. Mixed microorganism appeared as dominant microorganism throughout microbial aggregates of R2. It can be stated that the formations of microbial aggregates in R1 and R2 were different. Self-aggregation may occurred during the formation of microbial aggregate in R1 as mentioned before resulting on filamenteous microorganism as dominant microorganism in aggregate. However, the formation of microbial aggregate in R2 may strongly be caused of the effect of cationic polymer addition, dynafloc 8265. This cationic polymer could reduce negativity of all microorganism surface charge (**Figure 4.8** about zeta potential results) which resulted on the aggregation of all microorganism to form larger microbial aggregates. By this mechanism, mixed microorganism (cocci-, rod- and filament-shaped microorganism) aggregated each other because their surface charges were less negative. However, the effect of dynafloc 8265 was not too strong to maintain the microbial aggregates which resulted on weak and fragile structure. The results of size distribution of microbial aggregates in R2 also showed that average diameter size and nuclei ratio were slightly higher than that of microbial aggregate in R1.

The observation of microbial aggregates in R3 with various magnifications at day 58 can be seen in **Figure 4.24**. Chitosan affected on the structure of microbial aggregate in R3 which showed stronger and more compact structure than that of microbial aggregate in R1 and R2. The diameter size was found larger as approximately 500 – 600 µm. The dominant microorganism in microbial aggregate of R3 was found similar with that of microbial aggregates in R2. Mixed microorganism (such as cocci-, rod- and filament-shaped microorganism) also appeared as dominant microorganism in microbial aggregate of R3. Therefore, it can be concluded that the formations of microbial aggregate of R2 and R3 were similar in which cationic polymers, dynafloc 8265 and chitosan, were responsible for microbial aggregation. The main different was the structure of microbial aggregate of R3 which was stronger and more granule-like than that of microbial aggregate of R2. Cation from chitosan strongly affected on microbial aggregation and polymer-like characteristic of chitosan maintained that the compactness of microbial aggregate of R3. The structure of microbial aggregates of R3 was granule-like with pile of microbial nuclei or smaller

microbial aggregates. Lack of EPS production during nucleation phase may cause the roughness of microbial aggregate surface.

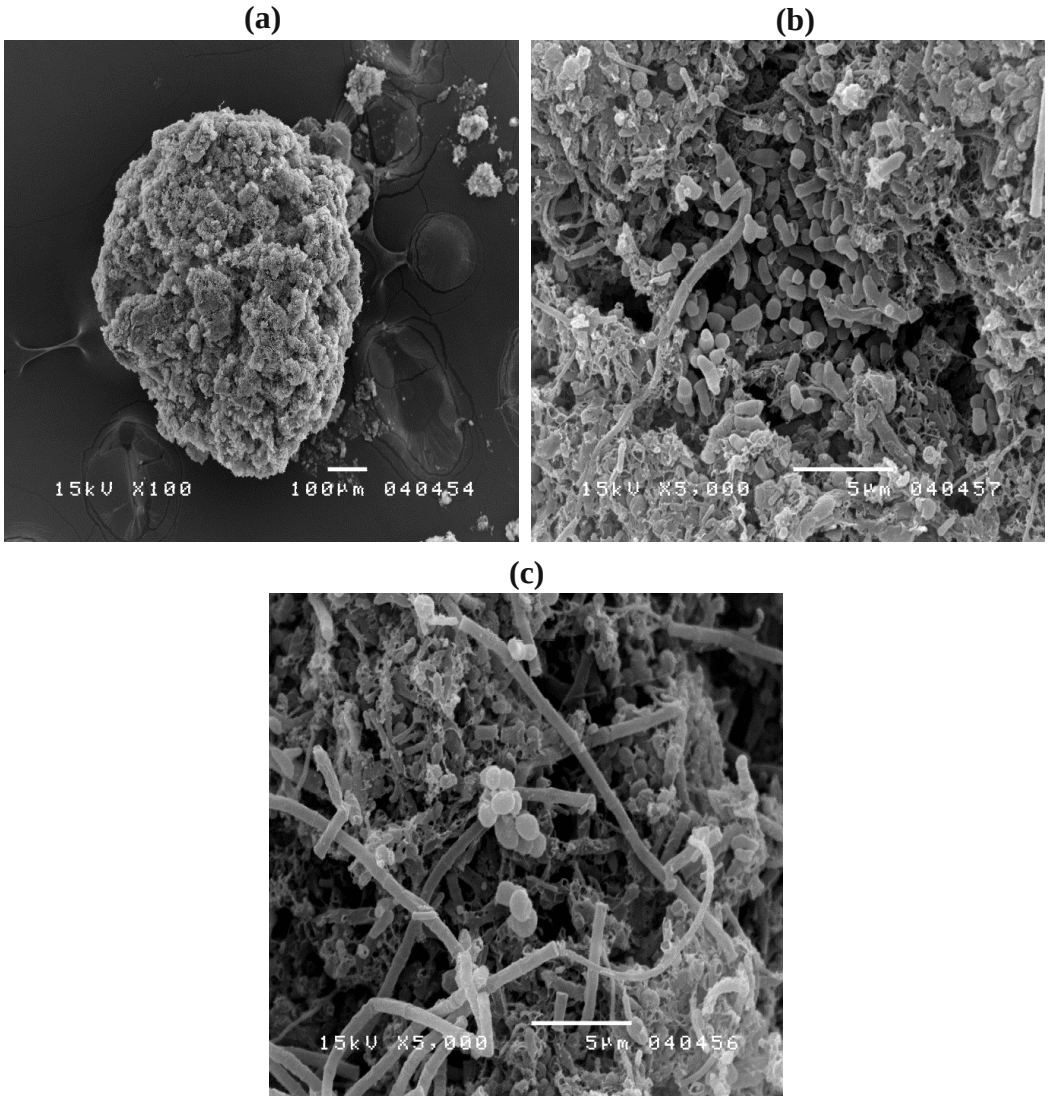


Figure 4.24 Morphology of microbial aggregates in R3 at the end of nucleation phase with magnifications of (a) 100x, (b) 1500x and (c) 5000x

4.7.3 Morphology of microbial aggregates at the end of early maturation phase

Substrate switching from mixed VFA to glucose was able to alter the dominant microorganisms inside the reactors. The morphology of microbial aggregates at day 118 was observed to investigate the effect of substrate switching to dominant microorganism and structure of microbial aggregates in all reactors. Based on the results of size distribution, the average diameter size and granule ratio in each reactor still increased during early maturation

phase. EPS composition may affect on the development of microbial nuclei to be larger microbial aggregates or granules.

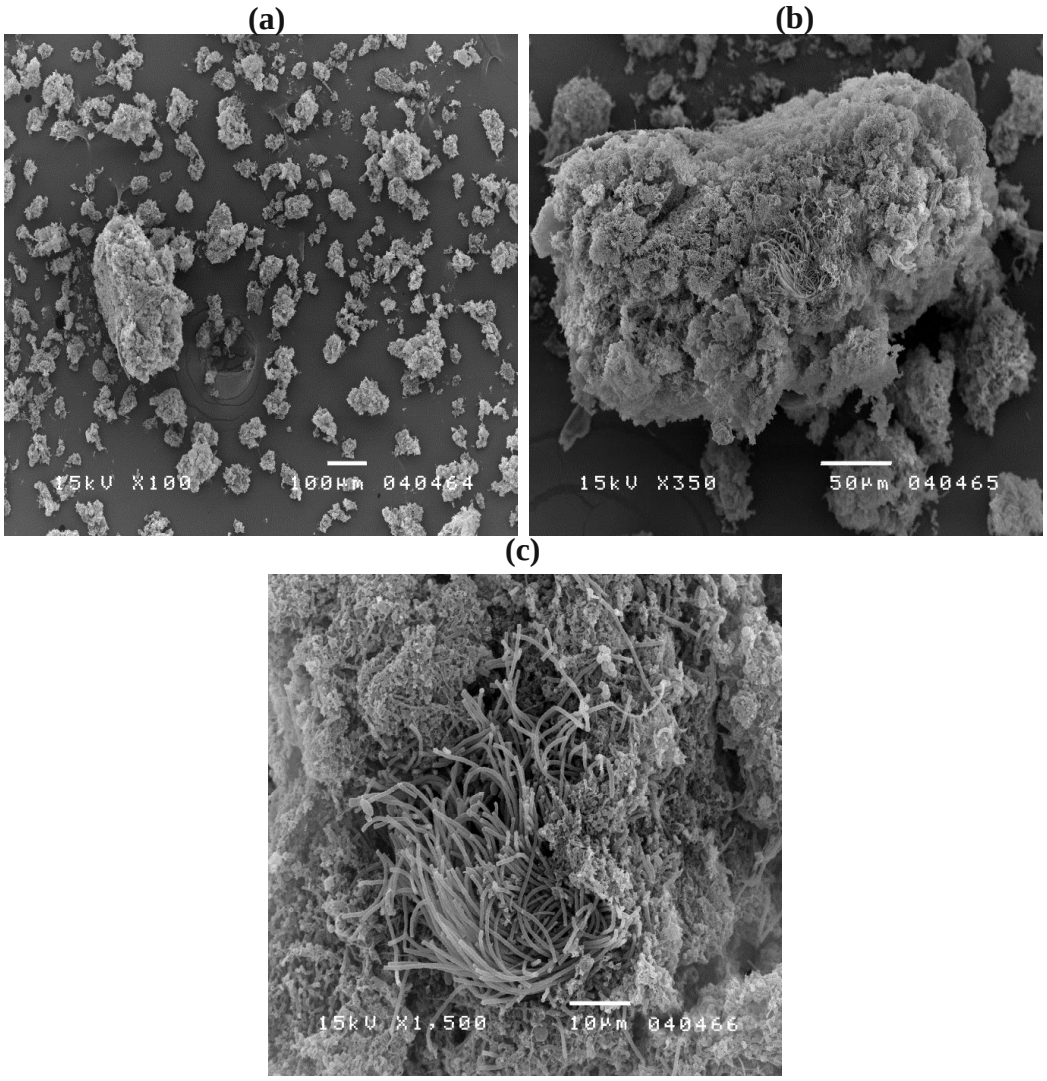


Figure 4.25 Morphology of microbial aggregates in R1 at the end of early maturation phase with magnifications of (a) 100x, (b) 350x and (c) 1500x

Figure 4.25 shows the morphology of microbial aggregates of R1 at the end of the early maturation phase (day 118) at various magnifications. The microbial aggregation of R1 in this phase seemed more perfect as nuclei compared to nucleation phase which the structure of this aggregate was larger and more compact. The diameter size of this microbial aggregate was approximately 150 – 200 µm. At magnification of 1500x, it can be seen that some group of filament microorganism appeared from inner part of microbial aggregate and

was surrounded by mixed microorganism. On nucleation phase, the formation of microbial aggregate in R1 was initiate by self-aggregation of filamentous microorganism and the structure of this aggregate was weak and fragile. However, that microbial aggregate, which was resulted from self-aggregation, became more perfect in the early maturation phase. Substrate switching from mixed VFA to glucose was able to trigger on the growth of microorganisms that acted as a "blanket" for weak filamentous-microbial aggregates. Glucose also enhanced on EPS production which helped for strengthening and maintaining the compactness of microbial aggregate.

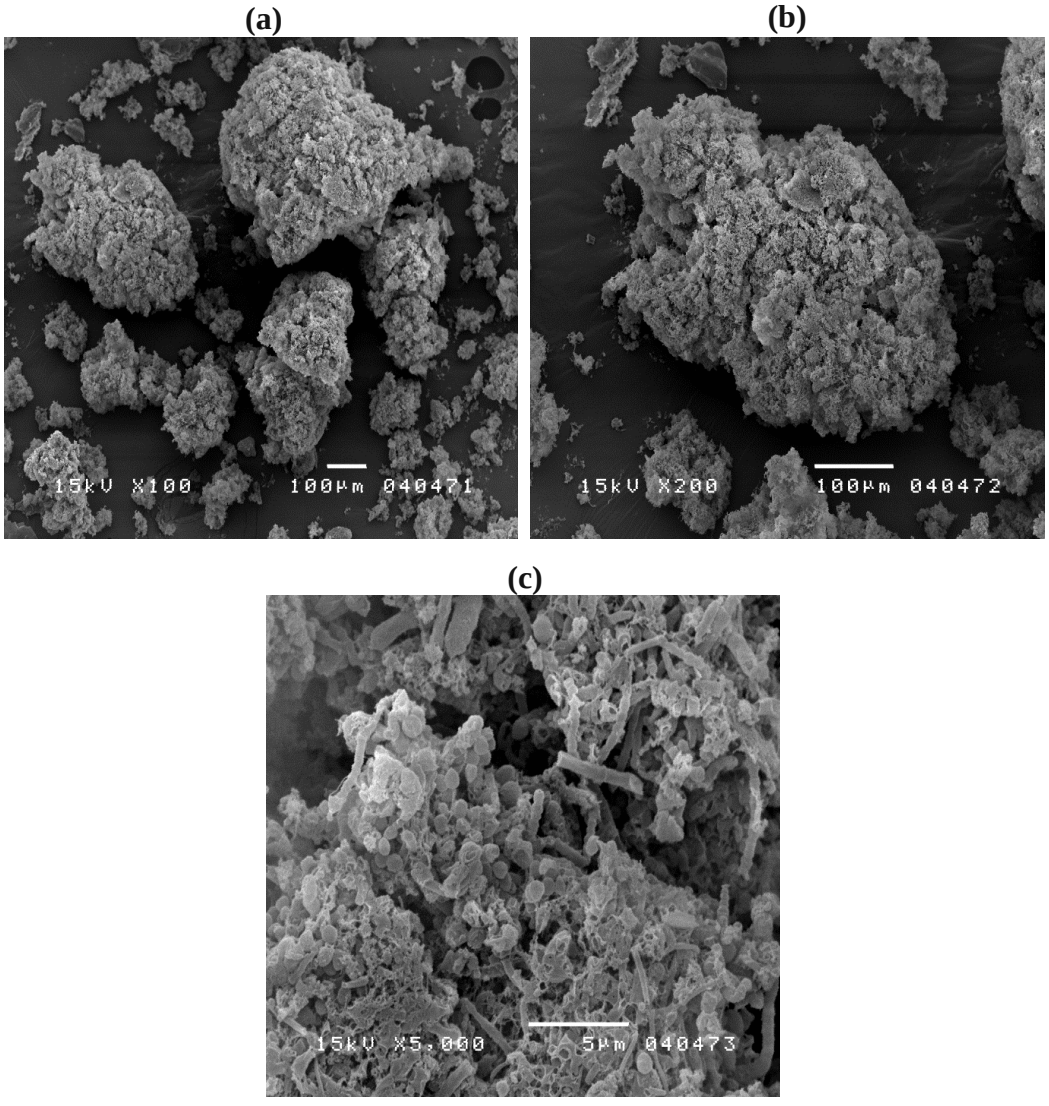


Figure 4.26 Morphology of microbial aggregates in R2 at the end of early maturation phase with magnifications of (a) 100x, (b) 200x and (c) 5000x

The morphology of the microbial aggregate in R2 was also observed at day 118 at various magnifications (**Figure 4.26**). The morphology of microbial aggregates of R2 at magnification 100x seemed larger than that of microbial aggregate in R1. The results of SEM observation for microbial aggregate in R2 were in agreement with the results of size distribution which showed that the ratio of larger microbial aggregates in R2 was higher than that in R1 (**Figure 4.3** – size distribution results). Therefore, the average diameter size of microbial aggregate in R2 was higher than that of microbial aggregate in R1 during early maturation phase. At day 118, the diameter size of microbial aggregate in R2 was observed as approximately 300 – 400 µm. The dominant microorganism found in microbial aggregate of R2 was still mixed microorganism, similar with its dominant microorganism at the end of nucleation phase. However, the structure of this microbial aggregates at the end of early maturation (day 118) seemed stronger and more compact than that at the end of nucleation phase (day 58). Triggering of EPS production during early maturation phase by substrate switching could help on bridging among microbial nuclei and strengthening the structure formed.

The structure of mature granules was observed in R3 at the end of the early maturation phase (**Figure 4.2**7). Physical characteristics, such as the morphology and diameter size, were completed by microbial aggregate of R3 at the end of early maturation phase (day 118). The diameter size of this microbial aggregate was observed as approximately 800 – 900 µm. Mixed microorganism, such as cocci- and rod-shaped microorganism, *Methanosaeta* – and *Methanosarcina* – like microorganism, appeared as dominant microorganism at surface of granule. The initial aggregation of small aggregates in R3 during nucleation phase was triggered by the addition of chitosan. Chitosan decreased the negativity of microbial surface charge (based on zeta potential results), thus caused microorganism and smaller aggregates in R3 aggregated each other to form nuclei with average diameter 100 – 600 µm. However, the morphology microbial nuclei at the end of nucleation (day 58) was rough and more like piles or clumps of smaller microbial aggregates. Those structures of microbial nuclei was then perfected during early maturation phase. Lack EPS production during nucleation phase was considered as main factor for rough and clumps structures of microbial nuclei. Therefore, EPS production was then enhanced to perfect the structure of microbial nuclei by switching the substrate from mixed VFA to glucose. As a result of SEM observation, the surface of microbial aggregate in R3 was smoother and the structure of it also had more compact and spherical forms.

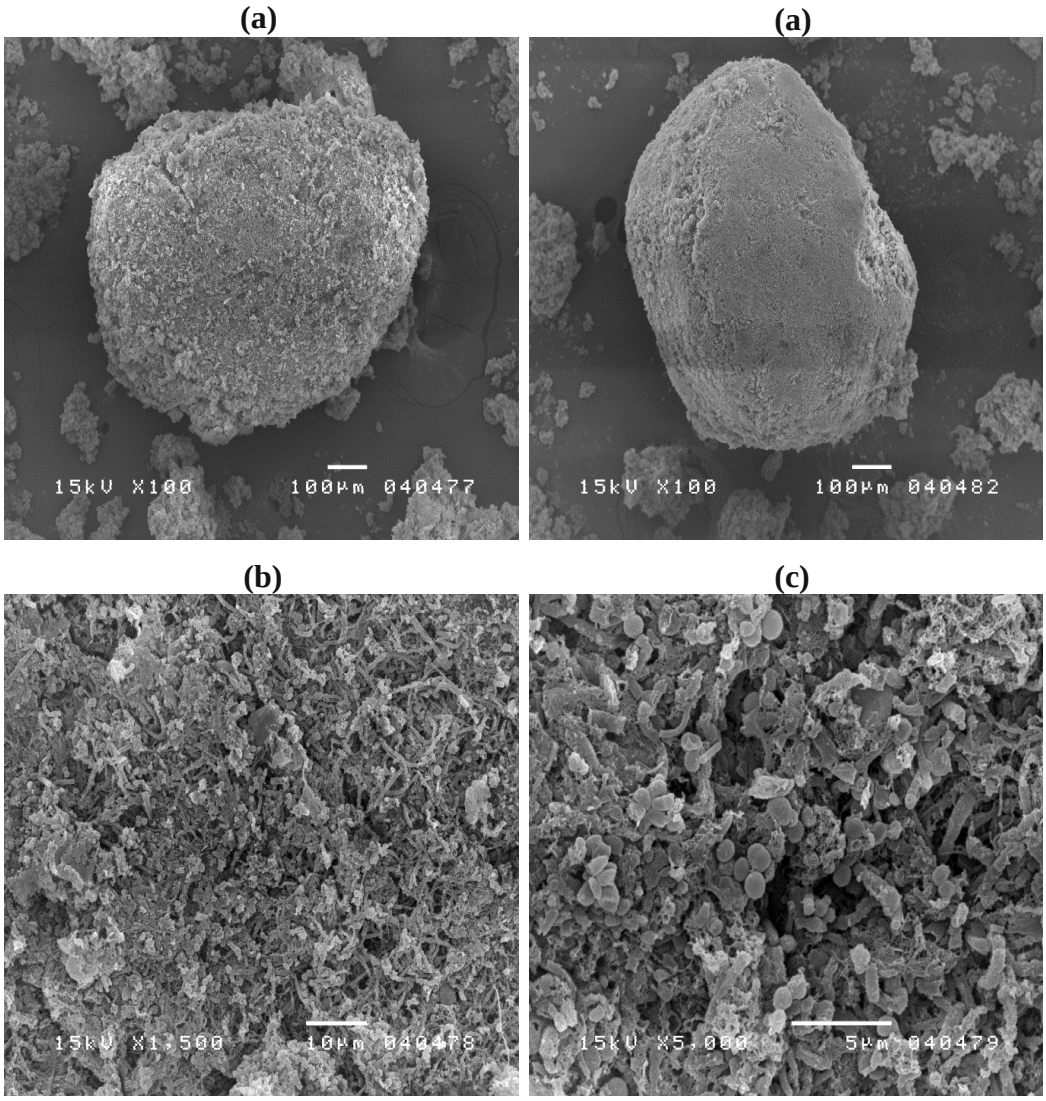


Figure 4.27 Morphology of microbial aggregates in R3 at the end of early maturation phase with magnifications of (a) 100x, (b) 200x and (c) 5000x

4.8 Microbial distribution in microbial aggregates during nucleation and early maturation phase

The microbial compositions inside the microbial aggregates or granules were observed by fluorescence in situ hybridization – confocal laser scanning microscopy (FISH-CLSM). FISH was used to determine the location of specific microbial groups in each microbial aggregate of R1, R2 and R3. The probes used for *Archaea* (presumptively methanogens) and *Eubacteria* (presumptively acidogenic and acetogenic microorganism) were ARC915 with red cy3 label and EUB338 with green FITC label, respectively. The

development of microbial compositions in microbial aggregates of all reactors were observed on the initial day, at the end of nucleation phase (day 58) and the end of the early maturation phase (day 118).

4.8.1 Microbial distribution in initial seed sludge

The microbial distribution in the initial seed sludge is shown in **Figure 4.28**. The microbial aggregates observed had diameter size approximately 50 – 60 µm. FISH result showed that the distribution of *Archaea* (red) and *Eubacteria* (green) was dispersed and randomly distributed throughout aggregates. The result of microbial distribution analysis by FISH was correlated with the results of morphology observation under SEM. At initial seed sludge, the microbial layered in microbial aggregate had not been formed. *Archaea* seemed more dominant than *Eubacteria* in initial seed sludge

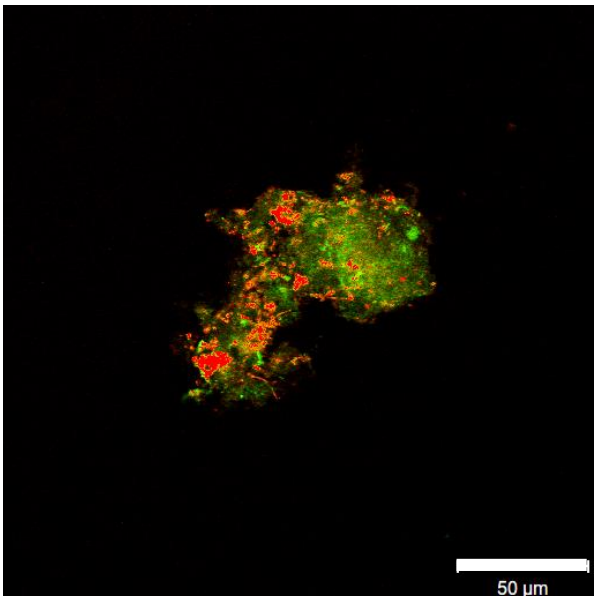


Figure 4.28 Microbial distribution in initial seed sludge (red cy3 probe – *Archaea*; green FITC probe – *Eubacteria*; white bar: 50 µm)

4.8.2 Microbial distribution in microbial aggregates at the end of nucleation phase

The microbial distributions inside the aggregates of all the reactors were also observed at the end of nucleation phase (day 58). The effect of cationic polymer addition on microbial distribution could be investigated by FISH-CLSM results. The morphology of microbial aggregate based on SEM analysis was possible to be confirmed by the result of FISH-CLSM.

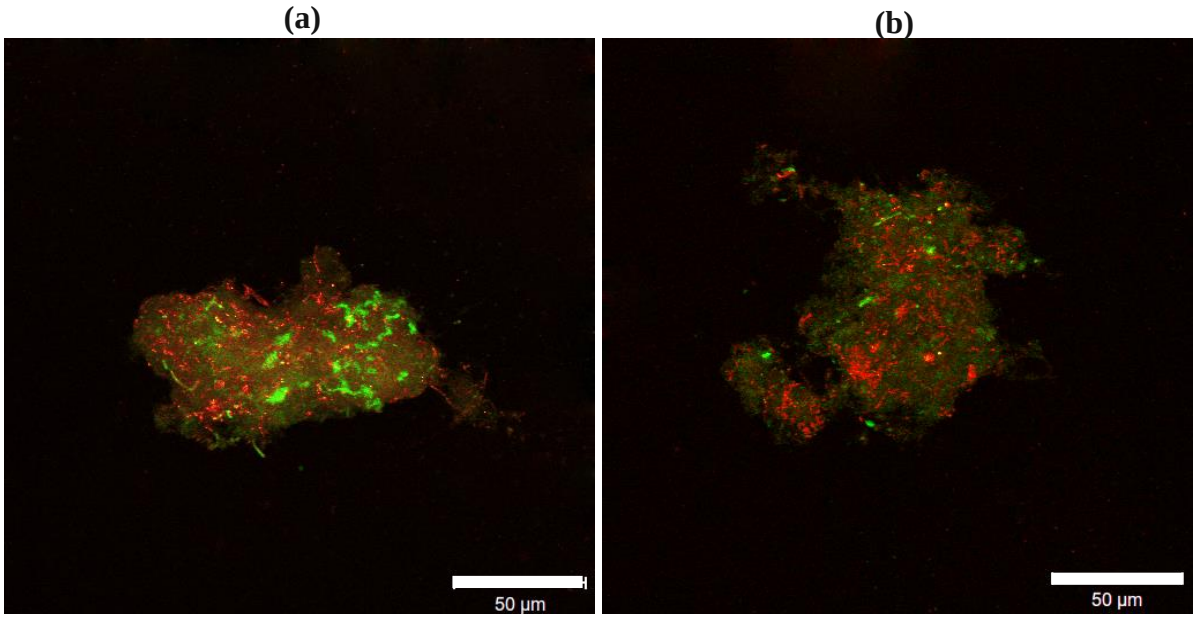


Figure 4.29 Microbial distribution in aggregates of (a) R1 and (b) R2 at the end of nucleation phase (day 58) (red cy3 probe – *Archaea*; green FITC probe – *Eubacteria*; white bar: 50 µm)

The microbial distribution in the aggregates of R1 and R2 at the end of the nucleation phase (day 58) showed that *Archaea* and *Eubacteria* were still randomly dispersed inside the aggregate (**Figure 4.29**). These results were similar with microbial distribution in initial seed sludge. More *Archaea* was observed in both aggregates while *Eubacteria* was around that red group *Archaea*. Mixed VFA feeding in nucleation was possible to enhance the population of active methanogen group (*Archaea*) and its specific activity. The results of specific microbial activities showed that the activity of BDM, PDM and ACM increased from initial day until day 58. Those results were confirmed by the result of FISH-CLSM which showed the balance composition between acetogenic microorganism (green *Eubacteria*) and methanogens (red *Archaea*).

The results of the size distribution showed that the average diameter size and nuclei ratio of the microbial aggregates of R3 were higher than those of the microbial aggregates of R1 and R2. These results were confirmed by SEM observations that showed the presence of microbial nuclei with rough and clump structures at the end of the nucleation phase (day 58). The result of FISH-CLSM analysis seemed in agreement with the results of the size distribution and SEM analysis. **Figure 4.30** shows the microbial distribution in microbial aggregates of R3 at the end of nucleation phase (day 58) with various magnification. The results showed that *Archaea* was dominant over *Eubacteria* in that aggregates which indicated that methanogen population was more than acetogenic microorganism. At higher magnification, it could be seen that the morphology of the methanogen was dominated by filament – shaped methanogen or *Methanosaeta sp.* – like methanogen. *Archaea* was located in the middle or inner part of microbial aggregate, although some colonies were found at outer part. While *Eubacteria* was found at middle to outer part of aggregate. However, it cannot be concluded that the layer of microorganism was formed in this phase because mixed microorganism groups, *Archaea* and *Eubacteria*, were still randomly dispersed. The result of FISH-CLSM supported the hypothesis of microbial aggregation due to chitosan addition by SEM analysis. The addition of chitosan was able to decrease the negativity of microbial surface charge in reactors. As a result, all microorganism tend to aggregate each other and form larger aggregates with random and dispersed composition of *Archaea* and *Eubacteria* as observed by FISH-CLSM analysis.

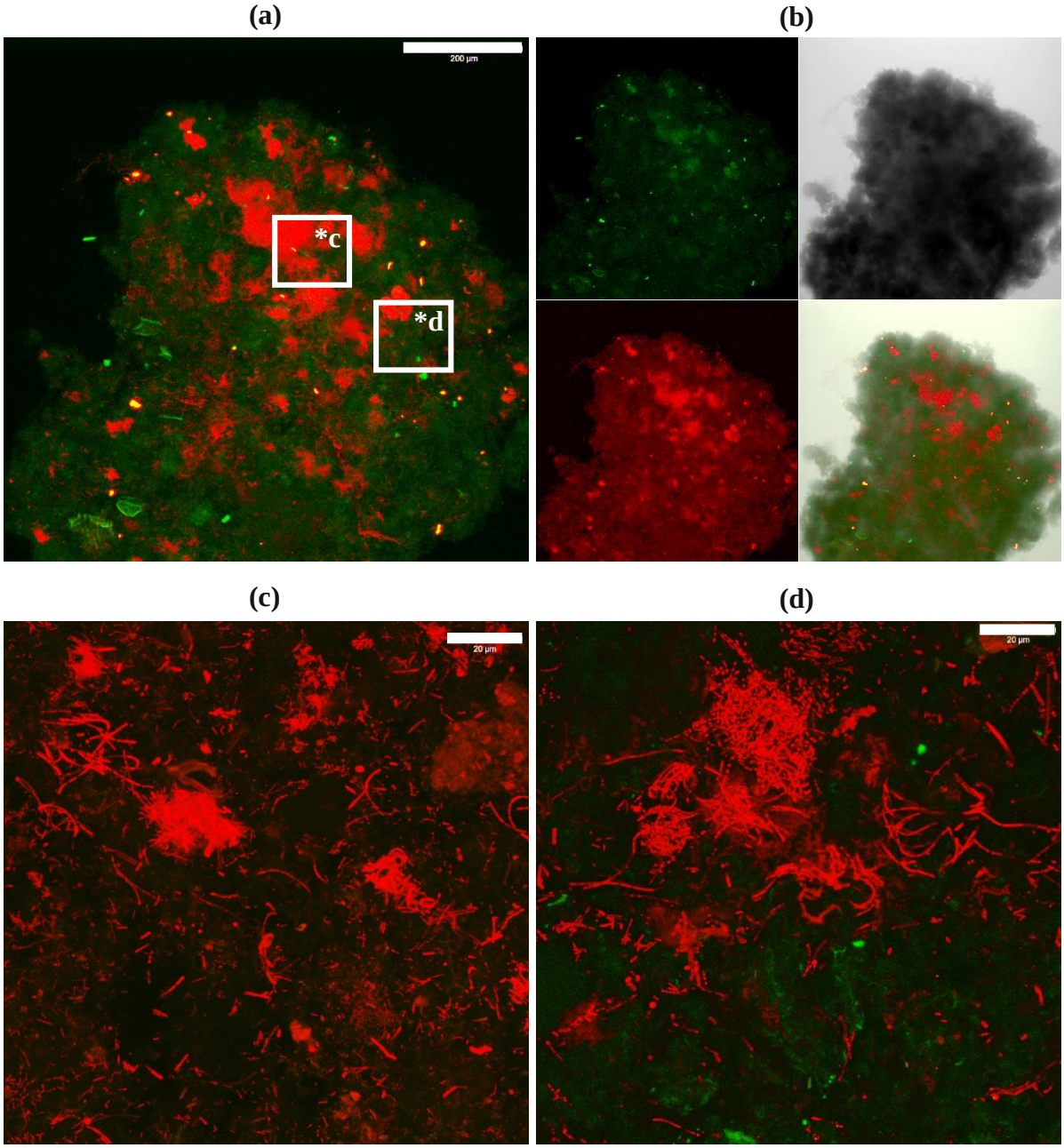


Figure 4.30 Microbial distribution of microbial aggregates in R3 at the end of nucleation phase (day 58) (red cy3 probe – *Archaea*; green FITC probe – *Eubacteria*; bar (a, b): 50 µm; bar (c,d): 20 µm)

4.8.3 Microbial distribution in microbial aggregates of R3 at the end of early maturation phase

The development of microorganisms inside the anaerobic reactors was affected by various factors, such as substrate types. The substrate switching from mixed VFA used in nucleation phase to glucose used in early maturation phase could affect on the microbial composition in anaerobic reactors. In nucleation phase, *Archaea* was dominant microorganism in microbial aggregates of R1, R2 and R3. Mixed VFA feeding triggered the population and specific activity of syntroph microorganism, including methanogen. However, mixed VFA was then switched to glucose as main substrate in early maturation phase. The substrate switching was aimed to trigger the EPS production for strengthening microbial nuclei which was produced from nucleation phase. However, glucose also enhanced the population of fast-growing acidogenic microorganism which may produce more organic acids and thus decreased pH inside reactors.

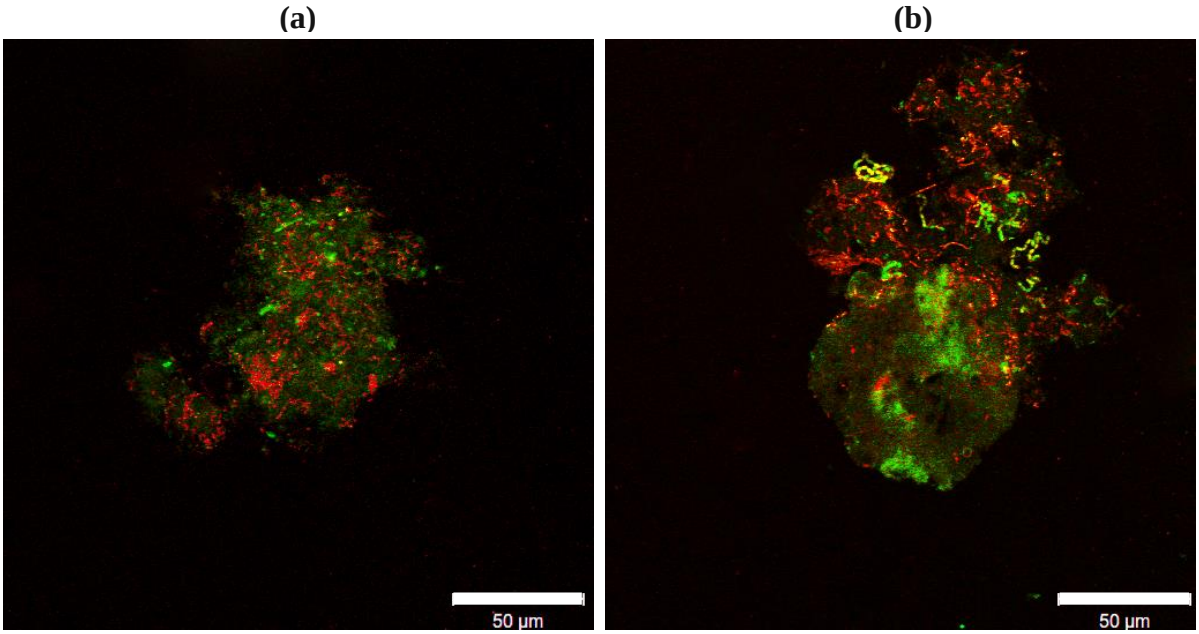


Figure 4.31 Microbial distributions in aggregates of (a) R1 and (b) R2 at the end of early maturation phase (day 118) (red cy3 probe – *Archaea*; green FITC probe – *Eubacteria*; white bar: 50 µm)

The microbial distributions of *Archaea* and *Eubacteria* in the aggregates of (a) R1 and (b) R2 at the end of the early maturation phase (day 118) are shown at **Figure 4.31**. It seemed that green *Eubacteria* was dominant than red *Archaea* groups. Those microbial

groups were still randomly dispersed inside reactors and the diameters of microbial aggregates were approximately 90 – 100 µm. Glucose as the main substrate in this phase had affected these microbial distributions. The utilization of glucose could enhance the production of organic acids which decreased pH system. High organic acids and low pH inhibited the activity of methanogen (the most sensitive microorganism) which randomly located inside aggregates. It means that methanogen was directly exposed to that organic acids and low pH. As a result, the activity and population of methanogen may decreased. While, the activity and population of *Eubacteria*, especially acidogenic microorganism increased until day 118. The ratio between the activity of ACM to BDM-PDM of microbial aggregates in R1 and R2 were low in the early maturation phase (**Figure 4.20**).

Different results of the FISH-CLSM analysis were observed in the microbial aggregate of R3. The substrate switching from mixed VFA to glucose was able to enhance EPS production which then positively acted on the formation of multilayered granule. EPS bridged microbial nuclei with other nuclei or small microbial aggregates to form mature granule. **Figure 4.32** showed the microbial distribution in aggregates of R3 at the end of early maturation (day 118). Red *Archaea* clumps, presumptive methanogen, was mostly located at middle and inner part of aggregates. Those *Archaea* clumps were surrounded by green *Eubacteria,* presumptive acidogenic or acetogenic microorganism. Acetogenic and methanogenic syntroph consortia was considered as *Eubacteria* (cell binding EUB338-FITC) and closely associated with *Archaea* (normally fine filaments binding ARC915-cy3), while acidogenic microorganism was considered as *Eubacteria* which was unassociated with *Archaea* [15]. In a good anaerobic digestion system, the distance between VFA degraders (acetogenic microorganism) and methanogen was close each other [107]. Therefore, it can be concluded that green *Eubacteria* clumps around red *Archaea* clumps were acetogenic microorganism (**Figure 4.32b)**. These positions had allowed the possibility of syntroph relationships inside microbial aggregate of R3. The accumulation of organic acids and low pH (as result of glucose degradation) could not adversely affect on the activity of methanogen since the distribution of red *Archaea* clumps, presumptive methanogen, was at inner part of microbial aggregate of R3. These microbial distribution in microbial aggregate of R3 was probably the main cause for better ACM activity and reactor performances during early maturation phase.

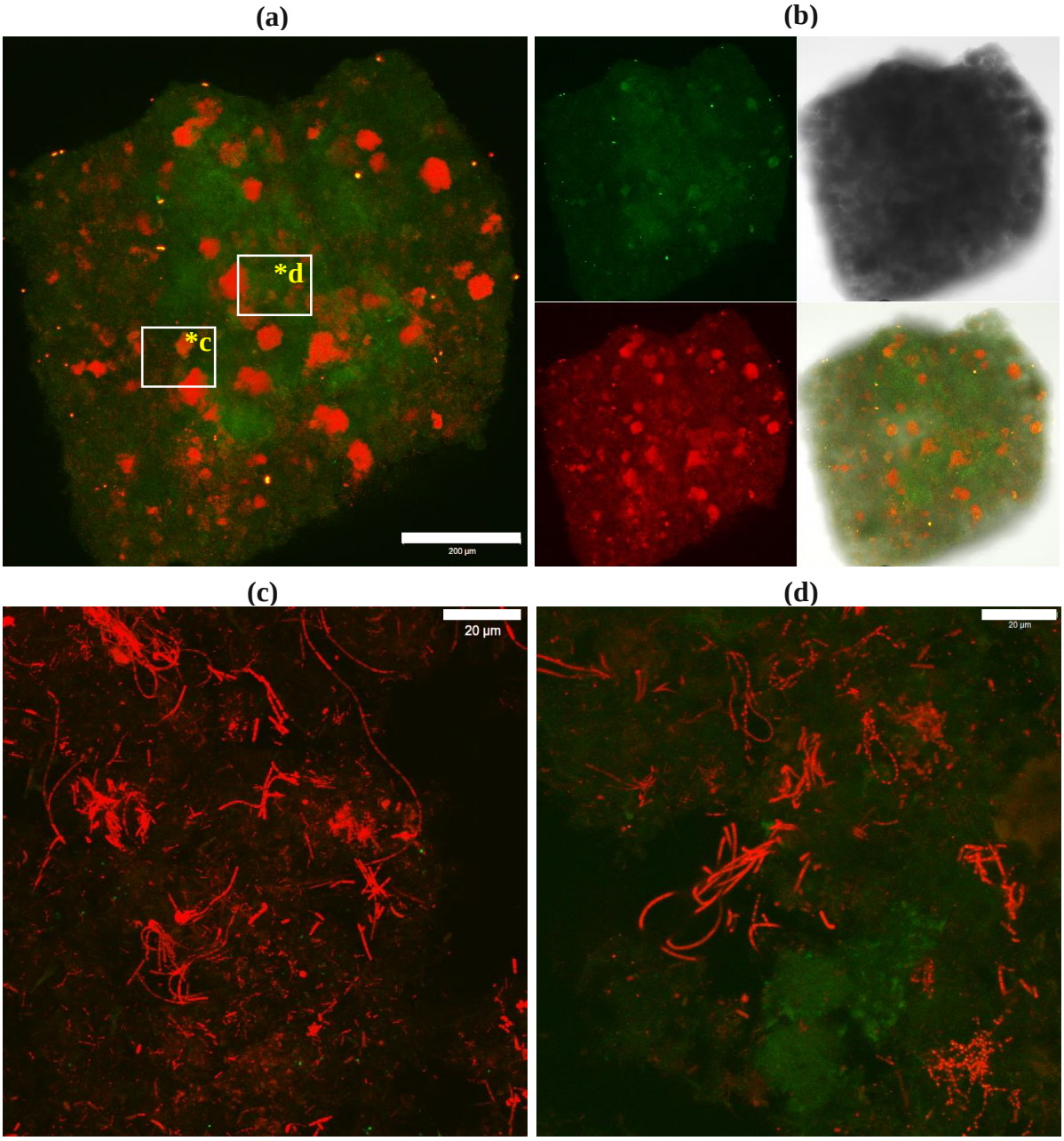


Figure 4.32 Morphology of microbial aggregates in R3 at the end of early maturation phase (day 58), red cy3 probe – *Archaea*; green FITC probe – *Eubacteria*; bar (a, b): 50 µm; bar (c, d): 20 µm

4.9 The mechanism of dynafloc 8265 and chitosan for microbial aggregation during nucleation phase

Dynafloc 8265 and chitosan represented synthetic and natural cationic polymers. Besides their cationic characteristics, these polymers were considered for their water absorbing ability. Water-absorbing polymers (WAP) swelled in water, which then exhibited a complex network structure for microbial attachment sites. Large structures of WAP in water could improve microbial contacts to particles due to its lower density than the densities of sand or other inert particles.

According to the results above, chitosan addition in R3 displayed better qualities of microbial aggregates than did dynafloc 8265 in terms of zeta potential, size distribution, settling ability and also nuclei structure at day 58. Chitosan could significantly increase the zeta potential values of microbial aggregates in R3 from -26.4 mV to -10.5 mV at day 0 to day 58, respectively. While, dynafloc 8265 slightly decreased the zeta potential values of microbial aggregates in R2. Based on the polymers stock solutions, dynafloc 8265 and chitosan has high positive zeta potential values as approximately +45 to +50 mV. However, the effects of those cationic polymers addition in R2 and R3 were significantly different.

Sodium polyacrylate is manufactured by the free-radical polymerization of a mixture of sodium acrylate and acrylic acid, and a cross linker such as trimethylol propanetriacrylate (**Fig. 4.33**). In the wastewater treatments, polyacrylate is known for its salt form with sodium resulting sodium polyacrylate or *waterlock*. This polymer has the ability to absorb as much as approximately 200-300 times its mass in water by the principle of osmosis, the passage of water through a membrane permeable only to the water. Osmotic pressure results from the difference of the sodium ion concentration between the inside of the polymer and the solution. This osmotic pressure forces water into the solid polymer matrix in an attempt to equilibrate sodium ion concentration inside and outside the polymer. The concentration of electrolytes or ions in the water determines the osmotic pressure which then affects on the amount of water absorbed by the polymer. For example, sodium polyacrylate will absorb approximately 800 times of its weight in distilled water, but will only absorb 300 times of its weight in tap water, due to the high ion concentration of tap water.

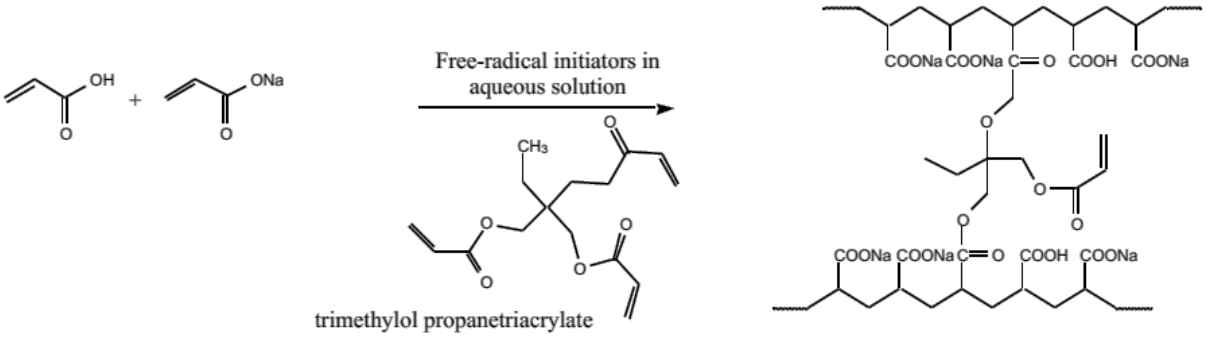


Figure 4.33 The formation of sodium polyacrylate from acrylic acid and sodium acrylate [108]

Dynafloc 8265 slightly increased the zeta potential values of microbial aggregates in R2 during the nucleation phase. From this result, it can be concluded that dynafloc 8265 was not efficient to decrease the negativity of microbial surface charges which then lead to slow development of granule nuclei in R2. In dry state, sodium polyacrylate consists of sodium linked to oxygen atoms (**Fig. 4.34**). This link can be broken when this polymer is dissolved in water due to salt dissolution which causes sodium ions disperse in water and linear chain of polyacrylate become negatively charged (**Fig. 4.34**). These long chain tend to unfold due to electrostatic repulsion which allows to absorb a large quantity of water and increases their volume (more surface for microbial attachment). Although the volume of polyacrylate significantly increased in water, microorganism was probably difficult to attach on that polymer due to the repulsion forces of similar charge between microbial surface charge and carboxyl groups of polyacrylate. Moreover, sodium ions from sodium polyacrylate which was dispersed in water could not significantly decrease the negativity of microbial surface charge in R2. Cations from sodium ions were probably washed out with the effluents during the reactor operation.

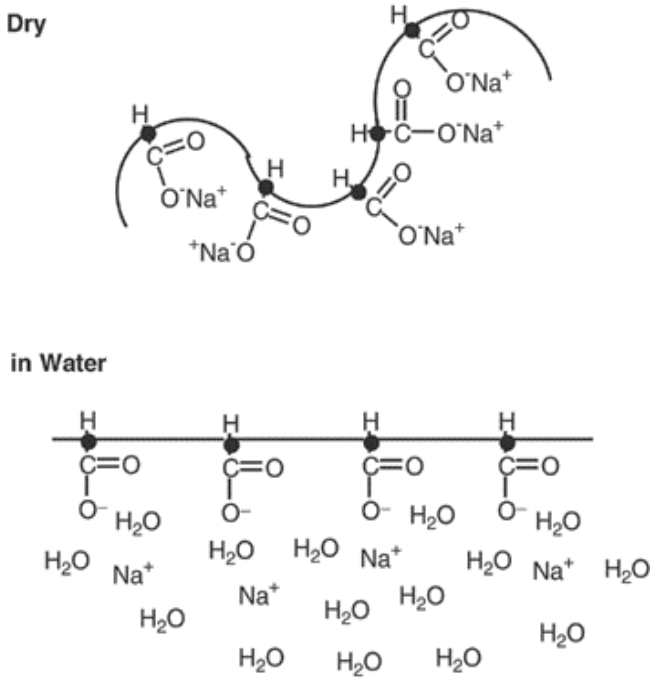


Figure 4.34 The structures of sodium polyacrylate in dry and wet matter [108]

Different results were obtained from in R3 in which chitosan significantly increased the zeta potential values. Chitosan has two main characteristics, water absorbing ability and cations that affected on the significant increase of granule nuclei during nucleation phase. Water absorbing ability of chitosan was similar with other water absorbing polymer which was able to swell in water and then provided complex network for microbial attachment. The protonization of amino groups of GlcN in solution resulted in positive charge for chitosan as shown at **Fig. 4.35** [58]. Cations from chitosan was assumed to decrease the negativity of microbial surface charge in R3 which was shown by significant increase of zeta potential values during nucleation phase. Then, microorganism in R3 tend to flocculate or aggregate each other and trapped by complex network resulted from water absorbing characteristic of chitosan. These double effects of chitosan, i.e., cation and water absorbing characteristics, resulted on stronger and larger microbial aggregates in R3.

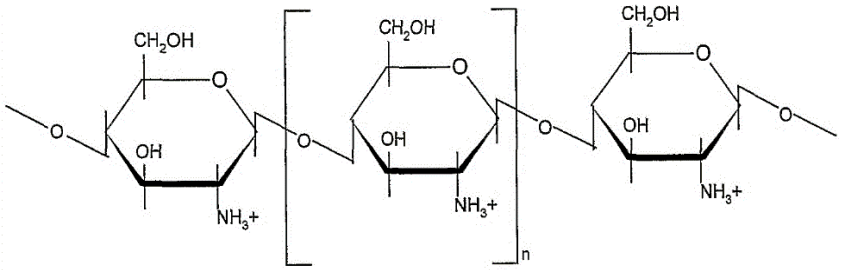


Figure 4.35 Structure of fully deacetylated chitosan [57]

Based on the mechanism of the two cationic polymers, it can be concluded that the double effects of chitosan, i.e. cations amount and water absorbing ability, were the main factors determining the success of the nucleation phase. Cations from chitosan decreased the negativity of microbial surface charge indicated by increase of zeta potential value in R3 which lead on microbial aggregation. Then, the nuclei ratio in R3 also increased due to more microbial aggregation. Large microbial aggregates linearly related to good settling ability which was shown by lowest SVI value in R3 compared to other reactors. The complex network as the effect of water absorbing characteristic of chitosan was able to maintain the structure of microbial aggregates in R3. Based on SEM result, the structure of microbial aggregates in R3 was stronger and more compact compared to that of R1 and R2 which showed lumpy, loose and weak structures.

4.10 The stability of anaerobic granules

The stability of the granules were considered to be one of the most important factors for the successful operation of an anaerobic granule-based reactor. This could be determined based on the development of physiochemical and microbial characteristics of granule during reactor operation. Based on this study, stability of microbial granule was considered on zeta potential, size distribution (nuclei and granule ratio), sludge volume index, specific microbial activities and structure of microbial granule. The stability of microbial granule was observed during the nucleation and early maturation phases in order to achieve accurate times of granulation.

The operational condition for the UASB reactor, such as OLR and substrate type, affected the stability of the microbial granules. Increase of OLR produced more biogas inside the reactor which was able to alter the hydrodynamic condition. Too high hydrodynamic condition inside reactor (due to feeding velocity and biogas production) could washed the

microorganism and smaller granule out from reactor. Disruption of anaerobic granule had been observed due to too high hydrodynamic shear forces applied inside reactor [10, 109]. High OLR could negatively affect on specific microbial activities since the balance of each microbial groups of anaerobic digestion must be maintained. Rapid ORL increase adversely affected methanogen activity which then lead to reactor failure. Type of substrate also affected on the stability of microbial activity in anaerobic digestion. The utilization of glucose caused rapid acid production in which the accumulation of acids could inhibit the microbial activities, especially methanogen. Another factor, good anaerobic granule must have main characteristics to overcome the factor which possible to disrupt granule quality. The round and spherical structure of granule could be an advantage to avoid sludge washout due to high hydrodynamic condition.

In this study, the anaerobic granules from R3 were focused on for their stability for since these granules displayed the best physicochemical and microbial characteristics. Those characteristics were shown by anaerobic granule of R3 which still developed during nucleation phase, such as nuclei and granule ratio, SVI, microbial activities and morphologies. Nuclei ratios of microbial aggregate in R3 were observed as 0.59, 24.6 and 55.1% at day 0, 33 and 58, respectively. While, granule ratios were approximately, 1.8 and 8.2% at day 0, 33 and 58, respectively. Settling ability also increased as shown by decrease of SVI value as 72, 66 and 43 $ml.g^{-1}SS$ at day 17, 33 and 58, respectively. The ratio between methanogen and non-methanogen activities of microbial aggregates in R3 also increased during nucleation phase as 0.07, 0.22 and 0.45 at day 0, 33 and 58. The morphology of anaerobic granule from R3 was observed as strong and compact aggregates. However, the surface of that granule was still clumpy and rough which was still possibly easy to break by high hydrodynamic condition inside reactor. Microbial distributions, i.e., *Archaea* and *Eubacteria*, were still dispersed throughout granule. From the results of nucleation phase, it can be concluded that anaerobic nuclei or granule still developed to be better granule. Therefore, the stability was still observed during the early maturation phase in which the substrate switched from mixed VFA to glucose. Glucose could produce acids rapidly which could affect the stability of anaerobic granule, especially specific microbial activities.

At the beginning of the early maturation phase (OLR 0.5 kg $COD.m^{-3}.d^{-1}$), the physicochemical and microbial characteristics of the granules in R3, e.g. granule ratio, SVI and activities ratio between methanogen and non-methanogen, still increased. Granule ratios were observed as 17.6, 30.8 and 34.2% at day 74, 88 and 118, respectively. SVI values were

approximately 37, 35 and 33 ml.g^{-1}SS at day 74, 88 and 118, respectively. Glucose as main substrate in this phase could increase the activity of GDM decreased the activity ratios of methanogen and non-methanogen of granule in R3 as 0.27 and 0.23 at day 88 and 118, respectively (which were lower than that at day 58). However, the activity ratios of methanogens and non-methanogens of granule in R1 and R2 significantly decreased during early maturation phase. Acids production from glucose fermentation during early maturation adversely affected on acetoclastic methanogens in R1 and R2, whereas the activity of non-methanogen significantly increased.

Based on these parameters, there was no significant difference between the physicochemical and microbial characteristics of the microbial aggregates in R3 observed on day 88 and 118. Therefore, it was possible to stop the early maturation phase at day 88, instead of day 118. Early decision for terminating early maturation phase could contribute on faster time which could possibly shorten the whole start-up period for UASB reactor operation. Based on morphology of granule in R3, surface and structure of granule at day 88 was tend to smooth and no-clump structure which was not much different compared to morphology of granule at day 118.

CHAPTER 5
CONCLUSIONS AND FUTURE WORKS

5.1 Conclusions

The concomitant adaptation of seed sludge to a syntroph specific substrate and nucleation by the addition of a cationic polymer, i.e. chitosan, was able to produce nuclei with high syntroph activities in a short time. Chitosan addition in R3 could decrease the negativity of microbial surface charge which was represented by increase zeta potential values of microbial aggregates to near zero (-26.4 mV at day 0 and -10.5 at day 58). This resulted on increase of nuclei and granule ratio as approximately 55.1% and 8.2% at day 58, respectively, significant higher than control reactor, R1 and dynafloc addition, R2. Higher size distribution of nuclei and granule (larger microbial aggregates) affected on good settling ability which was shown by significant decrease of SVI values of microbial aggregates in R3. During nucleation phase, the effect of cationic polymer addition, especially chitosan addition, on nuclei formation was very significant than the effect of EPS because EPS concentration ($EPS_{protein}$ and $EPS_{polysaccharides}$) were not significant different in all reactors. The specific microbial activities in all reactors at the end of nucleation phase were higher compared to that at initial seed sludge. It was assumed that the adaptation of seed sludge to syntroph specific substrate successfully increased the specific microbial activities, i.e., BDM, PDM and ACM. Moreover, the activity ratios of methanogen and non-methanogen of granule in R3 increased from 0.07, 0.22 and 0.45 at day 0, 33 and 58, respectively. These results were supported by good reactor performance of R3, such as pH, TVA/alkalinity ratio, methane production and yield, during nucleation phase. At the end of nucleation phase, day 58, the morphology of granule from R3 was stronger and more compact compared to that from R1 and R2 (weak and fragile). However, the surface and structure of granule was not smoother and clumpy which was sensitive to break in high hydrodynamic condition of reactor. Microbial distribution, i.e., *Archaea* and *Eubacteria,* was still dispersed throughout granule.

The mixed VFA was then switched to glucose for enhancing EPS production during the early maturation phase. EPS production was aimed to strengthen the structure of nuclei formed in nucleation phase. Glucose was able to increase EPS production, especially $EPS_{polysaccharide}$, which could affect on granulation since there was no cationic polymer addition

in early maturation phase. Zeta potential values in all reactor slightly increased during this phase. It indicated that EPS also decrease the negativity of microbial surface charge in all reactors. The effect of EPS to granulation was clearly shown by microbial aggregates in R3. During early maturation phase, nuclei and smaller microbial aggregates ratio to total microbial aggregates in R3 tend to slightly decrease which were followed by the increase of granule ratio as 17.6, 30.8 and 34.2% at day 74, 88 and 118, respectively. This can be assumed that EPS bridged nuclei to nuclei or smaller microbial aggregates (<100 μm) to form larger microbial aggregates (granule). This tendencies was different compared to that in R1 and R2. In these reactors, only the nuclei ratio to total microbial aggregates increased which was followed by a decrease in smaller microbial aggregates ratio (<100 μm) to total microbial aggregates. EPS trapped nuclei and smaller microbial aggregates to form other nuclei in R1 and R2. The quality and ratio of nuclei resulted from nucleation phase was important factor determining the granule formation during early maturation phase. The increase of granule ratio during early maturation phase also decreased SVI value of microbial aggregates in R3. However, glucose feeding affected on specific microbial activity, especially methanogen (ACM). The activity of ACM significantly decreased during early maturation phase. Rapid acids production by glucose fermentation and weak/fragile structures of microbial aggregates of R1 and R2 probably affected on the decrease of ACM activity. Accumulated acids adversely affect the methanogen which could not be protected by outer layer structure of microbial aggregates of R1 and R2. In other hand, multilayered structure of anaerobic granule in R3 was observed by FISH-CLSM analysis in which *Archaea* clumps were located at middle and inner layer, while *Eubacteria* clumps were observed at outer layer. This structure could protect methanogen from adverse effect of acid accumulation during early maturation phase which then resulted on good and stable reactor performance during this phase.

The stability of the physicochemical and microbial characteristics were also observed during the nucleation and early maturation phases. During nucleation phase, microbial aggregates still developed to be better aggregates in terms of nuclei ratio, granule ratio, SVI and specific microbial activities. Substrate switching from mixed VFA to glucose during early maturation phase affected on characteristics of microbial aggregates. Early maturation phase was terminated at day 118 which was considered on stable development of granule in R3 from day 88 and 118. From results of this study, it can be concluded that the early maturation phase was possible to terminate at day 88 considered on non-significant increase

of granule characteristics in R3, such as granule ratio, SVI, microbial activities, activity ratio of methanogen to non-methanogen and granule morphology from days 88 to 118. The decision for terminating the early maturation phase was important to shorten the whole start-up period of the UASB reactor.

5.2 Future works

5.2.1 Direct substrate switching from mixed VFA to glucose adversely affected the acetoclastic methanogens in the reactors, especially R1 and R2, which led to a decrease of COD removal and methane productions. Therefore, the substrate switching should gradually be conducted by mixing between mixed VFA with glucose (vary the concentration ratio) before use glucose as sole carbon source.

5.2.2 The methanogen and non-methanogen activities in R3 were well balanced. Therefore, it is possible to use this microbial aggregates to treat organic wastewater, such as wastewater from tapioca and palm oil processing industries.

5.2.3 Multilayered anaerobic granules were produced at the end of the early maturation phase in R3. However, it would be interesting to study for the microbial communities by other types of analyses (FISH with more specific probe, DGGE, etc.) and in situ microbial activity in granules by microsensor analysis.

REFERENCES

[1]. Lettinga, G. (1995), Anaerobic Digestion and Wastewater Treatment Systems, *Antonie van Leeuwenhoek*, **67**, pp. 3-28.

[2]. Van Lier, J.B. (2007). Current Trends in Anaerobic Digestion: Diversifying from Waste(Water) Treatment to Resource Oriented Conversion Techniques. *The 11th IWA World Congress on Anerobic Digestion* September 23-26, Brisbane, Australia

[3]. Britz, T.J., Lamprecht, C., and Sigge, G.O. (2008), "Dealing with Environmental Issues", In *Advanced Dairy Science and Technology*, Blackwell Publishing Ltd, p.^pp 262-293.

[4]. Ahring, B., Angelidaki, I., de Macario, E., Gavala, H., Hofman-Bang, J., Macario, A., Elferink, S., Raskin, L., Stams, A., Westermann, P., and Zheng, D. (2003), Perspectives for Anaerobic Digestion, *Advanced in Biochemical Engineering/Biotechnology*, **81**, 1, pp. 1-30.

[5]. Gerardi, M.H. (2003), *The Microbiology of Anaerobic Digesters*. John Wiley & Sons, New Jersey.

[6]. Gupta, S.K. (2005), Morphological Study of the Granules in Uasb and Hybrid Reactors, *Clean Technol Environ Pol*, **7**, 5 April 2005, pp. 203-212.

[7]. Tiwari, M.K., Guha, S., Harendranath, C.S., and Tripathi, S. (2005), Enhanced Granulation by Natural Ionic Polymer Additives in Uasb Reactor Treating Low-Strength Wastewater, *Water Res*, **39**, 16, pp. 3801-3810.

[8]. Del Nery, V., Pozzi, E., Damianovic, M.H.R.Z., Domingues, M.R., and Zaiat, M. (2008), Granules Characteristics in the Vertical Profile of a Full-Scale Upflow Anaerobic Sludge Blanket Reactor Treating Poultry Slaughterhouse Wastewater, *Bioresource Technology*, **99**, 6, pp. 2018-2024.

[9]. Oleszkiewicz, J.A. and Thadani, V.J. (1988), Effects of Biofilter Media on the Performance of Anaerobic Hybrid Reactors, *Environ Technol Lett*, **9**, 2, pp. 89-100.

[10]. Wu, J., Zhou, H.-M., Li, H.-Z., Zhang, P.-C., and Jiang, J. (2009), Impacts of Hydrodynamic Shear Force on Nucleation of Flocculent Sludge in Anaerobic Reactor, *Water Research*, **43**, 12, pp. 3029-3036.

[11]. Chaiprasert, P., Suvajittanont, W., Suraraksa, B., Tanticharoen, M., and Bhumiratana, S. (2003), Nylon Fibers as Supporting Media in Anaerobic Hybrid Reactors: It's Effects on System's Performance and Microbial Distribution, *Water Res*, **37**, 19, pp. 4605-4612.

[12]. Liu, Y. and Tay, J.-H. (2004), State of the Art of Biogranulation Technology for Wastewater Treatment, *Biotechnology advances*, **22**, 7, pp. 533-563.

[13]. Hulshoff Pol, L.W., de Castro Lopes, S.I., Lettinga, G., and Lens, P.N.L. (2004), Anaerobic Sludge Granulation, *Water Res*, **38**, 6, pp. 1376-1389.

[14]. Schmidt, J.E. and Ahring, B.K. (1996), Granular Sludge Formation in Upflow Anaerobic Sludge Blanket (Uasb) Reactors, *Biotechnology and Bioengineering*, **49**, 3, pp. 229-246.

[15]. Batstone, D.J., Keller, J., and Blackall, L.L. (2004), The Influence of Substrate Kinetics on the Microbial Community Structure in Granular Anaerobic Biomass, *Water Res*, **38**, 6, pp. 1390-1404.

[16]. Santegoeds, C.M., Damgaard, L.R., Hesselink, G., Zopfi, J., Lens, P., Muyzer, G., and de Beer, D. (1999), Distribution of Sulfate-Reducing and Methanogenic Bacteria in Anaerobic Aggregates Determined by Microsensor and Molecular Analyses, *Applied and Environmental Microbiology*, **65**, 10, pp. 4618-4629.

[17]. Satoh, H., Miura, Y., Tsushima, I., and Okabe, S. (2007), Layered Structure of Bacterial and Archaeal Communities and Their in Situ Activities in Anaerobic Granules, *Applied and Environmental Microbiology*, **73**, 22, pp. 7300-7307.

[18]. MacLeod, F.A., Guiot, S.R., and Costerton, J.W. (1990), Layered Structure of Bacterial Aggregates Produced in an Upflow Anaerobic Sludge Bed and Filter Reactor, *Applied and Environmental Microbiology*, **56**, 6, pp. 1598-1607.

[19]. Batstone, D.J. and Keller, J. (2001), Variation of Bulk Properties of Anaerobic Granules with Wastewater Type, *Water Res*, **35**, 7, pp. 1723-1729.

[20]. Molina, F., Garcia, C., Roca, E., and Lema, J.M. (2008), Characterization of Anaerobic Granular Sludge Developed in Uasb Reactors That Treat Ethanol, Carbohydrates and Hydrolyzed Protein Based Wastewaters, *Water Sci Technol*, p. 837.

[21]. Lamprecht, C., (2009), Uasb Granulation Enhancement by Microbial Inoculum Selection and Process Induction, Food Sci, Stellenbosch University, Matieland.

[22]. Bhunia, P. and Ghangrekar, M.M. (2008), Effects of Cationic Polymer on Performance of Uasb Reactors Treating Low Strength Wastewater, *Bioresource Technology*, **99**, 2, pp. 350-358.

[23]. Show, K.Y., Wang, Y., Foong, S.F., and Tay, J.H. (2004), Accelerated Start-up and Enhanced Granulation in Upflow Anaerobic Sludge Blanket Reactors, *Water Res*, **38**, pp. 2293 - 2304.

[24]. Zhou, W., Imai, T., Ukita, M., Sekine, M., and Higuchi, T. (2006), Triggering Forces for Anaerobic Granulation in Uasb Reactors, *Process Biochem*, **41**, 1, pp. 36-43.

[25]. El-Mamouni, R., Leduc, R., and Guiot, S.R. (1998), Influence of Synthetic and Natural Polymers on the Anaerobic Granulation Process, *Water Sci Technol*, **38**, 8-9, pp. 341-347.

[26]. Ghangrekar, M.M., Asolekar, S.R., and Joshi, S.G. (2005), Characteristics of Sludge Developed under Different Loading Conditions During Uasb Reactor Start-up and Granulation, *Water Res*, **39**, pp. 1123 - 1133.

[27]. Liu, Y.-Q., Liu, Y., and Tay, J.-H. (2004), The Effects of Extracellular Polymeric Substances on the Formation and Stability of Biogranules, *Applied Microbiology and Biotechnology*, **65**, 2, pp. 143-148.

[28]. Show, K.Y., Tay, J.Y., Yang, L., Wang, Y., and Lua, C.H. (2004), Effects of Stressed Loading on Startup and Granulation in Upflow Anaerobic Sludge Blanket Reactors, *J Environ Eng*, **130**, 7, pp. 743 - 750.

[29]. Uyanik, S., Sallis, P.J., and Anderson, G.K. (2002), The Effect of Polymer Addition on Granulation in an Anaerobic Baffled Reactor (Abr). Part I: Process Performance, *Water Res*, **36**, 4, pp. 933-943.

[30]. Wang, J.S., Hu, Y.Y., and Wu, C.D. (2005), Comparing the Effect of Bioflocculant with Synthetic Polymers on Enhancing Granulation in Uasb Reactors for Low-Strength Wastewater Treatment, *Water SA*, **31**, 2, pp. 177-182.

[31]. Wang, Y., Show, K.Y., Tay, J.H., and Sim, K.H. (2004), Effects of Cationic Polymer on Start-up and Granulation in Upflow Anaerobic Sludge Blanket Reactors, *J Chem Technol Biotechnol*, **79**, 3, pp. 219-228.

[32]. Zhou, W., Imai, T., Ukita, M., Li, F., and Yuasa, A. (2007), Effect of Loading Rate on the Granulation Process and Granular Activity in a Bench Scale Uasb Reactor, *Bioresource Technology*, **98**, 7, pp. 1386-1392.

[33]. El-Mamouni, R., Leduc, R., and Guiot, S.R. (1997), Influence of the Starting Microbial Nucleus Type on the Anaerobic Granulation Dynamics, *Applied Microbiology and Biotechnology*, **47**, pp. 189 - 194.

[34]. Del Nery, V., de Nardi, I.R., Damianovic, M.H.R.Z., Pozzi, E., Amorim, A.K.B., and Zaiat, M. (2007), Long-Term Operating Performance of a Poultry Slaughterhouse Wastewater Treatment Plant, *Resour Conser Recy*, **50**, 1, pp. 102-114.

[35]. Schmidt, J.E.E. and Ahring, B.K. (1994), Extracellular Polymers in Granular Sludge from Different Upflow Anaerobic Sludge Blanket (Uasb) Reactors, *Applied Microbiology and Biotechnology*, **42**, 2, pp. 457-462.

[36]. Lettinga, G., van Velsen, A.F.M., Hobma, S.W., de Zeeuw, W., and Klapwijk, A. (1980), Use of the Upflow Sludge Blanket (Usb) Reactor Concept for Biological Wastewater Treatment, Especially for Anaerobic Treatment, *Biotechnology and Bioengineering*, **22**, 4, pp. 699-734.

[37]. Yu, H.Q., Tay, J.H., and Fang, H.H.P. (1999), Effects of Added Powdered and Granular Activated Carbons on Start-up Performance of Uasb Reactors, *Environ Technol*, **20**, pp. 1095-1101.

[38]. Tay, J.-H., Xu, H.-L., and Teo, K.-C. (2000), Molecular Mechanism of Granulation. I: H+ (Translocation-Dehydration Theory), *J Environ Eng*, **126**, 5, pp. 403-410.

[39]. Chen, J. and Lun, S.Y. (1993), Study on Mechanism of Anaerobic Sludge Granulation in Uasb Reactors, *Water Sci Technol*, **28**, 7, pp. 171-178.

[40]. Yu, H.Q., Tay, J.Y., and Fang, H.H.P. (2001), The Roles of Calcium in Sludge Granulation During Uasb Reactor Start-Up, *Water Research*, **35**, 4, pp. 1052 - 1060.

[41]. Vlyssides, A., Barampouti, E.M., and Mai, S. (2008), Granulation Mechanism of a Uasb Reactor Supplemented with Iron, *Analytical Biochemistry*, **14**, 5, pp. 275-279.

[42]. Francese, A., Córdoba, P., Durán, J., and Siñeriz, F. (1998), High Upflow Velocity and Organic Loading Rate Improve Granulation in Upflow Anaerobic Sludge Blanket Reactors, *World J Microb Biot*, **14**, 3, pp. 337-341.

[43]. Liu, Y. and Tay, J.-H. (2002), The Essential Role of Hydrodynamic Shear Force in the Formation of Biofilm and Granular Sludge, *Water Res*, **36**, 7, pp. 1653-1665.

[44]. Sam-Soon, P.A.L.N.S., Loewenthal, R.E., Dold, P.L., and Marais Gv, R. (1987), Hypothesis for Pellisation in the Upflow Anaerobic Sludge Bed Reactor, *Water SA*, **13**, 2, pp. 69-80.

[45]. Wu, W., Jain, M., and Zeikus, J. (1996), Formation of Fatty Acid-Degrading, Anaerobic Granules by Defined Species, *Applied and Environmental Microbiology*, **62**, 6, pp. 2037-2044.

[46]. Zheng, D., Angenent, L.T., and Raskin, L. (2006), Monitoring Granule Formation in Anaerobic Upflow Bioreactors Using Oligonucleotide Hybridization Probes, *Biotechnology and Bioengineering*, **94**, 3, pp. 458-472.

[47]. van Loosdrecht, M.C., Lyklema, J., Norde, W., and Zehnder, A.J. (1990), Influence of Interfaces on Microbial Activity, *Microbiol Mol Biol R*, **54**, 1, pp. 75-87.

[48]. Tiwari, M., Guha, S., Harendranath, C., and Tripathi, S. (2006), Influence of Extrinsic Factors on Granulation in Uasb Reactor, *Applied Microbiology and Biotechnology*, **71**, 2, pp. 145-154.

[49]. Speece, R.E. (1996), *Anaerobic Biotechnology*. Archae Press, Tennesse.

[50]. Morvai, L., Miháltz, P., Czakó, L., and Holló, J. (1991), Application of Uasb-Reactors to Industrial Waste-Water Treatment; Performance Data and Results in Granulation Control, *Acta Biotechnologica*, **11**, 5, pp. 409-418.

[51]. Grotenhuis, J.T., Smit, M., Plugge, C.M., Xu, Y.S., Van Lammeren, A.A., Stams, A.J., and Zehnder, A.J. (1991), Bacteriological Composition and Structure of Granular Sludge Adapted to Different Substrates, *Applied and Environmental Microbiology*, **57**, 7, pp. 1942-1949.

[52]. El-Mamouni, R., Guiot, S.R., Leduc, R., and Costerton, J.W. (1995), Characterization of Different Microbial Nuclei as Potential Precursors of Anaerobic Granulation, *J Biotechnol*, **39**, 3, pp. 239-249.

[53]. Jhung, J.K. and Choi, E. (1995), A Comparative Study of Uasb and Anaerobic Fixed Film Reactors with Development of Sludge Granulation, *Water Research*, **29**, 1, pp. 271-277.

[54]. Uyanik, S., Sallis, P.J., and Anderson, G.K. (2002), The Effect of Polymer Addition on Granulation in an Anaerobic Baffled Reactor (Abr). Part Ii: Compartmentalization of Bacterial Populations, *Water Res*, **36**, 4, pp. 944-955.

[55]. Resmanto, A.M., (2010), Granulation of Substrate-Adapted Microorganism with Cationic Polymer Addition, Biotechnology, King Mongkut's University of Technology Thonburi.

[56]. Imai, T., Ukita, M., Liu, J., Sekine, M., Nakanishi, H., and Fukagawa, M. (1997), Advanced Start up of Uasb Reactors by Adding of Water Absorbing Polymer, *Water Sci Technol*, **36**, 6-7, pp. 399-406.

[57]. Rinaudo, M. (2006), Chitin and Chitosan: Properties and Applications, *Progress in Polymer Science*, **31**, 7, pp. 603-632.

[58]. Strand, S.P., Nordengen, T., and Østgaard, K. (2002), Efficiency of Chitosans Applied for Flocculation of Different Bacteria, *Water Research*, **36**, 19, pp. 4745-4752.

[59]. Franco, A., Gresia, G., Roca, E., Rozzi, A., and Lema, J.M. (2002), Influence of Pulsation on Start up of Uasb Reactors, *Water Sci Technol*, **45**, 10, p. 6.

[60]. Franco, A., Roca, E., and Lema, J.M. (2003), Improvement of the Properties of Granular Sludge in Uasb Reactors by Flow Pulsation, *Water Sci Technol*, **48**, 6, p. 6.

[61]. Sheng, G.-P., Yu, H.-Q., and Li, X.-Y. (2010), Extracellular Polymeric Substances (Eps) of Microbial Aggregates in Biological Wastewater Treatment Systems: A Review, *Biotechnol Adv*, **28**, 6, pp. 882-894.

[62]. Laspidou, C.S. and Rittmann, B.E. (2002), A Unified Theory for Extracellular Polymeric Substances, Soluble Microbial Products, and Active and Inert Biomass, *Water Research*, **36**, 11, pp. 2711-2720.

[63]. Tay, T.-L.S., Ivanov, V., Kim, I.S., Feng, L., and Tay, J.-H. (2001), Quantification of Ratios of Bacteria and Archaea in Methanogenic Microbial Community by Fluorescence in Situ Hybridization and Fluorescence Spectrometry, *World J Microb Biot*, **17**, 6, pp. 583-589.

[64]. Nielsen, P. and Jahn, A. (1999), "Extraction of Eps", In *Microbial Extracellular Polymeric Substances*. Wingender, J., Neu, T., and Flemming, H.-C., Springer Berlin Heidelberg, p.^pp 49-72.

[65]. Goodwin, J.A.S. and Forster, C.F. (1985), A Further Examination into the Composition of Activated Sludge Surfaces in Relation to Their Settlement Characteristics, *Water Research*, **19**, 4, pp. 527-533.

[66]. Durmaz, B. and Sanin, F.D. (2003), Effect of Carbon to Nitrogen Ratio on the Physical and Chemical Properties of Activated Sludge, *Environmental Technology*, **24**, 11, pp. 1331-1340.

[67]. Jorand, F., Boué-Bigne, F., Block, J.C., and Urbain, V. (1998), Hydrophobic/Hydrophilic Properties of Activated Sludge Exopolymeric Substances, *Water Science and Technology*, **37**, 4–5, pp. 307-315.

[68]. Flemming, H.-C. and Leis, A. (2003), "Sorption Properties of Biofilms", In *Encyclopedia of Environmental Microbiology*, John Wiley & Sons, Inc., p.^pp.

[69]. Liu, H. and Fang, H.H.P. (2002), Extraction of Extracellular Polymeric Substances (Eps) of Sludges, *Journal of Biotechnology*, **95**, 3, pp. 249-256.

[70]. Mayer, C., Moritz, R., Kirschner, C., Borchard, W., Maibaum, R., Wingender, J., and Flemming, H.-C. (1999), The Role of Intermolecular Interactions: Studies on Model Systems for Bacterial Biofilms, *International Journal of Biological Macromolecules*, **26**, 1, pp. 3-16.

[71]. Miqueleto, A.P., Dolosic, C.C., Pozzi, E., Foresti, E., and Zaiat, M. (2010), Influence of Carbon Sources and C/N Ratio on Eps Production in Anaerobic Sequencing Batch Biofilm Reactors for Wastewater Treatment, *Bioresource Technology*, **101**, 4, pp. 1324-1330.

[72]. Fang, H.H.P., Chen, T., Li, Y.Y., and Chui, H.K. (1996), Degradation of Phenol in Wastewater in an Upflow Anaerobic Sludge Blanket Reactor, *Water Res*, **30**, 6, pp. 1353-1360.

[73]. Morgan, J.W., Forster, C.F., and Evison, L. (1990), A Comparative Study of the Nature of Biopolymers Extracted from Anaerobic and Activated Sludges, *Water Research*, **24**, 6, pp. 743-750.

[74]. Liu, X.-M., Sheng, G.-P., Luo, H.-W., Zhang, F., Yuan, S.-J., Xu, J., Zeng, R.J., Wu, J.-G., and Yu, H.-Q. (2010), Contribution of Extracellular Polymeric Substances (Eps) to the Sludge Aggregation, *Environmental Science & Technology*, **44**, 11, pp. 4355-4360.

[75]. Liao, B.Q., Allen, D.G., Droppo, I.G., Leppard, G.G., and Liss, S.N. (2001), Surface Properties of Sludge and Their Role in Bioflocculation and Settleability, *Water Res*, **35**, 2, pp. 339-350.

[76]. Sheng, G.-P. and Yu, H.-Q. (2007), Formation of Extracellular Polymeric Substances from Acidogenic Sludge in H_2-Producing Process, *Applied Microbiology and Biotechnology*, **74**, 1, pp. 208-214.

[77]. Zheng, Y.-M. and Yu, H.-Q. (2007), Determination of the Pore Size Distribution and Porosity of Aerobic Granules Using Size-Exclusion Chromatography, *Water Research*, **41**, 1, pp. 39-46.

[78]. Jia, X.S., Fang, H.H.P., and Furumai, H. (1996), Surface Charge and Extracellular Polymer of Sludge in the Anaerobic Degradation Process, *Water Sci Technol*, **34**, 5-6, pp. 309-316.

[79]. Li, X.Y. and Yang, S.F. (2007), Influence of Loosely Bound Extracellular Polymeric Substances (Eps) on the Flocculation, Sedimentation and Dewaterability of Activated Sludge, *Water Research*, **41**, 5, pp. 1022-1030.

[80]. Wilén, B.-M., Jin, B., and Lant, P. (2003), Relationship between Flocculation of Activated Sludge and Composition of Extracellular Polymeric Substances, *Water Sci Technol*, **47**, 12, p. 9.

[81]. Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C.M., and Heijnen, J.J., 1994, Model of the Anaerobic Metabolism of the Biological Phosphorus Removal Process: Stoichiometry and Ph Influence. Wiley Subscription Services, Inc., A Wiley Company, Vol. 43, p.^pp. 461-470.

[82]. APHA. (2005), *Standard Method for the Examination of Water and Wastewater*. 21st, American Public Health Association, Washington, D.C.

[83]. Adav, S.S. and Lee, D.J. (2008), Extraction of Extracellular Polymeric Substances from Aerobic Granule with Compact Interior Structure, *Journal Hazardous Materials*, **154**, 13, pp. 1120-1126.

[84]. Bradford, M.M. (1976), A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, *Analytical Biochemistry*, **72**, 12, pp. 248-254.

[85]. Walker, J.M. and Kruger, N.J. (2002), "The Bradford Method for Protein Quantitation", In *The Protein Protocols Handbook*, Humana Press, p.^pp 15-21.

[86]. Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.-I., and Lee, Y.C. (2005), Carbohydrate Analysis by a Phenol–Sulfuric Acid Method in Microplate Format, *Analytical Biochemistry*, **339**, 1, pp. 69-72.

[87]. Miller, G.L. (1959), Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar, *Analytical Biochemistry*, **31**, 3, pp. 426-428.

[88]. Angenent, L.T., Zheng, D., Sung, S., and Raskin, L. (2010), Methanosaeta Fibers in Anaerobic Migrating Blanket Reactors, *Water Science and Technology*, **41**, 4, pp. 35–39.

[89]. Raskin, L., Poulsen, L.K., Noguera, D.R., Rittmann, B.E., and Stahl, D.A. (1994), Quantification of Methanogenic Groups in Anaerobic Biological Reactors by Oligonucleotide Probe Hybridization, *Applied and Environmental Microbiology*, **60**, 4, pp. 1241-1248.

[90]. Jupraputtasri, W., Boonapatcharoen, N., Cheevadhanarak, S., Chaiprasert, P., Tanticharoen, M., and Techkarnjanaruk, S. (2005), Use of an Alternative Archaea-Specific Probe for Methanogen Detection, *J Microbiol Meth*, **61**, 1, pp. 95-104.

[91]. Switzenbaum, M.S., Giraldo-Gomez, E., and Hickey, R. (1990), Monitoring of the Anaerobic Methane Fermentation Process, *Enzyme and Microbial Technology*, **12**, p. 9.

[92]. Horiuchi, J., Shimizu, T., Kanno, T., and Kobayashi, M. (1999), Dynamic Behavior in Response to Ph Shift During Anaerobic Acidogenesis with a Chemostat Culture, *Biotechnology Techniques*, **13**, 3, pp. 155-157.

[93]. Van Lier, J., Grolle, K.C., Fritjers, C.T., Stams, A.J., and Lettinga, G. (1993), Effects of Acetate, Propionate and Butyrate on the Thermophilic Anaerobic Degradation of Propionate by Methanogenic Sludge and Defined Cultures, *Applied and Environmental Microbiology*, **59**, 4, p. 10.

[94]. Angelidaki, I. and Sanders, W. (2004), Assessment of the Anaerobic Biodegradability of Macropollutants, *Rev Environ Sci Biotechnol*, **3**, 2, pp. 117-129.

[95]. Sheng, G.-P., Yu, H.-Q., and Li, X.-Y. (2010), Extracellular Polymeric Substances (Eps) of Microbial Aggregates in Biological Wastewater Treatment Systems: A Review, *Biotechnology advances*, **28**, 6, pp. 882-894.

[96]. Yan, Y.G. and Tay, J.H. (1997), Characterisation of the Granulation Process During Uasb Start-Up, *Water Res*, **31**, 7, pp. 1573-1580.

[97]. Ismail, S.B., de La Parra, C.J., Temmink, H., and van Lier, J.B. (2010), Extracellular Polymeric Substances (Eps) in Upflow Anaerobic Sludge Blanket (Uasb) Reactors Operated under High Salinity Conditions, *Water Research*, **44**, 6, pp. 1909-1917.

[98]. Martínez O, F., Lema, J., Méndez, R., Cuervo-López, F., and Gómez, J. (2004), Role of Exopolymeric Protein on the Settleability of Nitrifying Sludges, *Bioresource Technology*, **94**, 1, pp. 43-48.

[99]. Tay, J.H., Liu, Q.S., and Liu, Y. (2001), The Role of Cellular Polysaccharides in the Formation and Stability of Aerobic Granules, *Letters in Applied Microbiology*, **33**, 3, pp. 222-226.

[100]. Dinopoulou, G., Sterritt, R.M., and Lester, J.N. (1988), Anaerobic Acidogenesis of a Complex Wastewater: Ii. Kinetics of Growth, Inhibition, and Product Formation, *Biotechnology and Bioengineering*, **31**, 9, pp. 969-978.

[101]. Schink, B. and Stams, A.J.M. (2006), Syntrophism among Prokaryotes, *The Prokaryotes*, **2**, pp. 309-335.

[102]. Rittmann, B.E. and McCarty, P.L. (2001), *Environmental Biotechnology: Principles and Applications*. McGraw-Hill.

[103]. Gonzalez-Gil, G., Lens, P.N.L., Van Aelst, A., Van As, H., Versprille, A.I., and Lettinga, G. (2001), Cluster Structure of Anaerobic Aggregates of an Expanded

Granular Sludge Bed Reactor, *Applied and Environmental Microbiology*, **67**, 8, pp. 3683-3692.

[104]. Collins, G., Woods, A., McHugh, S., Carton, M.W., and O'Flaherty, V. (2003), Microbial Community Structure and Methanogenic Activity During Start-up of Psychrophilic Anaerobic Digesters Treating Synthetic Industrial Wastewaters, *FEMS Microbiology Ecology*, **46**, 2, pp. 159-170.

[105]. Meepian, K., (2003), Microbial Characterization in Suspended Zone and Packed Zone of Anaerobic Hybrid Reactor, Biotechnology, King Mongkut's University of Technology Thonburi, Bangkok, 180.

[106]. Wiegant, W.M. (1987), "The 'Spaghetti Theory' on Anaerobic Sludge Formation, or the Inevitability of Granulation", In *Granular Anaerobic Sludge: Microbiology and Technology*. Lettinga, G., et al., The Netherlands: Pudoc, Wageningen, p.^pp 146-152.

[107]. Thiele, J.H. and Zeikus, J.G. (1988), Control of Interspecies Electron Flow During Anaerobic Digestion: Significance of Formate Transfer Versus Hydrogen Transfer During Syntrophic Methanogenes, *Applied and Environmental Microbiology*, p. 30.

[108]. Hu, Y., Beach, J., Raymer, J., and Gardner, M. (2000), Disposable Diaper to Collect Urine Samples from Young Children for Pyrethroid Pesticide Studies, *J Expo Anal Environ Epidemiol*, **14**, 5, pp. 378-384.

[109]. Wu, J., Bi, L., Zhang, J.B., Poncin, S., Cao, Z.P., and Li, H.Z. (2012), Effects of Increase Modes of Shear Force on Granule Disruption in Upflow Anaerobic Reactors, *Water Research*, **46**, 10, pp. 3189-3196.

APPENDIX A
DATA

Table A1. Data of reactor performances during nucleation and early maturation phases

OLR (kg COD.m^{-3}.d^{-1})	Day	Reactor	Reactor performance				
			pH	TVA/alk.	COD removal (%)	CH_4 production (ml.d^{-1})	CH_4 yield (m^3 CH_4. kg COD $_{rem.}$)
1.0 (NUCLEATION)	33	R1	7.50 – 7.70	0.33 – 0.37	82 – 84	1022 – 1113	0.17
		R2	7.52 – 7.63	0.30 – 0.37	86 – 88	1199 - 1352	0.19
		R3	7.48 – 7.60	0.25 – 0.39	88 – 90	1622 - 1643	0.23
1.5 (NUCLEATION)	58	R1	7.49 – 7.82	0.27 – 0.41	79 – 82	1424 - 1472	0.25
		R2	7.55 – 7.82	0.27 – 0.41	82 – 84	1532 - 1574	0.26
		R3	7.40 – 7.63	0.27 – 0.40	89 – 91	2034 - 2153	0.33
0.5 (MATURATION)	74	R1	7.10 – 7.13	0.28 – 0.29	76 – 79	198 – 202	0.09
		R2	7.14 – 7.20	0.27 – 0.28	80 – 84	202 – 209	0.09
		R3	7.14 – 7.21	0.25 – 0.29	86 – 88	248 – 298	0.11
0.7 (MATURATION)	88	R1	6.72 – 6.81	0.44 – 0.50	68 – 74	282 – 315	0.12
		R2	6.76 – 6.82	0.45 – 0.52	68 – 77	347 – 353	0.13
		R3	7.15 – 7.20	0.32 – 0.33	84 – 86	448 – 478	0.15
1.0 (MATURATION)	118	R1	6.83 – 6.85	0.41 – 0.49	66 – 69	475 – 510	0.13
		R2	6.88 – 6.91	0.42 – 0.46	75 – 77	563 – 585	0.15
		R3	7.02 – 7.05	0.33 – 0.35	83 – 85	1019 - 1038	0.24

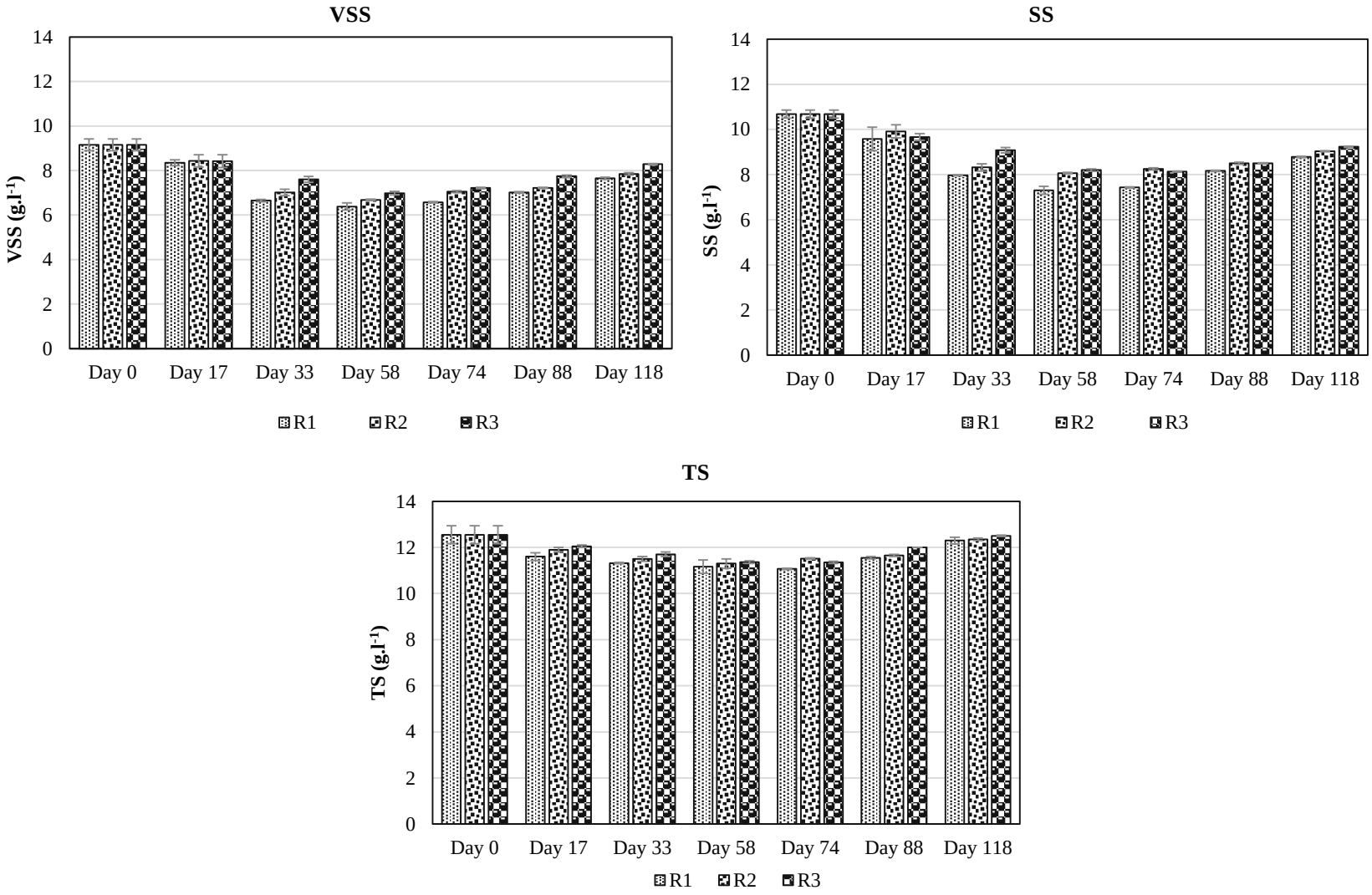


Figure A2. Biomass concentration during nucleation and early maturation phases

Table A2. Summary for physical characteristics of microbial aggregates in each reactor

Parameters	**Initial day (day 0)**			**End of Nucleation (day 58)**			**End of early maturation (day 118)**		
	R1	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
Av. sludge diameter (μm)	47	47	47	76	89	115	97	118	190
Nuclei ratio (%)	0.6	0.6	0.6	24.2	25.9	55.1	29.8	41.7	45.1
Granule ratio (%)	0	0	0	0.8	2.6	8.2	3.7	3.8	34.2
Zeta potential (mV)	-26.4	-26.4	-26.4	-24.5	-22.2	-10.5	-20.3	-19.1	-6.6
SVI ($ml.g^{-1}SS$)	92.7	91.8	74.9	82	71	43	66	55	33
$EPS_{protein}/EPS_{poly.}$ ratio	3.17	3.17	3.17	6.31	6.92	5.37	1.37	1.36	1.42
GDM ($g\ COD.g^{-1}VSS.d^{-1}$)	1.70	1.70	1.70	1.39	1.30	1.24	3.38	2.98	2.83
BDM ($g\ COD.g^{-1}VSS.d^{-1}$)	0.29	0.29	0.29	0.45	0.42	0.39	0.53	0.52	0.46
PDM ($g\ COD.g^{-1}VSS.d^{-1}$)	0.17	0.17	0.17	0.53	0.51	0.52	0.45	0.52	0.54
ACM ($g\ COD.g^{-1}VSS.d^{-1}$)	0.12	0.12	0.12	0.48	0.51	0.56	0.27	0.32	0.66
Methanogen/non-methanogen ratio	0.05	0.05	0.05	0.20	0.23	0.26	0.06	0.08	0.17
Methanogen to acetogens ratio	0.25	0.25	0.25	0.49	0.55	0.61	0.28	0.30	0.66

APPENDIX B
ANALYTICAL METHODS

1. Fluorescence in situ hybridization (FISH)

Fixation and Sample preparation step

1. Fixed granule or aggregates with fixative solution (4% paraformaldehyde in PBS)
2. Incubated at 4^{o}C, 2 hours or overnight
3. Remove supernatant
4. Re-suspended with PBS 1X three times
5. Re-suspended in 1:1 (v/v) of PBS 1X – ethanol 99%
6. Stored at -20^{o}C before analyzing

Slide preparation

1. Concave slide was used
2. Slides were cleaned in acid alcohol (1-2% HCl in 70% ethanol) for 5 min
3. Air-dried
4. The slides were placed into 0.01% poly-L-Lysine at room temperature for 5 min
5. The coated slides were air dried overnight or 1 hour at 60^{o}C before used.

Fluorescent *in situ* hybridization

1. Placed fixed-granule or aggregate on coated slide and air-dried or dried in 37^{o}C incubator.
2. Dehydration by dipping in ethanol series (50, 70, 96% ethanol), 3 min each then air-dried.
3. Subsequently, mixture of v9:1 hybridization solution **(*a)** and FITC- or Cy3-labeled probe **(*b)** (50 ng/µl) were applied to each sample (~15 µl/ each spotted).
4. Incubated at 46^{o}C in a moisture chamber for 3 hours
5. After hybridization, the slides were rinsed twice with pre-warmed (48^{o}C) washing solution **(*c)** [depend on % Formamide] and then washed for 15 min at 48^{o}C in Falcon tubes 50 ml.
6. The slides were stained with 300 mM/ µl of 4',6-diamidino-2-phenylindole (DAPI) (6.26 µg/ml in 0.1 M Tris-HCl and 0.9 M NaCl, pH 7.2; Sigma, Deisenhofen, Germany) for 5-10 min and rinsed with distilled water.
7. Air-dried, the slides were mounted with anti-fading solution.
8. Capture on an epi-fluorescence microscope.

*** a) Hybridization buffer (1.0 ml) (0.9 M NaCl, 20 mM Tris-HCl, 0.01% SDS)**

Stock solution

1% SDS (sodium dodecyl sulfate)

3.0 M NaCl

1.0 M Tris-HCl pH 7.2

% Formamide	1% SDS (µl)	1.0 M Tris-HCl (µl)	3.0 M NaCl (µl)	Formamide (µl)
15	10	20	300	150
35	10	20	300	350

***b) Oligonucleotide probes**

Probe name	Target group	*E. coli* numbering	Probe sequence (5'-3')	Formamide (%)
ARC915	*Archaea* domain	915–934	GTGCTCCCCCGCCAATTCCT	15
EUB338	*Bacteria* domain	338-355	GCTGCCTCCCGTAGGAGT	15

*** c) Washing Solution (100 ml)**

% Formamide	1% SDS (ml)	1.0 M Tris-HCl (ml)	3.0 M NaCl (ml)	NaCl (M)
15	1.0	2.0	10.6	0.318
35	1.0	2.0	2.6	0.080

2. Volatile fatty acids (VFAs) by gas chromatography

The individual VFA (butyric, propionic and acetic acids) was determined as follows: 1 ml of sample media was taken and transferred to 1.5 ml in an eppendorf tube. Then 200 µl of oxalic acid was added into the sample and centrifuged at 10.000 rpm for 10 minutes. The supernatant was transferred into tubes. 3 ml of ethyl acetate was added into tubes. Mixture between acids and ethyl acetate was then mixed for one minutes for VFA extraction. The mixture was then resided until water phase clearly separated with non-polar phase. 1 ml of the non-polar phase was sampled for prior GC analysis. The concentration of VFA after GC analysis was re-calculated based on dilution factor during extraction.

The conditions for analyzing VFA are as follows:

- Column : Stabiwax – DA with length 30 m, inner diameter 0.25 mm and film thickness 0.25 µm
- Column temperature : 60^0C
- Detector : Flame ionization detector (FID)
- Detector temperature : 250^0C
- Injection temperature : 250^0C

3. Biogas production and gas composition

Biogas production was measured by a gas counter (in the reactor) and water replacement method (in the vial). While, the biogas composition was analyzed by taking 10 µl of gas from the headspace of the vial or U-tube of reactor using 100 µl syringe, the gas was injected into the gas chromatography (GC-TCD) (Shimadzu, GC-9A). The percentage of methane and carbon dioxide in biogas were calculated by comparing with standard peak area.

The conditions for analyzing biogas composition as follow:

- Column : Porapak-N 80/100 with 2 meter long and 1/8 inch in diameter
- Detector : Thermal conductivity detector (TCD)
- Detector temperature : 120^0C
- Column temperature : 70^0C
- Injection temperature : 70^0C
- Carrier gas: Helium with flow rate of 50 ml.