Formation and Characterization of Aerobic Granules in Sewage Treatment

A Dissertation Report

Submitted in partial fulfillment of the requirements
for the award of degree of

Master of Technology
in
Environmental Science and Technology

Submitted
By
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July 2012

CERTIFICATE

This is to certify that thesis entitled; **"Formation and Characterization of Aerobic Granules in Sewage Treatment"** by **Ms. Parinita Chowdhry** in partial fulfillment of the requirements for the award of **Master of Technology** degree in **Environmental Science and Technology** at **Thapar University,** Patiala (Deemed University) is an authentic piece of work carried out by her under our guidance and supervision.

To the best of our knowledge, the matter embodied in this thesis has not been submitted to any other university/institute for award of any degree/diploma.

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Declaration

I, the undersigned, hereby declare that the research work presented in the M.Tech project entitled "**Formation and Characterization of Aerobic Granules in Sewage Treatment**" has been carried out by me under the supervision and guidance of Dr. Subhankar Basu and Dr. Malini Balakrishnan, The Energy and Resources Institute, New Delhi and Mr. Anoop Verma, Assistant Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala.

Further, I declare that no part of this Dissertation has been submitted for a degree or any other qualification of any other university or examining body in India/elsewhere.

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Abstract

Aerobic granulation is an emerging technology for wastewater treatment. However, long start-up periods are required for the development of granules. In this study, aerobic granules were developed in ambient conditions in four identical sequencing batch reactors (SBRs). The reactors were fed with a mixture of domestic wastewater and leachate from the acidification reactor of a two-phase anaerobic digester treating food waste. They were seeded with three different inert nuclei to compare their effect on the process of granulation. The results show that when the reactor is seeded with large flocs and selective discharge of the microbial consortia is allowed, the presence of inert nuclei is not essential for the formation of granules, neither has it has any major influence on the properties of the granules. The SEM images show absence of filamentous organisms in all the reactors. Organic removal efficiency has been found to be highest (90%) in the reactor inoculated with commercial activated carbon. The results indicate that the reactor without any particle addition formed the granule with the highest EPS content and protein, which is essential for bacterial agglomeration. The granules were found to be very well settling with settling velocity of 40m/h and SVI of around 20mL/g in all the four reactors. The integrity coefficient and hydrophobicity of the sludge in all the reactors were found to be similar.

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1. Introduction

Application of aerobic granular sludge system for the treatment of wastewater has gained a lot of attention in recent years. In contrast to suspended growth typically encountered in activated sludge process (ASP), membrane bioreactors (MBR) etc., biogranules are characterized by (i) regular, smooth structure and nearly round in shape, (ii) better sedimentation, (iii) dense and strong microbial structure, (iv) high biomass retention, (v) ability to withstand fluctuations in organic loading rate and (iv) tolerant to toxic substances [**Liu and Tay, 2004**]. The granules involve cell-to-cell interactions that include biological and physicochemical phenomena. Microorganisms are capable of attaching to each other and aggregating in selective environment, thereby granules are formed by self-immobilization or immobilization of microorganisms onto seeding materials. The granules are dense microbial consortia packed with different bacterial species and generally contain millions of organisms per gram of biomass [**Liu and Tay 2004**].

Biogranulation technology can be applied in both aerobic and anaerobic treatment systems. There has been extensive research on the formation of anaerobic granules, best recognized in Upflow Anaerobic Sludge Blanket (UASB) reactor. Many industrial and municipal wastewater treatment plants involve biogranulation technology [**Alves et al., 2000**]. Anaerobic granular sludge is a dense microbial community; the presence of different species in the microbial community results in almost complete degradation of industrial wastes and treatment of high strength wastewater. Large size and high density of individual granules causes them to settle rapidly, which improves the separation of treated effluent from the biomass. Granulation under anaerobic conditions has limitations viz. (i) long start-up period, (ii) relatively higher operation temperature. This resulted in the development of aerobic granular technology which has become popular in recent years [**Liu and Tay 2004**].

The development of aerobic granules was first reported by **Mishima and Nakamura** (1991) in a continuous aerobic UASB reactor. Since then, aerobic granulation has been reported in sequencing batch reactors (SBRs) by many researchers [**Morgenroth et al., 1997; Beun et al., 1999; Kreuk et al., 2005; Iaconi et al; 2007; Etterer and Wilderer, 2001; Tay et al., 2001 a;**

Liu and Tay, 2002; Tay et al., 2007; Abdullah et al., 2011]. Aerobic biogranules have been mostly developed under controlled laboratory conditions, in column-type upflow reactors with glucose or acetate as carbon source in synthetic wastewater or diluted industrial wastewater [**Tay et al., 2001 a; Li et al., 2011; Abdullah et al., 2011**]. The objective of this study was to explore the possibility of developing aerobic granules in the aeration tank of the activated sludge system using sewage under ambient conditions. This thesis provides the hydrodynamic and biomass characteristics of the aerobic granules in the reactor.

Aerobic granules usually takes several months to mature [**Yilmaz et al., 2008; Ni et al. 2009**], but some researchers have reported that addition of support media e.g. activated carbon or crushed aerobic granules enhance granule formation and reduce the startup time. The support media is characterized by high surface area, fast settling velocity, irregular surface and a characteristic adsorption property that provide a favorable environment to expedite the aerobic granulation process [**Li et al., 2011; Gansen et al., 2007; Pijuan et al., 2011; Verawaty et al., 2011**]. This study therefore focuses on the effect of two different support media (activated carbon and zeolite) that have different surface area and particle size range for aerobic granulation under the conditions mentioned above.

2. Objectives

The main objectives of this study are:

1. To investigate the formation of aerobic granules under ambient conditions in an aeration tank operating in the SBR mode; the feed was domestic sewage mixed with leachate from the acidification reactor of a two-phase anaerobic digester treating food waste.
2. To study the effect of two different support materials (activated carbon and zeolite) having different particle sizes and surface areas on the start-up time and the properties of aerobic granules.
3. To study the hydrodynamic and physicochemical characteristics of the aerobic granules.

3. Literature Review

3.1 Granular sludge

Granular sludge is a compact bacterial aggregate, typically having millions of microorganisms per gram of biomass **[Liu and Tay, 2004]**. The different species of bacteria act concurrently to degrade organics, nutrients and other pollutants present in wastewater. Sludge granulation can occur under aerobic or anaerobic conditions. In aerobic systems like activated sludge treatment, granulation improves sludge settling.

3.1.1 Anaerobic granulation

Anaerobic granular sludge has been well studied and applied in UASB reactors. However, anaerobic granulation requires long start-up periods and a relatively high operation temperature [**Liu and Tay, 2004**]. In addition, anaerobic granulation technology has not been found suitable for the removal of nutrients (nitrogen and phosphorus) from wastewater.

3.1.2 Aerobic granulation

Aerobic granules are tight clusters of bacterial cells which are embedded within an extracellular polymeric matrix [**Tay et al., 2004b**].It not only makes the treatment plant more compact by eliminating the requirement for external settlers [**Beun et al., 1999**], but also makes high organic loading feasible e.g. organic loading as high as 62.76 kg COD/m^3.d is possible [**Ganesan et al., 2007**]. Systems with aerobic granules have been known to provide up to 98% COD removal at a loading rate of 6 kg/m^3.d [**Tay et al., 2002**]. The aerobic granules show capability to handle toxic substances like phenol [**Tay et al., 2004a**].

3.2Aerobic granular sludge

3.2.1 Strategies for the formation of aerobic granular sludge

A variety of microorganisms are present in the sludge inoculated in a bioreactor. From this mixed consortia, certain bacterial species with ability to agglomerate have to be selected so that they propagate [**Beun et al., 2002**]. The pressure of elimination induces the tendency to adhere in cells. This selection of cells that occurs is in accordance with Darwin’s theory of natural selection.

Granulation can also be better described as defense strategy of microbes against external selection pressure applied. The force applied for selection of such microbes can be short settling time [**Beun et al., 1999; Adav et al., 2009**], periodical starvation [**Tay et al., 2001a**], intermittent feeding [**Mc. Swain et al., 2004**] or even manipulating the presence of certain ions in the feed [**Wang et al., 2006a**]. This depends on the system and the operating conditions feasible. All these factors attain one goal in the long run: creating clusters of tightly bound bacterial cells, better known as aerobic granules.

3.2.1.1Periodic starvation

The introduction of feast and famine periods during the operation of the bioreactor induces the formation of biogranules. Periodic starvation brings about changes in the morphology and the metabolism of cells. Feed interruption is known to be associated with formation of connecting fibrils [**Varon and Choder, 2000]** and also with the increased production of extracellular polymeric substances (EPS) [**Qin et al., 2004**], both of which are known to increase hydrophobicity of cells and ultimately lead to cell-to-cell binding [**Tay et al., 2001a; Adav et al., 2009**]. The feeding strategy is further discussed in 3.2.1.5.

3.2.1.2 Treatment system

Most studies related to aerobic granulation have been conducted using sequential batch reactors (SBRs) [**Buen 1999; Wang 2004; Tay et al., 2001b; Moy et al., 2004**]. The operation of a typical SBR is shown in Fig. 3.1.The main reason why SBRs are popular for the development of aerobic granules is that they are systems with inbuilt feed interruption, which in turn regulates the amount of EPS secreted by the bacterial cells and leads to agglomeration [**Tay et al., 2001a; Beun et al., 2002**].

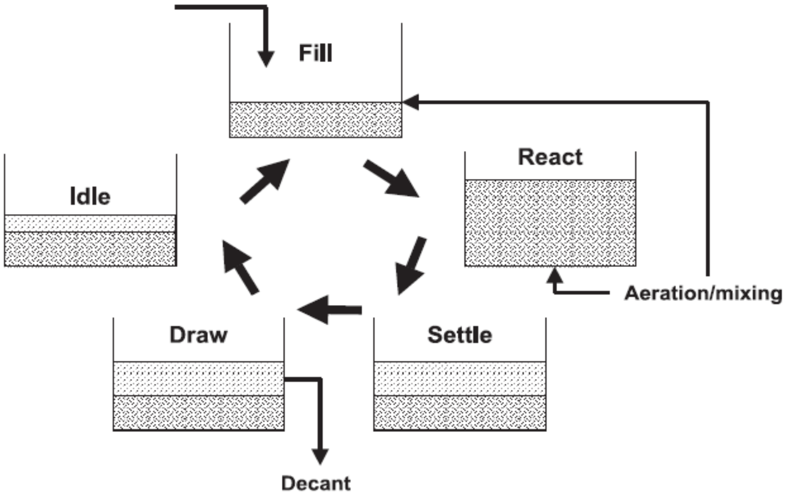


Fig. 3.1 Sequencing Batch Reactor (SBR) [USEPA, 2000]

3.2.1.3Extracellular-Polymeric Substances (EPS)

Extracellular Polymeric Substances (EPS) produced by the microorganisms include all categories of substances like polysaccharides, proteins, glycoproteins, nucleic acids, phospholipids and humic acids [**Wingender et al., 1999**]. EPS change the traditional surface negative charge of bacteria. They also act as the matrix in which the cells are embedded. So, they are responsible for adhesion of cells to one another and also to inert particulate matter [**Tay et al., 2001a**].

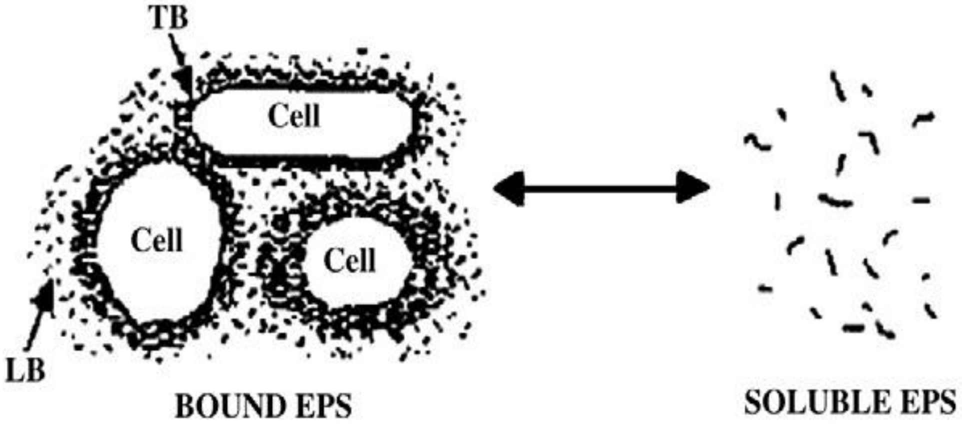


Fig 3.2 Sketch of EPS structure **[Nielsen and Jahn, 1999**]

EPS can be broadly divided into two fractions, soluble and bound (Fig.3.2).The soluble fraction(also called Soluble Microbial Product or SMP) is dissolved in the solution whereas the bound fraction is closely associated with microbial biomass and is able resist the shear forces except under some unfavorable circumstances [**Laspidou and Rittmann, 2002; Sheng et al., 2010]**

3.2.1.4Presence of divalent ions in the feed

Microbes accumulate ions like calcium and magnesium and they fill empty spaces in the granules [**Ren et al., 2008**]. They are mainly found to be present in interiors of the granules. Both the ions are known to have two basic ways by the means of which they are found to have an influence on the process of granulation [**Jiang et al., 2003**]. Firstly, being positively charged, they bind to the negatively charged bacteria [**Costerton 1987; Loosdrechth 1987; Isruchs, 1992**]. Also, calcium forms calcium-EPS-calcium bridges between cells [**Wang et al., 2006a**]. Studies also indicate that in presence of the ions granules accumulate higher amounts of polysaccharides without any known increase in the protein content of the cells.

The above factors cumulatively lead to the formation of a bigger and compact granule with higher sludge volume index (SVI), shear strength and compression strength [**Ren et al., 2008**]. They also decrease the start-up time taken by microbes for granulation. Studies conducted show that the time for granulation reduces to almost half the time when the dosed concentration of calcium ion in the feed was doubled [**Li et al., 2009]**.

3.2.1.5 Feeding strategy

Growing in suspension is natural for microbes due to higher and easy availability of substrate as compared to flocs or granules **[Buen et al., 2002; Li et al., 2011]**. Within a granule, bacteria tend to experience diffusion limitations of both substrate and oxygen. When a bioreactor is fed intermittently, it forces bacterial cells to store and accumulate food. This in a way results in enrichment of cells that have higher substrate kinetics than the rest [**McSwain et al., 2004**]. This prepares the cells for what lies ahead.

3.2.1.6 Settling and selective discharge of the microbial consortia

As described above, growth in the form of a suspension is easier for the cells. So, another way of eliminating the cells which form suspensions is the application of selective discharge to the microbial community present in the bioreactor [**Li et al., 2011; Sheng et al., 2010**]. In other words, it means to provide a short settling time and those bacteria which do not have a tendency to settle get eliminated [**Beun et al., 2009, Fig. 3.3**]. This reduces competition in terms of substrate for those bacteria which agglomerate [**Sheng et al., 2010**].

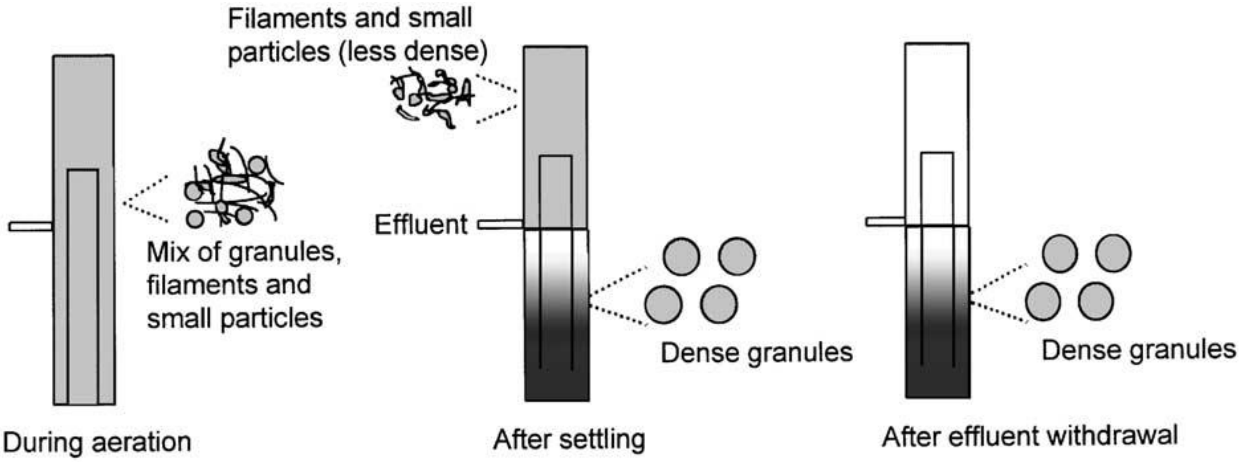
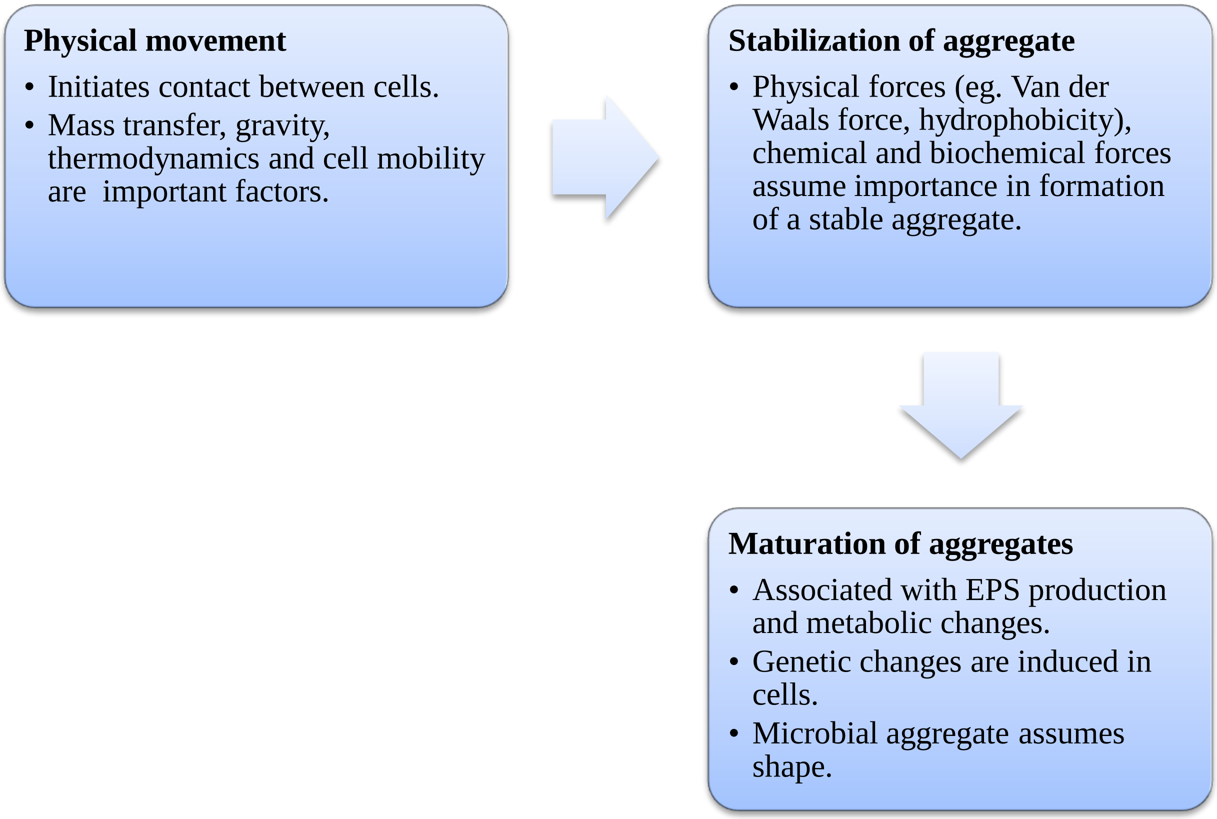


Fig. 3.3 Role of settling on formation of granular sludge [**Beun et al., 2002**]

3.2.2 Mechanism of formation of aerobic granular sludge

There are two general theories regarding the formation of aerobic granules in bioreactors. The mechanism proposed for the formation of bacterial aggregates by Hwan Oh, 2007 is presented in Fig.3.4.



Physical movement

- Initiates contact between cells.
- Mass transfer, gravity, thermodynamics and cell mobility are important factors.

Stabilization of aggregate

- Physical forces (eg. Van der Waals force, hydrophobicity), chemical and biochemical forces assume importance in formation of a stable aggregate.

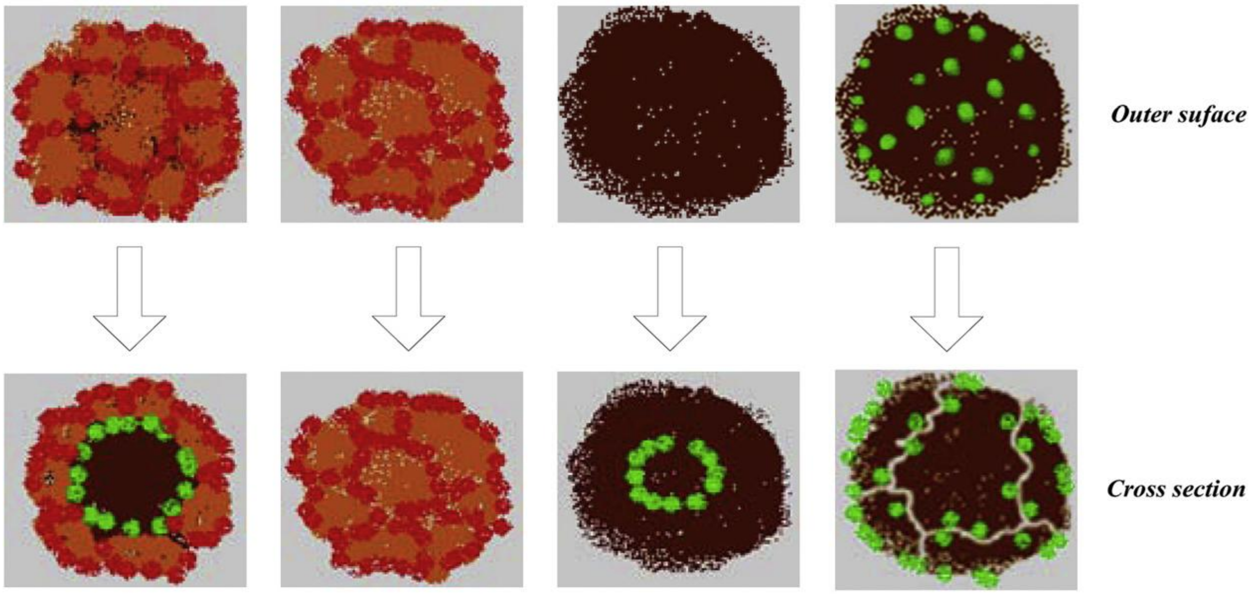
Maturation of aggregates

- Associated with EPS production and metabolic changes.
- Genetic changes are induced in cells.
- Microbial aggregate assumes shape.

Fig. 3.4 Mechanism for aerobic granulation **[Hwan Oh, 2007]**

The mechanism proposed by **Beun et al. [1999]** suggests that the initial microbial consortia are dominated by fungi which serve as the support matrix for the growth of bacteria. The bacterial species grow and become dominant and attain a compact structure.

Verawaty et al. [2011] worked on formation of aerobic granular sludge using crushed granules as the nuclei. The possible hypotheses proposed by them for formation of bacterial aggregates are shown in Fig.3.5.



1. Flocs attached to the crushed granules
2. Flocs attached together
3. Crushed granules growth
4. Small crushed granules attach to bigger one

Fig. 3.5 Possible hypothesis for aggregation of bacteria **[Verawaty et al., 2011]**.Labeled surfaces of crushed granules and flocs in fluorescence microscopy- the flocs are red and granules are green

3.2.3 Characteristics of granular sludge

3.2.3.1 Average diameter

The reported size of aerobic granules is variable. Glucose fed granules with sizes ranging from 1 to 10 mm was found to be formed in a continuous reactor [**Ganesan et al., 2007]**.However, small granules of 0.3 to 0.5 mm with very clear outline have also been reported [**Peng et al. in 1999]**.

3.2.3.2 Sludge Volume Index (SVI)

SVI is a measure of settleability of the sludge. As the SVI value decreases, the settleability improves. The SVI of granular sludge should ideally be less than 50 mL/g. An SVI value as low as 30 mL/g has been reported for aerobic granules developed in an airlift reactor fed on sodium acetate [**Liu and Tay, 2006].**

3.2.3.3 Settling velocity

Studies conducted show that a granule of size 4.2 mm has a settling velocity of 112 m/h. The study also indicates positive correlation between the size of the granule and its settling velocity [**Moy et al**., **2010**]. The same is also proved by the work of **Anuar et al., 2007**.

3.2.3.4 COD removal efficiency

The work of **Tay et al., 2002** shows that COD removal as high as 97 to 98% is feasible with the use of granular sludge in aerobic systems. **Wang et al. [2006a]** also obtained 92 to 99% COD removal with loading rate of 6 kg COD/m^3.d.

3.2.3.5 Morphology

Aerobic granules are usually dominated by rod shaped and coccid bacteria. The granules are very regular and spherical in shape with smooth surfaces [**Adav et al., 2009; Qin et al., 2004]**. In some cases, granules formed showed filamentous growth on the surface of the granule **[Tay et al., 2001a; Adav et al., 2009]**. Fig. 3.6 and 3.7 show granules cultivated in an SBR fed on glucose. The granules were fluffy at loading rate of 6kgCOD/m^3.d but at higher organic loading rates, they had irregular yet compact structure.

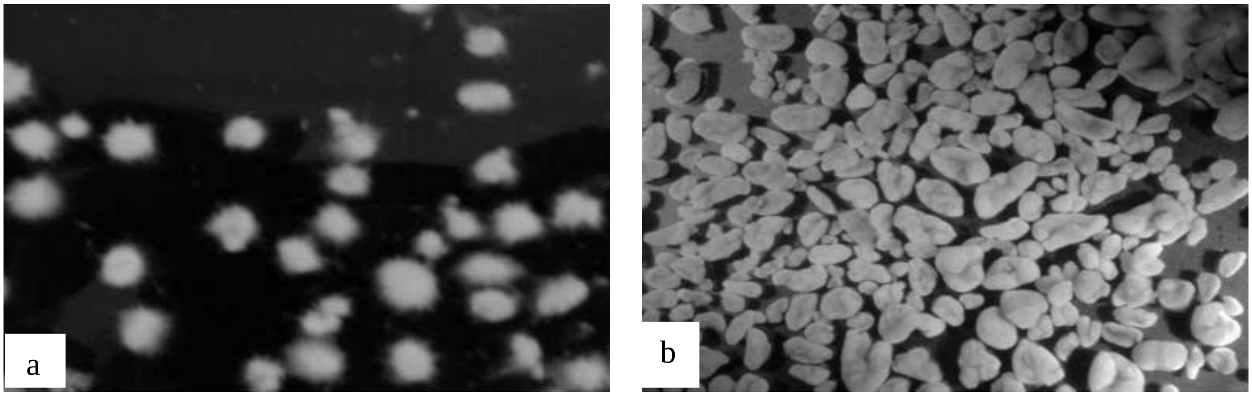


Fig. 3.6 Morphology of glucose-fed granules at different organic loading rates (OLRs) by image analyzer. (a) 6kgCOD/m^3.d; (b) 15kgCOD/m^3.d **[Moy et al., 2002]**

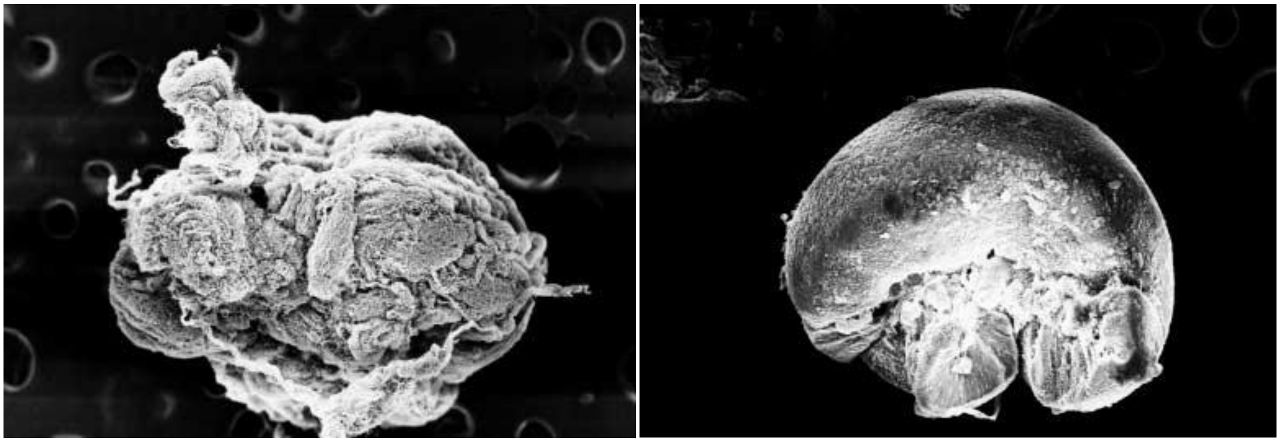


Fig. 3.7 SEM images of glucose-fed granules. (a) 6kgCOD/m^3.d; (b) 15kgCOD/m^3.d **[Moy et al., 2002]**

3.2.3.6 Integrity coefficient

The physical strength of the granules is measured in the terms of integrity coefficient (IC) **[Ghangrekar et al., 1996]**. Although the integrity coefficient does not give the exactstrength of a granule, it provides an overall idea of ability the sludge particles to maintain stable structure under the action of a shear force. Integrity coefficient is indirectly related to the strength of the granule.

3.2.3.7 Hydrophobicity

Hydrophobic interactions are known to play an important role in bacterial adherence **[Rosenberg, et al., 1983]**. The increase in hydrophobicity reduces the surface charges on bacterial surfaces, which leads to their agglomeration. Low value of cell surface hydrophobicity can disrupt the entire granulation process **[Tay et al., 2004b]**. Hydrophobicity is usually expressed as the percentage of cells adhering to hexadecane after 15 min of partitioning. Hydrophobicity of sodium acetate fed granules was found to be as high as 70% **[Qin et al., 2004]**.

4. Methodology

4.1Preparation of seed sludge

Activated sludge was obtained from Okhla wastewater treatment plant, New Delhi. The sludge was segregated into two fractions viz. small-loose sludge flocs with a slow-settling velocity and larger, denser sludge flocs with a fast-settling velocity. The two fractions were separated based on their settling velocities using a 1L graduated cylinder.

Prior to sludge separation, the sample was filtered through a 1mm screen to remove large debris. For each run of the sludge separation (filtrate), 50mL of the sludge suspension was transferred gently to the surface of the water column. After 2min of settling, the top 900 mL of suspension, which contained small and slow-settling sludge flocs, was decanted from the settling column, and the remaining 100mL of sludge suspension left at the bottom containing the denser and fast-settling flocs, was collected. More raw sludge was processed following the settling column procedure to obtain a sufficient amount of seed sludge for inoculation of the bioreactors. The fast-settling flocs were found to be larger in size, more compact in structure and faster in settling velocity than the slow-settling flocs. The MLSS of the separated fast-settling sludge was 44g/L.

4.2 Experimental set-up and operating conditions

Four cylindrical polypropylene reactors each with effective volume of 1.5L were operated in a 24 h cyclic fed-batch mode for six days and no feeding on the seventh day of the week (Fig. 4.1). The reactors were inoculated with the seed sludge (described in Section 4.1). Operation was initiated with a sludge concentration of 8g/L MLVSS. The organic loading (measured as COD) within the reactor was 8 to 10 kgCOD/m^3.d. The reactors were fed twice daily with an interval of 8h and 16h respectively in a sequence (120min for filling, 1200min for aeration,60min for settling, 30min for decanting and 30min idle)with a 50% volumetric exchange. The reactors were fed with a 3/1 v/v mixture of sewage and leachate from a two-phase anaerobic digester treating food waste. The leachate was, obtained from the 50 kg/d TEAM plant operational at RETREAT, TERI Gram, Gual Pahari, Haryana. The reactors were kept under ambient conditions (February 2012 to May 2012), with an average temperature of 12±3°C during February to 38±3°C in May.

The reactors were aerated continuously through submerged fine air bubble diffusers. The dissolved oxygen (DO) at start-up was 4.5mg/L but it reduced to 0.9mg/L after 3 months of continuous operation. The pH of the feed wastewater was maintained around 6.5-7.5 using 10N NaOH solution. The reactor volume was increased to 5L after forty seven days of operation.

Three reactors were nucleated with different nuclei viz (a) unburned carbon fraction separated from bagasse ash (obtained from Simbhaoli Sugars Limited, U.P.) (b) commercial activated carbon (Merck Chemicals) and (c) zeolite-4A (SRD Chemicals, New Delhi). The fourth reactor was the control and no particle addition was done. The characteristics of the support media used as nuclei for microbial granulation are mentioned in Table 4.1. 20g/L of support media was added in each of the three reactors. The overflow from the reactors was sieved through 0.2-0.4 µm sieves to recover the particles washed-out.

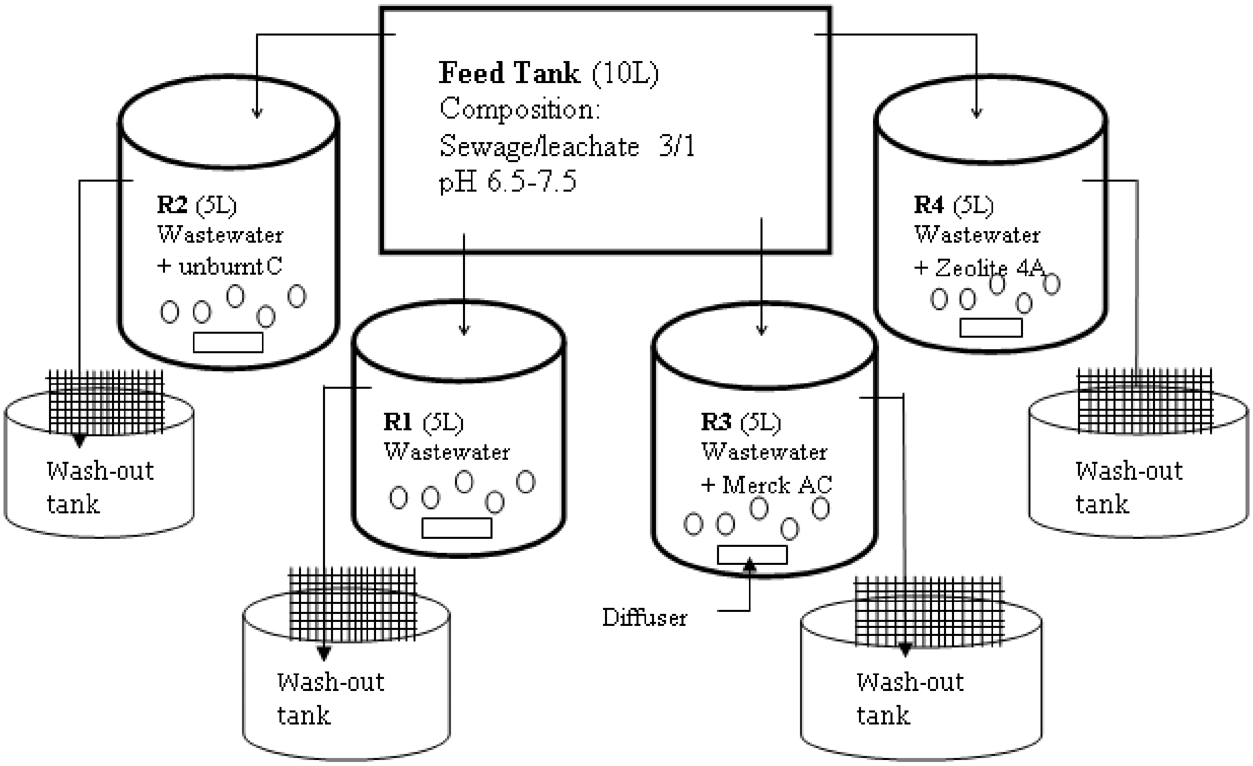


Fig.4.1 Schematic representation of the reactors

Table 4.1 Characteristics of the support media

Particles	BET surface area (m^2/g)	Particle size(D_{50}) µm
Unburned carbon (UC)	[redacted]	[redacted]
Merck activated carbon (MAC)#	923	12-15
Zeolite 4A (ZA)*	[redacted]	2.1-2.6

*Zeolite as specified by the supplier

Merck activated carbon determined experimentally

4.3 Analytical methods

4.3.1 Sludge size distribution

The sludge size distribution was determined by sieving method **[Laguna et al., 1999]**. Screening was performed with four stainless steel sieves of 5cm diameter having respective mesh openings of 0.2, 0.4, 0.6 and 0.9 mm. 25 mL sludge from the reactor was sampled with a calibrated cylinder and then screened by a series of standard sieves. The sample was subsequently washed with distilled water. The sludge was then divided into five ranges according to particle diameter (d) as: less than 0.2 mm, 0.2-0.4 mm, 0.4-0.6 mm, 0.6-0.9 mm and larger than 0.9 mm. Sludge with d smaller than 0.2 mm was defined as floc sludge, whereas the other sludge ranges (d>0.2 mm) were regarded as granules. Each fraction was collected in different beakers to determine the total suspended solids (TSS), represented as percentage of the total weight that they represent.

4.3.2 Sludge settling velocity

The settling velocity was determined by recording the average time taken for individual granule to settle at a certain height in a glass column filled with tap water **[Anuar et al., 2007]**. The column was 6cm in diameter and 42cm in height. The settling velocity was calculated by using equation (i).All settling tests were carried out in triplicate.

Settling velocity (m/h) = Distance of travel (m) / Settling time (h) (i)

4.3.3 MLSS and MLVSS

A known volume of sample (25 mL) was centrifuged at 8,000 rpm for 20 min (Remi Instruments, Mumbai, India).The supernatant was discarded and the pellet was washed with water. The pellet was resuspended in 25 mL RO water and centrifuged again. The supernatant was discarded and the pellet was placed in pre-weighed and pre-conditioned crucible. It was dried in hot air oven at 105°C overnight (until a constant weight was obtained).

The residue produced by the above method was transferred to a pre-weighed silica crucible and ignited in a muffle furnace at a temperature of 550°C for 4 h. The sample was transferred to a desiccator for final cooling in a dry atmosphere. The crucible was weighed after it cooled to room temperature.

4.3.4 Sludge volume index (SVI_{30})

The sludge volume index was measured in an Imhoff cone of 1L following standard method (APHA, 1999). The Imhoff cone was filled with a well-mixed sludge sample and allowed to settle for 30min. The experiment was done

$$SVI_{30}\ (\text{mL/g}) = \frac{\text{Volume occupied by 1L of sample (mL/L)}}{\text{MLSS (mg/L)}} \times 1000 \quad \text{(ii)}$$

4.3.5 Sludge hydrophobicity

Cell surface hydrophobicity was determined using the method described by **Rosenberg et al. [1980]** using p-xylene as the hydrophobic phase. Hydrophobicity was expressed as the percentage of cells adhered to the p-xylene after 15 min of phase separation. The experiment was done in duplicates.

4.3.6 Integrity coefficient

The granular strength was determined as described by **Ghangrekar et al. [2005]** and expressed as integrity coefficient (%). The measurement was based on the ratio of the weight of residual granules after shaking at 200 rpm for 5 min on a platform shaker versus the weight of the test sample. The experiment was done in duplicates.

4.3.7 Chemical oxygen demand (COD)

COD was measured by open reflux method using COD digestion apparatus (Model 2015M, Spectralab, Mumbai) following APHA, 1999.To 20 mL of appropriately diluted sample, 30 mL of sulphuric acid- silver sulphate reagent, 10mL $K_2Cr_2O_7$ and a pinch of $HgSO_4$ were added and the sample was refluxed for 2 h at 150^{o}C. After cooling, the sample was titrated with standardized 0.10M ferrous ammonium sulfate (FAS) using 4 to 5 drops of ferroin indicator. All chemicals were analytical grade and were procured locally.

4.3.8 Dissolved oxygen (DO)

Dissolved oxygen in the reactor tank was measured by azide modifications after copper-sulphate sulphamic acid flocculation modification (APHA, 1999). 1ml of $MnSO_4$ and alkali-iodide-azide reagent was added in 300 ml BOD (biochemical oxygen demand) bottles to fix the DO, followed by addition of 1ml concentrated H_2SO_4 and titration with 0.025 M sodium thiosulphate and starch as indicator. All the chemicals were of analytical grade and were procured locally.

4.3.9 Optical microscopy

The morphology of the granules was examined under a stereomicroscope equipped with digital camera (Zeiss Scope A1, Germany). The samples were prepared using HIMEDIA Gram Stains-Kit (K001-1KT).

4.3.10 Scanning electron microscopy (SEM)

Scanning electron microscopy was employed to observe the cultivated granules. The granules were fixed in a solution of 2.5% glutaraldehyde for 8h, followed by washing in sodium phosphate buffer (0.1M solution of pH 7.4) three times for 10 min each. Sequential dehydration of the granules was achieved using water-acetone solution (30%, 50%, 70%, 80%, 85%, 90%, 95% and 100%). After chemical drying with hexamethyldisilazane, the samples were coated with palladium in an argon atmosphere using a vacuum evaporator and examined with a Zeiss-EVO/MA10 instrument.

4.3.11 EPS extraction

EPS was determined using thermal treatment method (**Chang and Lee, 1998**).Mixed liquor was centrifuged at 3200rpm for 30 minutes and the supernatant was collected. This fraction constituted ‘Soluble EPS’ or ‘Soluble Microbial Product’ (SMP). The remaining pellet was washed and re-suspended in saline (0.9% NaCl solution). The suspension was subjected to heat treatment (100°C, 1 h) and the extracted solution was centrifuged again under the same conditions. The supernatant obtained is the bound EPS (bEPS).

The SMP and bound EPS were analyzed for MLSS and VSS, without centrifugation. The EPS content of each fraction was calculated by the following equation

EPS (mg VSS $gMLSS^{-1}$) = VSS (g/L) x 1000/MLSS (g/L) (iii)

4.3.12 Carbohydrate determination

The total carbohydrate content was measured by phenol-sulphuric acid method **[Dubois et al., 1956]**. To 1 mL ofappropriately diluted sample, 1 mL of phenol solution and 5 mL of 96% sulphuric acid were added and shaken in a vortex mixer. The mixture was allowed to stand for 1hfor color development. The absorbance was read at 480 nm using Shimadzu, UV-1700 spectrophotometer. The amount of total carbohydrate present was calculated using the standard curve for glucose.

4.3.13 Protein determination

The protein concentration was determined using Lowry's method **[Lowry et al., 1951].**The standard curve for Lowry's assay was constructed using known amounts of bovine serum albumin (BSA) The protein content of the unknown solution was determined by comparing the color produced by the unknown with the standard curve.

Reagent A: 0.57g sodium hydroxide and 2.86g sodium carbonate were mixed in 100 mL RO water. Reagent B: 1.42g copper sulphate to 100mL RO water. Reagent C: 3.49g sodium potassium tartrate to 100mL distilled water. Lowry’s Solution: Reagent A: Reagent B: Reagent C =10:0.1:0.1, Folin’s Reagent (1N).

To 2 ml of appropriately diluted sample, 2.8 mL of Lowry’s reagent and 0.4 mL of Folin-Ciocalteu reagent was added. The solution was mixed in a vortex and incubated at room temperature (away from light) for 45 min. The absorbance of the sample was read at 750 nm.

4.3.14 Volatile Fatty Acids

Volatile fatty acid content (VFA) was determined by gas chromatography (GC) on Nucon gas chromatograph 5700 (Nucon Engineers, New Delhi, India) attached to a flame ionization detector (FID).

5. Results and Discussion

5.1 Reactor start-up

The feed used initially was domestic sewage. But the COD of sewage was around 198mg/L which was found to very low to initiate the granulation process. So to facilitate growth of microorganisms, leachate from the two phase anaerobic digester was added. The composition of leachate is presented in Fig. 5.1.

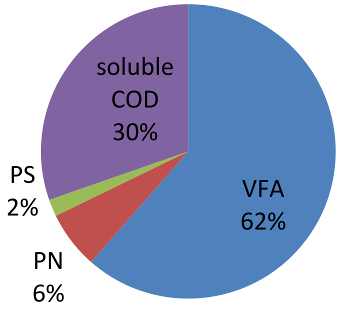


Fig. 5.1 Composition of leachate (COD: chemical oxygen demand; VFA: volatile fatty acids; PN: protein; PS: polysaccharide)

The characteristics of sludge collected from Okhla wastewater treatment plant are presented in Table 5.1. From this, the fast settling fraction was extracted, the properties of which are presented in Table 5.2. The reactor was inoculated with 8g/L of the extracted sludge. The seed sludge was dominated by filamentous bacteria and it had fluffy and irregular morphology (Fig. 5.2).

The micro-organisms were allowed to acclimatize to the wastewater for 30 days and the reactor was operated for 80 days, making it a total of 110 days.

Table5.1 Characteristics of sludge collected from Okhla wastewater treatment plant

Property	Value
MLVSS	56 g/L
SVI	150 mL/g SS

Table5.2 Characteristics of seed sludge (fast settling fraction)

Property	Value
MLVSS	44 g/L
MLVSS/MLSS	.8
Hydrophobicity	59.64 %
Particle Size(D_{50})	<0.2mm
PN/PS	4.6
Settling velocity	15 m/h

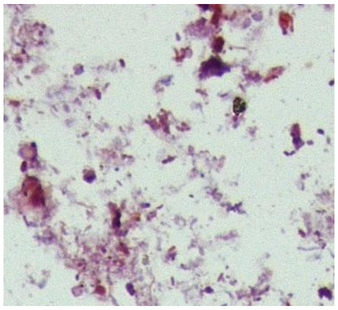


Fig. 5.2 Morphology of seed sludge

5.2 Granule formation and morphology

A loose granule structure was formed in all the four reactors by the end of the acclimatization phase, i.e. by the end of 30 days. Acclimatization can be defined as the time taken for the initiation of granulation (**Wang et al., 2004**). Fig. 5.3 shows the growth of the granule in the reactors. The granules are mainly dominated by rod-shaped and coccid bacteria. The granules are irregular in shape but they have a very clear outline.

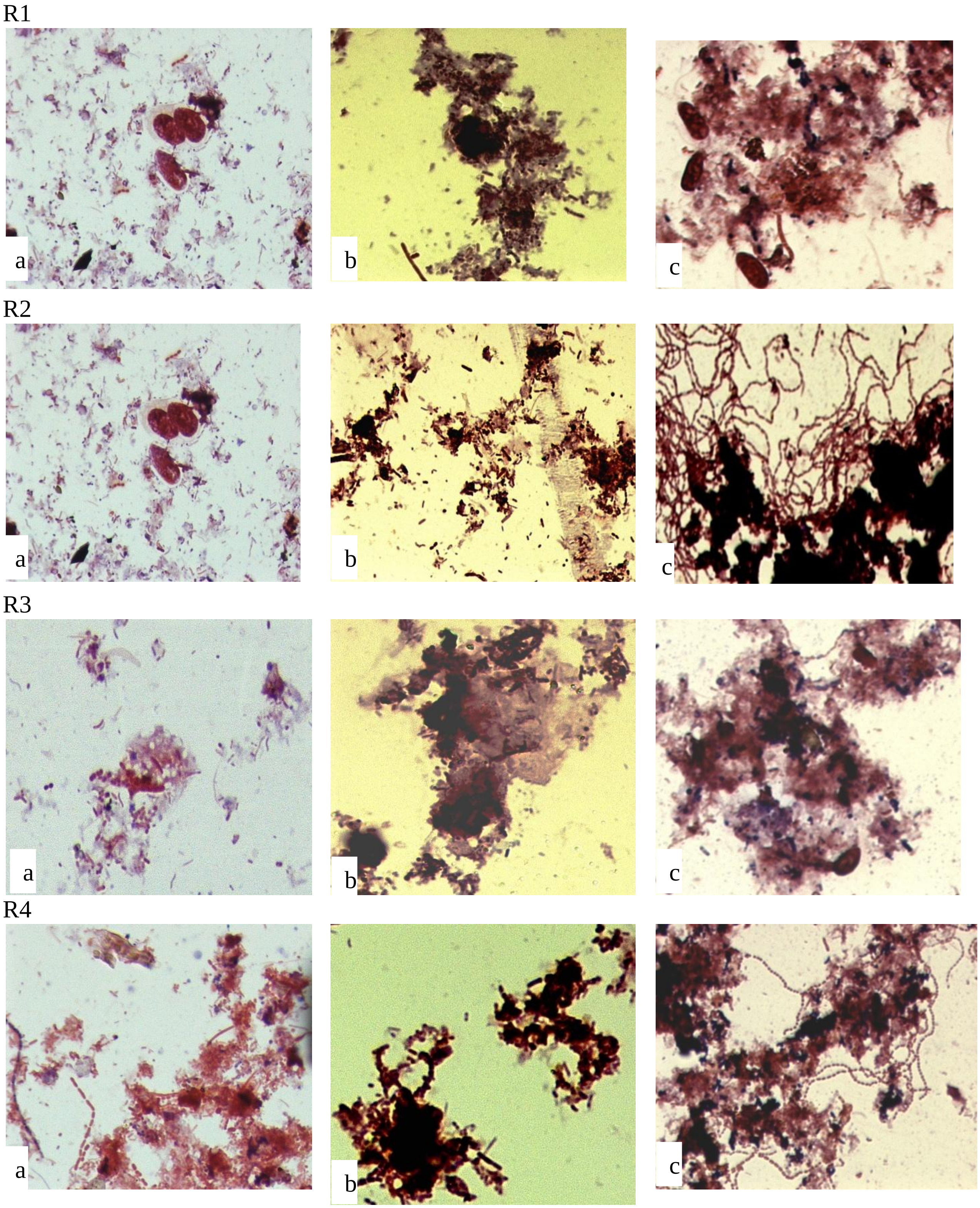


Fig. 5.3 The growth of aerobic granules. Microscopic observation of aerobic granules (a) at the end of acclimatization period (b) after 40 days (c) after 80 days of reactor operation (R1: control; R2: unburned carbon; R3: commercial activated carbon; R4: zeolite; the biomass sampled from

the reactor was directly used for microscopic analysis without separating any granules from flocs)

Fig.5.4 shows the SEM images of the granules on the 80th day. The surface of the granules exhibit compact bacterial colonies dominated by rod shaped bacteria in all the three reactors, except for zeolite, where the coccid bacteria are found to be dominant. The granule has irregular shape. Similar irregular yet compact granules have been reported in other studies [e.g. **Moy et al., 2002**]. The irregularities on the surface of the granule can help in the better penetration of nutrients inside the granules as compared to spherical-shaped granules. The filamentous bacteria are found to be absent, as reported in previous works [**Adav et al., 2008, Tay et al., 2001a**]. It is clear from the images that the core of the granule has less density of cells as compared to the surface of the granule. Also, there are voids present in the granules. These can be attributed to the presence of an inert core material and also to the extracellular polymeric substances secreted by the cells. Similar results have been reported by other researchers as well [e.g. **Chen et al., 2009**].

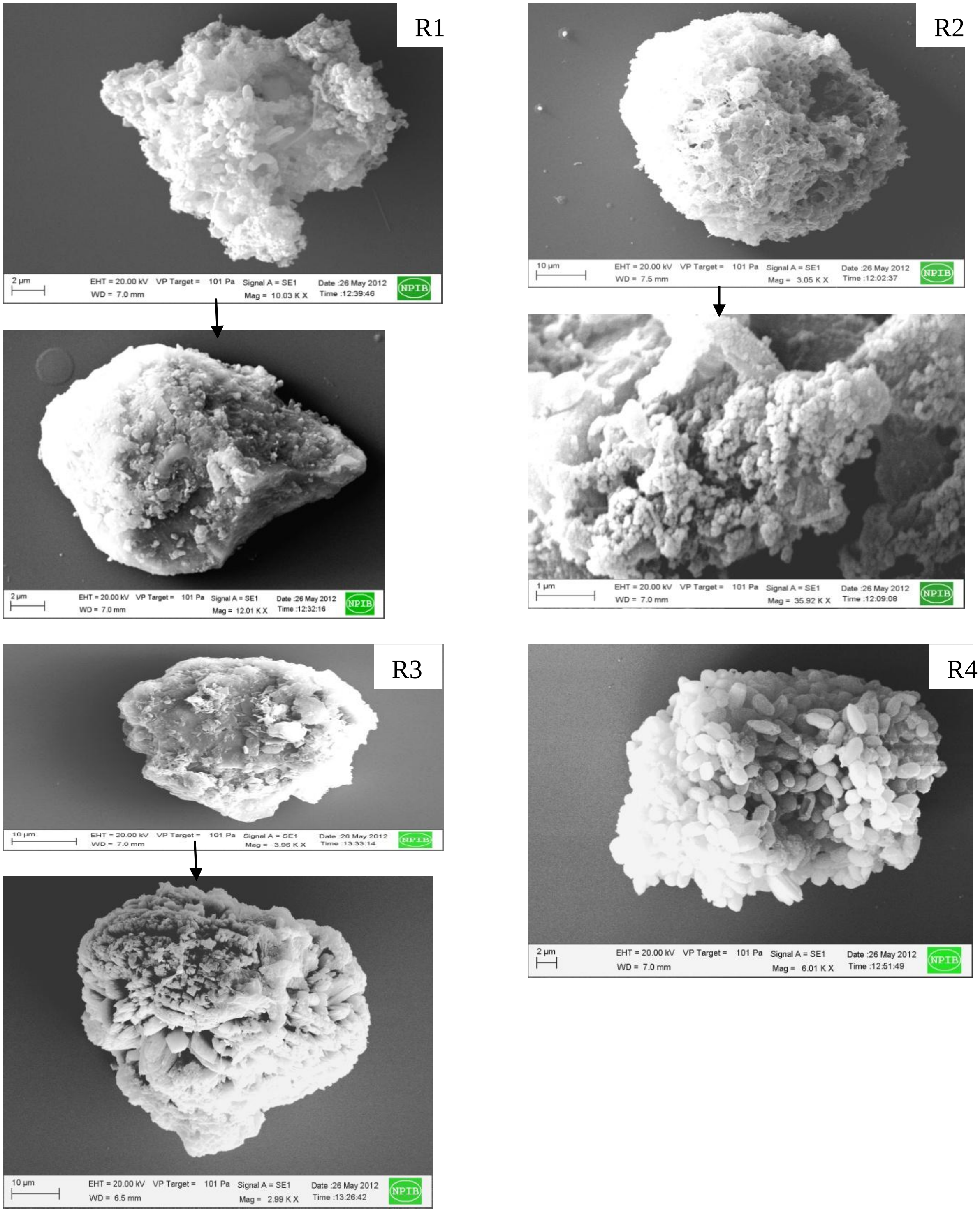


Fig. 5.4 Microstructure of the granules by observed by SEM at the end of the study (R1: control; R2: unburned carbon; R3: commercial activated carbon; R4: zeolite).

5.3 Reactor performance and organic matter utilization

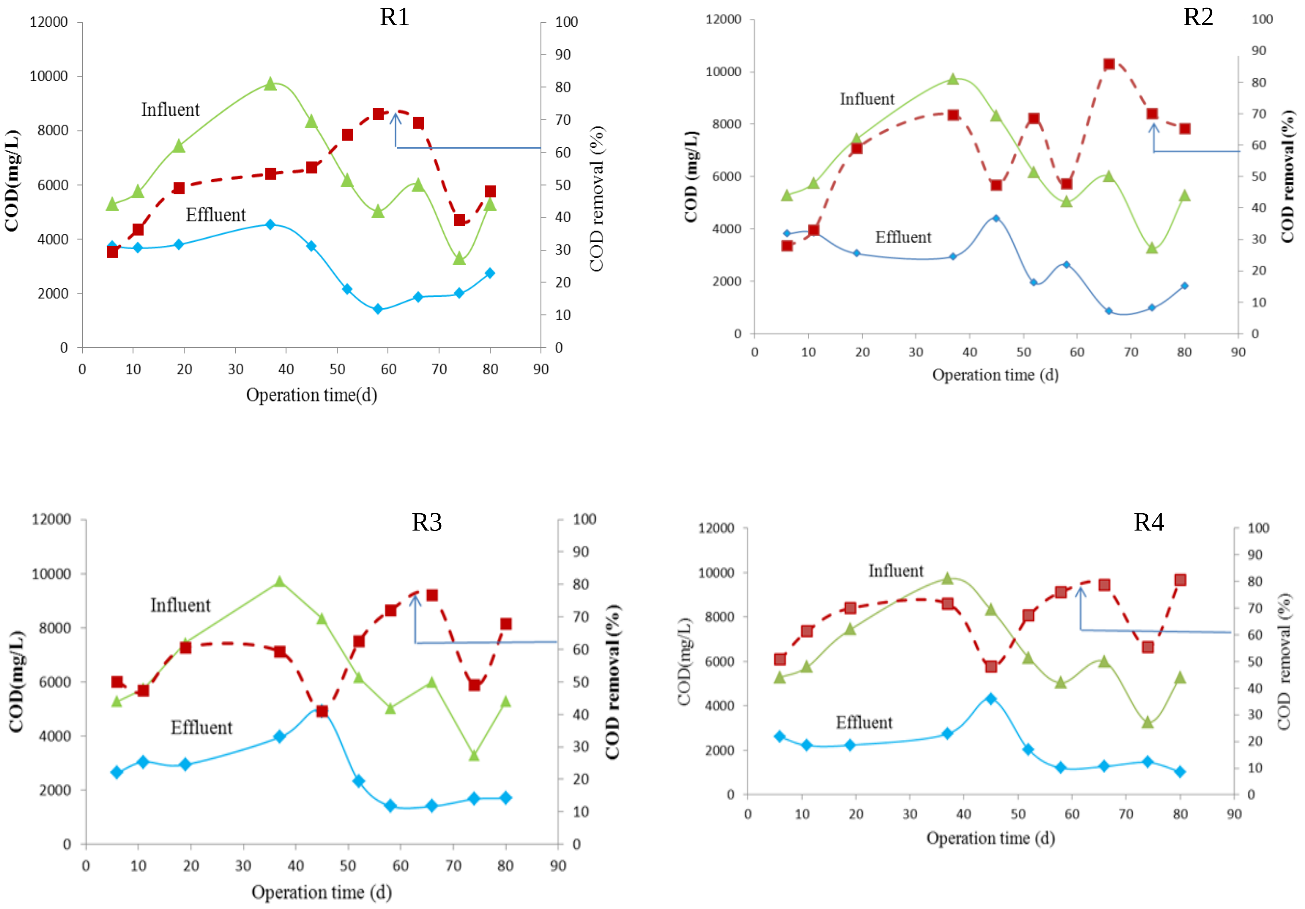


Fig. 5.5 Profile of COD removal (%) during reactor operation (R1: control; R2: unburned carbon; R3: commercial activated carbon; R4: zeolite).

The COD of the feed wastewater varied during the operation time as the composition of the sewage and the leachate was different on different days. All the four reactors showed considerable decrease in COD in the effluent during the granulation process (Fig. 5.5). The COD removal in all the four reactors increased over time, with maximum removal of 90% in R2, an average of 70-75% COD reduction was achieved in the other three reactors after two months of continuous operation.

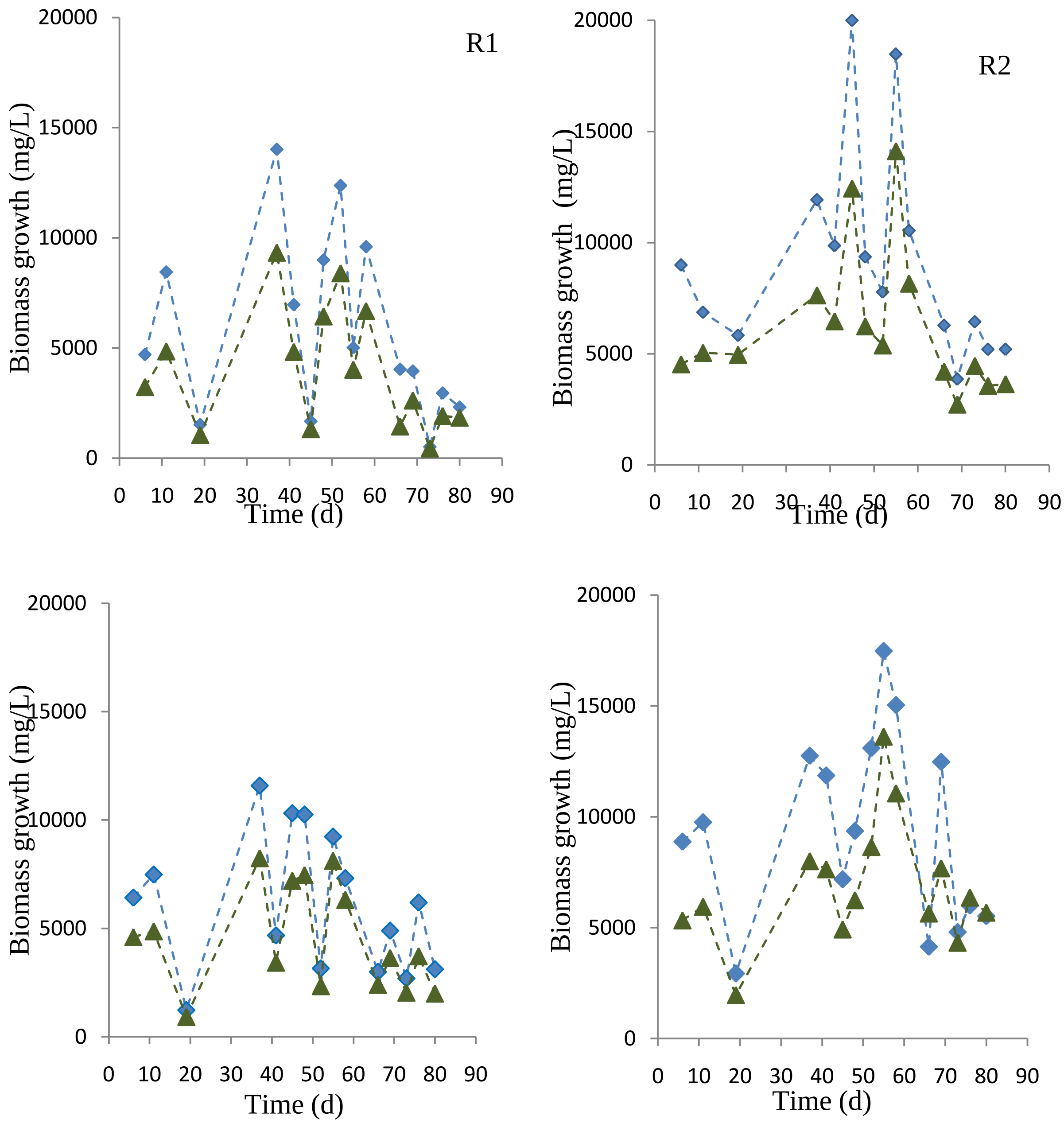


Fig. 5.6 Profile of biomass growth during reactor operation (R1: control; R2: unburned carbon; R3: commercial activated carbon; R4: zeolite; --◆-- MLSS, --▲-- MLVSS).

The presence of easily biodegradable components in the leachate (Fig.5.1) enhanced the uptake rate thereby facilitating microbial growth within the reactor (Fig. 5.6) .The biomass concentration measured in terms of MLSS and MLVSS also increased with time in all the four reactors (Fig.5.6). During granule cultivation, the MLVSS/MLSS ratios of the sludge in all the reactors increased to about 0.85-0.90, suggesting an increased biomass fraction in the granular sludge. This shows that granulation is a way of achieving higher biomass retention in the bioreactor which has also been proven in previous studies [**Sheng et al., 2010**]. The sudden fluctuation in MLSS and MLVSS concentration is due to sludge loss during reactor maintenance.

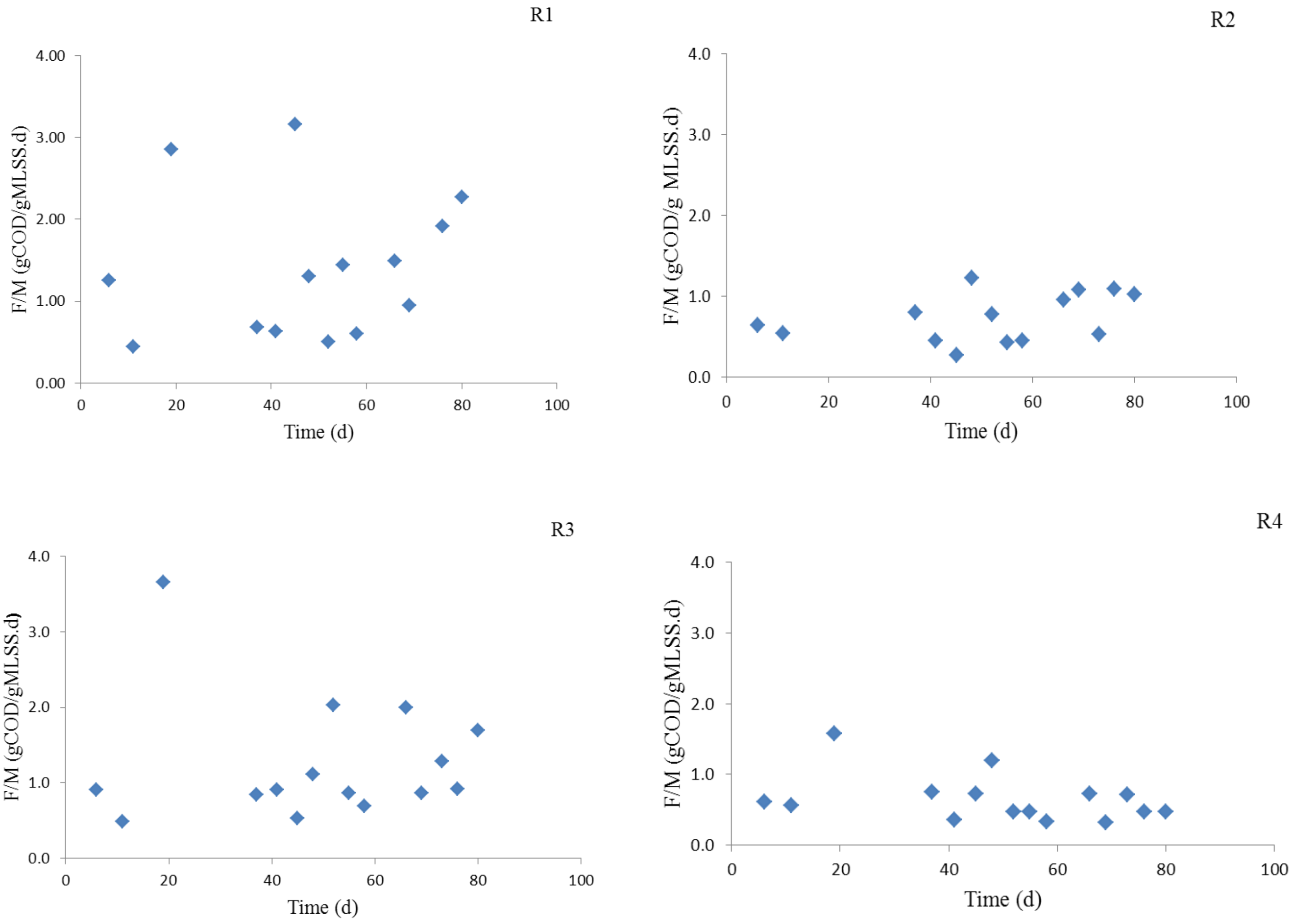


Fig. 5.7 F/M ratio (R1: control; R2: unburned carbon; R3: commercial activated carbon; R4: zeolite).

The food-to-microorganisms (F/M ratio) inside the reactor remained between 0.5-1.5 throughout the operation period in all the four reactors (Fig. 5.7). This indicates a steady condition in the reactor after a month of operation, despite the fluctuations in the ambient temperature between day-night and months and COD composition of the feed wastewater.

5.4 Extracellular Polymeric Substances in the aerobic granules

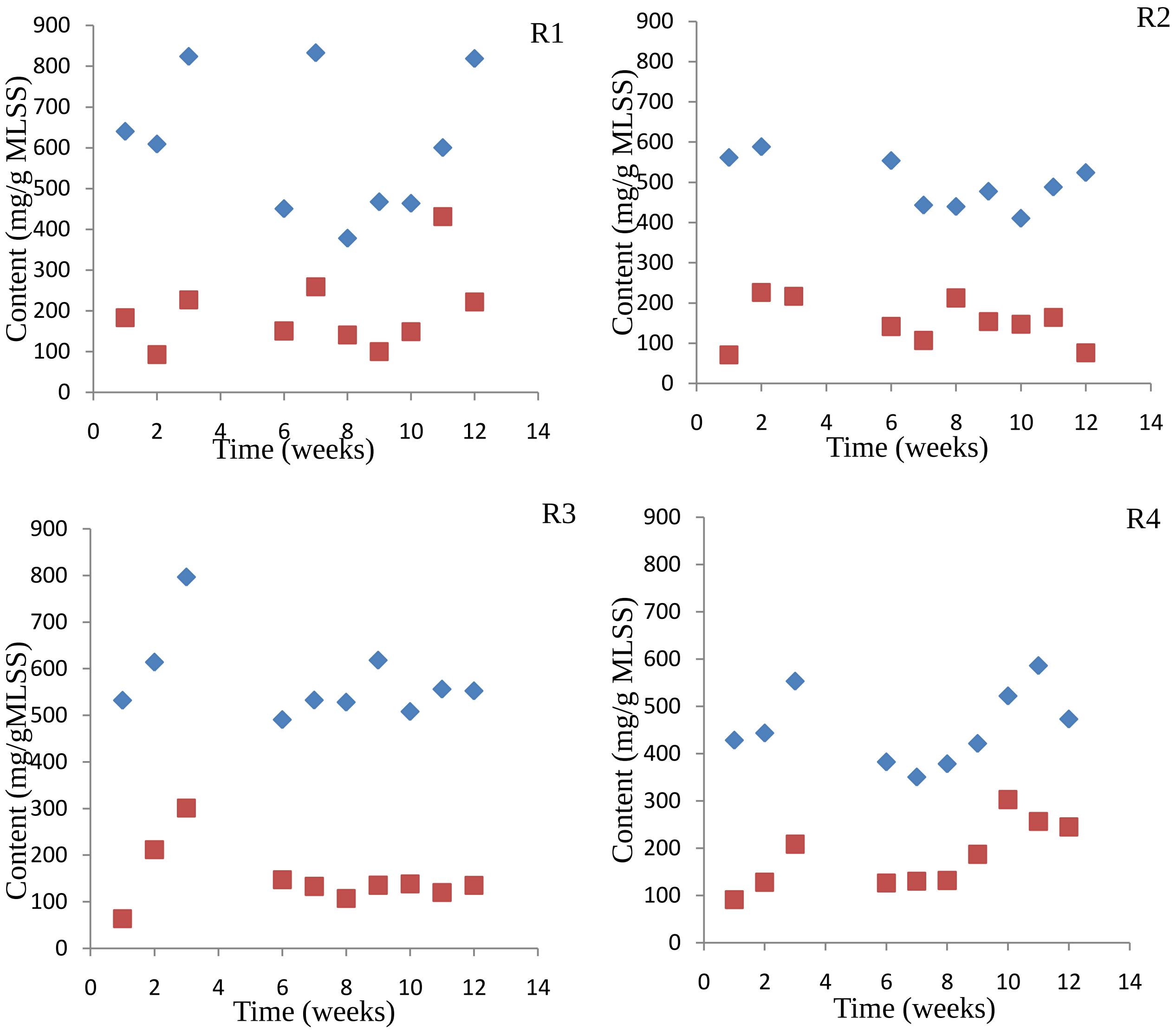


Fig. 5.8 Variation of EPS content of aerobic granular sludge with time (R1: control; R2: unburned carbon; R3: commercial activated carbon; R4: zeolite; ◆ soluble EPS, ■ bound EPS).

The EPS content of the aerobic granules is found to be increasing with increasing time during the granulation process, following which it stabilizes to a value of around 1000 mg/g MLSS in R1 and an average value of 700 to 800 mg/g MLSS in other three reactors after 2 months of continuous operation (Fig. 5.8). The bound EPS content in all the four reactors is much less than that of the soluble EPS. The soluble EPS content is almost three times more than that of the bound EPS. This can be attributed to the diffusion limitations of substrate experienced by the cells in the granules **[Aquino and Stuckey, 2003]**. Enzymes in nutrient limited conditions degrade bound EPS to the loosely-binding soluble EPS [**Lapsidou and Rittmann, 2002**].

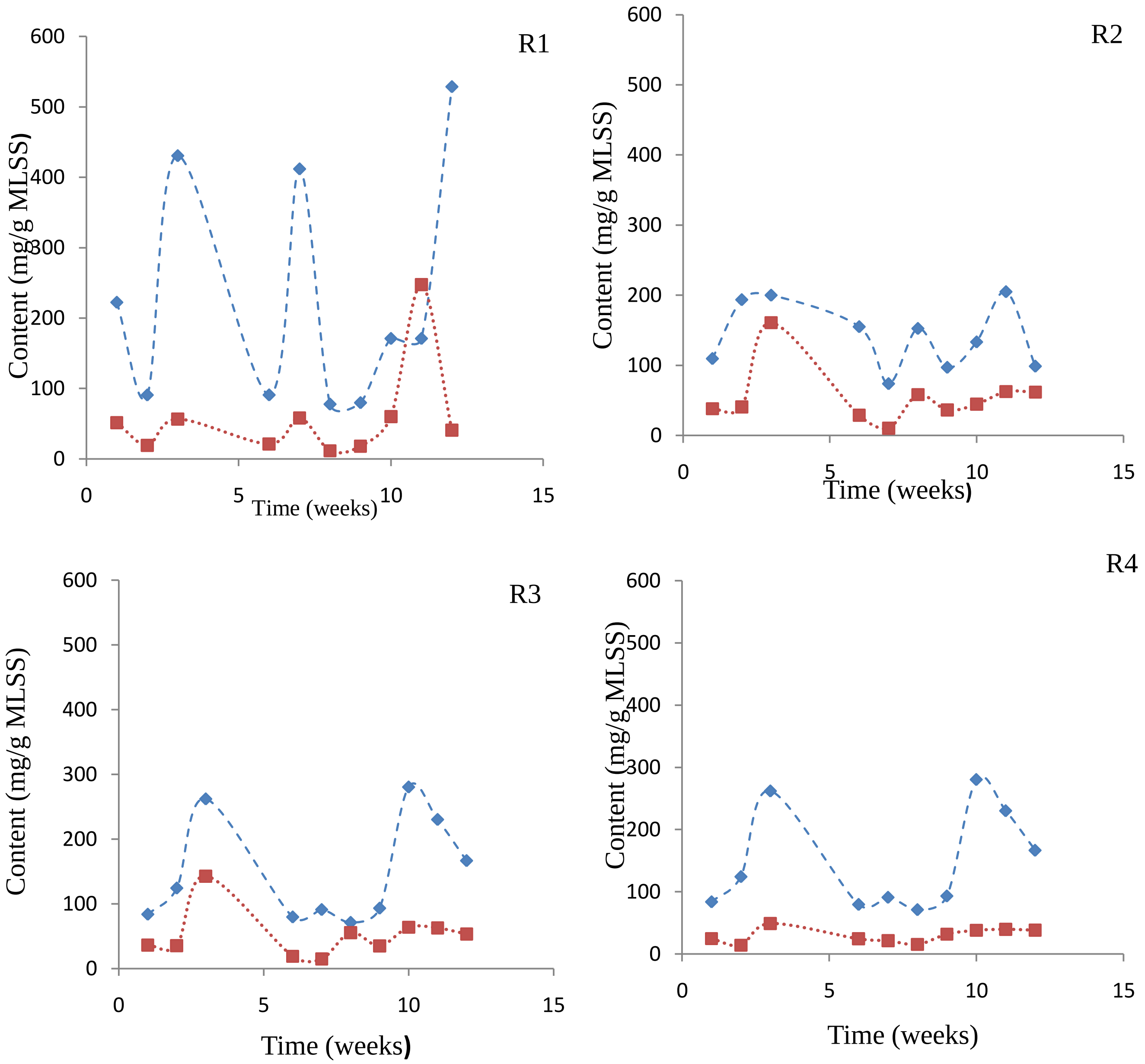


Fig. 5.9 Variation of protein (PN) and polysaccharide (PS) content during reactor operation (R1: control; R2: unburned carbon; R3: commercial activated carbon; R4: zeolite; --◆-- Protein, --■-- Polysaccharide)

EPS are organic macromolecule polymers secreted by bacterial cells, and the major substances are protein and polysaccharide [**Horan and Eccles, 1986**]. EPS is suggested to facilitate cell agglomeration by forming polymeric matrix [**Wang et al., 2006b; Wang et al., 2005**]. The changes in polysaccharide and protein content of the extracted EPS are presented in Fig 5.9.The content of protein in all the four reactors is greater than that of the polysaccharide. The protein in EPS also increased over the operation time. The average protein in EPS increased to between 150-200 mg/g MLSS from an initial value of 50 mg/g MLSS. The increase in protein content is likely to be related to the development of granules. Increased protein in EPS accentuates microbial cells to form a cross-linked network by attraction of organic and inorganic materials, which helps granulation [**Liu and Tay, 2004**]. The protein content increased during the first one and half months and then decreased for several weeks and then resumed increasing again, this indicates the imitation of the granulation process. The increase of polysaccharide in EPS from 5-10 mg/g MLSS to an average value of 50-80 mg/g MLSS in all the four reactors could help microbial cell agglomeration by bridging cells together during microbial aggregation [**Tay et al., 2001b**]. However, the change in protein content in EPS is more important than polysaccharide in aerobic granule formation [**Gao et al., 2011**]. After 80 days of continuous operation, the content of EPS, protein and polysaccharides of the granular sludge in all the reactors were higher than the seeded sludge inoculated (Figure 5.10). R1 has the highest protein content compared to the other three reactors.

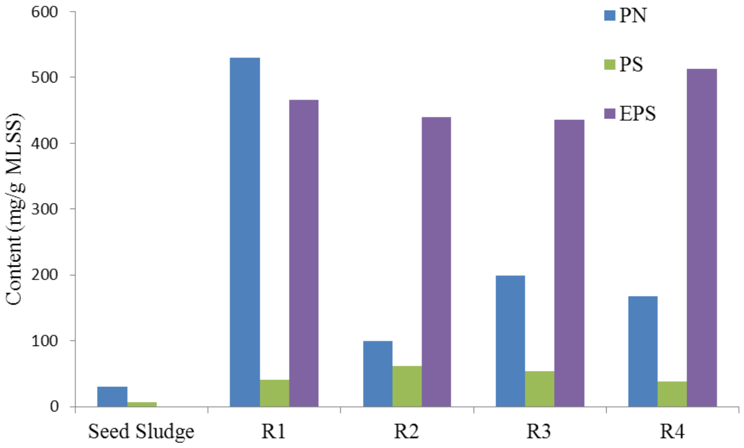


Fig. 5.10 Comparison between protein, polysaccharide and EPS content of seed sludge and sludge cultivated in the four reactors at the end of the study (R1: control; R2: unburned carbon; R3: commercial activated carbon; R4: zeolite)

5.5 SVI

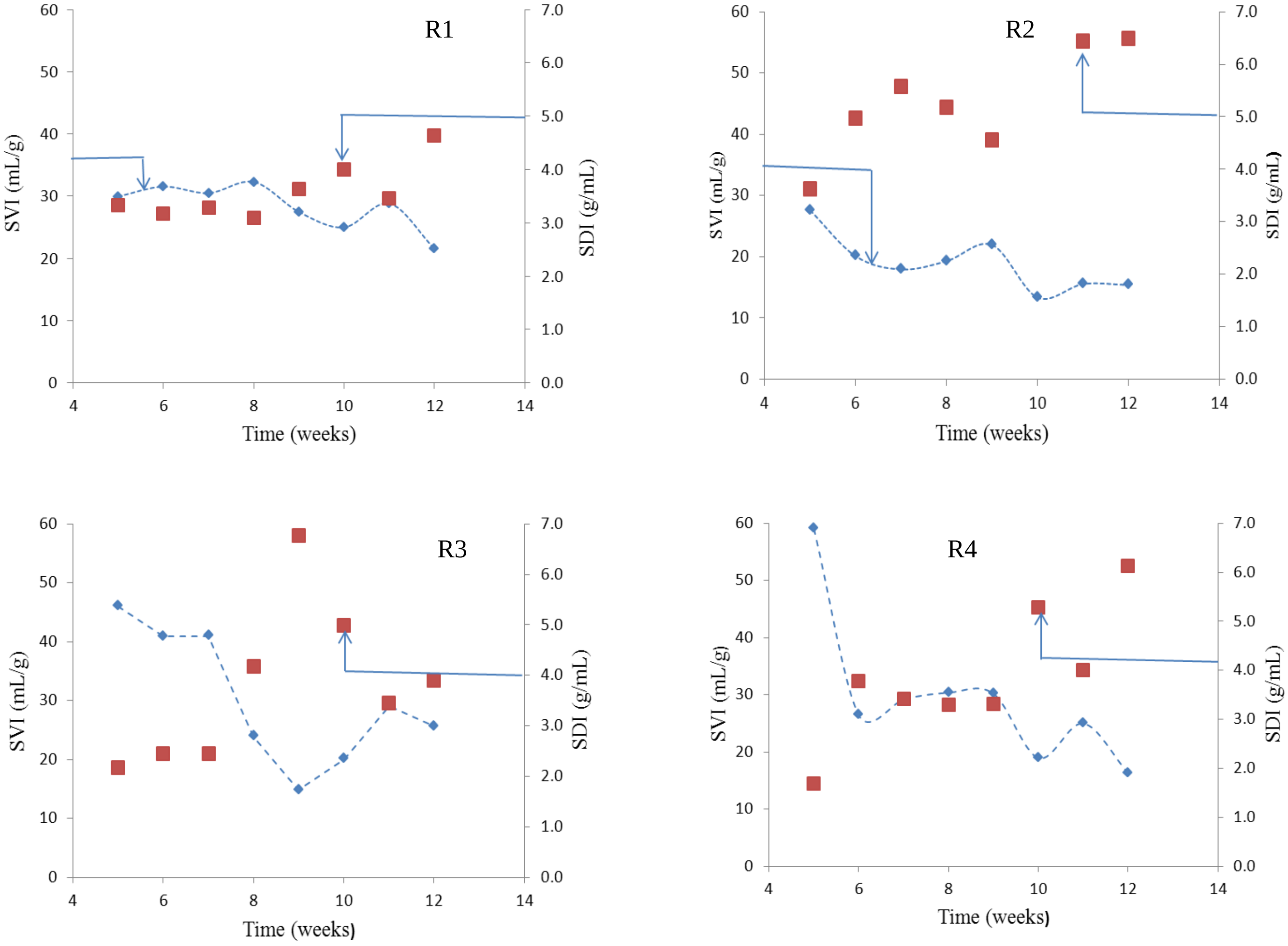


Fig. 5.11 The change in SVI over the reactor operation (R1: control; R2: unburned carbon; R3: commercial activated carbon; R4: zeolite).

The SVI in all the four reactors decreased during the operation (Fig. 5.12). Similarly, sludge density index (SDI = 100/SVI) increased in all the four reactors. The SVI values in R1 and R2 after one month of operation is around 30 mL/g and it decreased further to 15-20 mL/g. R3 and R4 also showed similar lower SVI values as R1 and R2 after 3 months of operation. This indicates biomass agglomeration over time and attributed to denser and heavier granules, which is a manifestation of good settling properties of the granules. The biomass settled very well, leaving behind a clear supernatant in the reactors. Thus, it appears that the operation conditions has caused biomass to be retained in the reactor and encouraged the formation of granules and better sludge accumulation in the reactor. Similar lower SVI values were reported earlier by many other workers [**Abdullah et al., 2011; Sheng et al., 2010**].

5.6 Particle size distribution

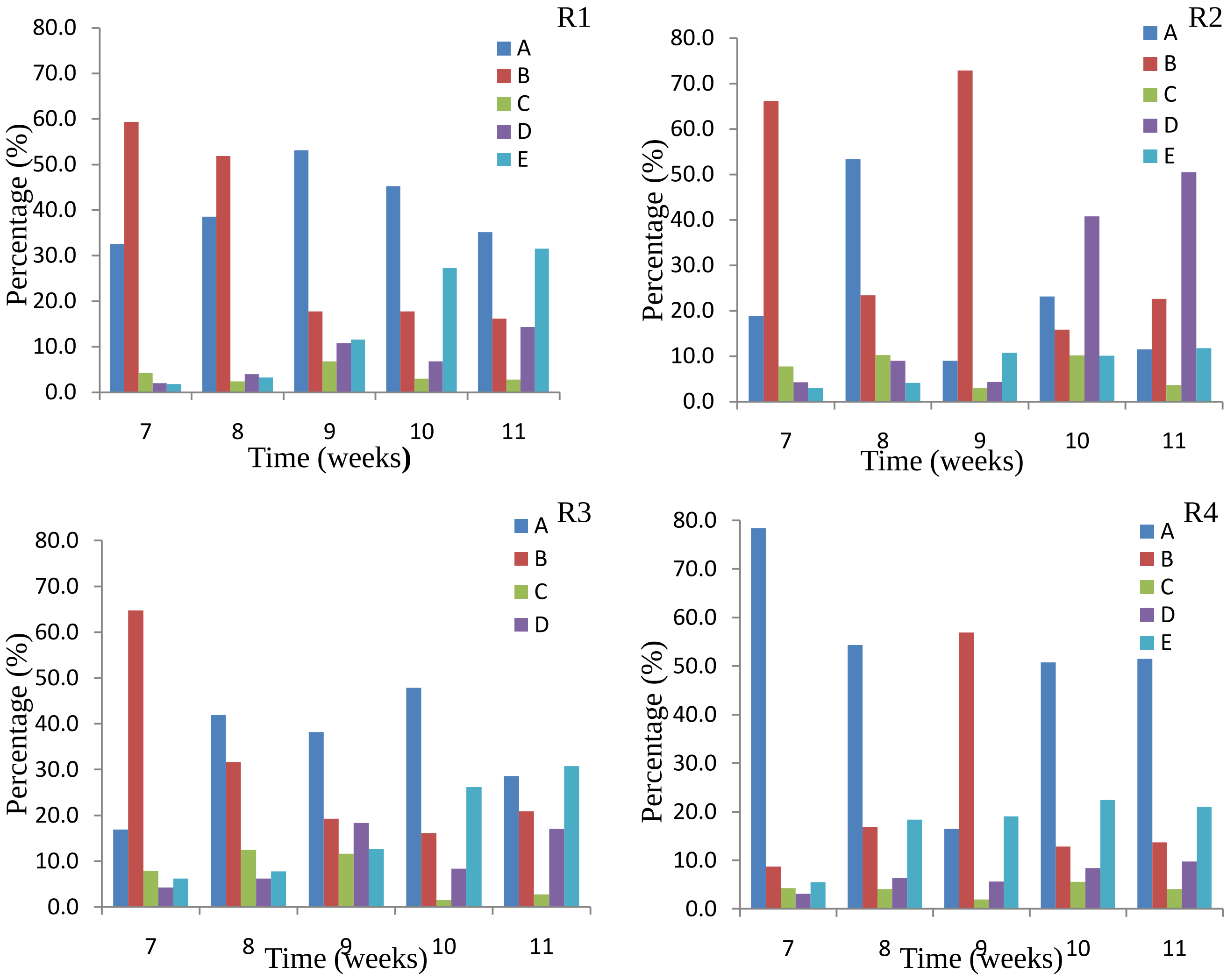


Fig. 5.12 The change in particle size distribution after 50 days of reactor operation (R1: control; R2: unburned carbon; R3: commercial activated carbon; R4: zeolite; A :< 0.2mm; B: 0.2-0.4mm; C: 0.4-0.6mm; D: 0.6-0.9mm; E :> 0.9mm)

The particle size of the granules increased during the continuous operation in all the four reactors (Fig. 5.12). However, the particle size distribution in each of the reactors is different. In R1, about 50-60% of the particles are in the size of 0.2-0.4mm at the end of 2 months; this slowly decreased to 20% while particles less than 0.2mm increased, in comparison particles greater than 0.9mm increased in the last month of operation to more than 30%. When the granules grew bigger, oxygen diffusion in the inner part of the granules became limited, resulting in breakage of the granules, similar results were also found in earlier studies [**Chen et al., 2009; Gao et al., 2011**]. Also, high shear force causes frequent collisions among developing granules, preventing generation of bigger granules. However, the increased percentage of particles between 0.6-0.9 mm and 1cm indicate deep penetration of substrate and oxygen in some granules. The fast-famine condition maintained inside the reactor resulted in longer starvation period and the granules experienced longer endogenous respiration phase at slower microbial growth rate and secreted EPS. This facilitated cell-cell agglomeration. Similar results are also found in R2 and, R3. The granulation process in R4 seems to have occurred much later than the other three reactors.

5.7 Settling velocity

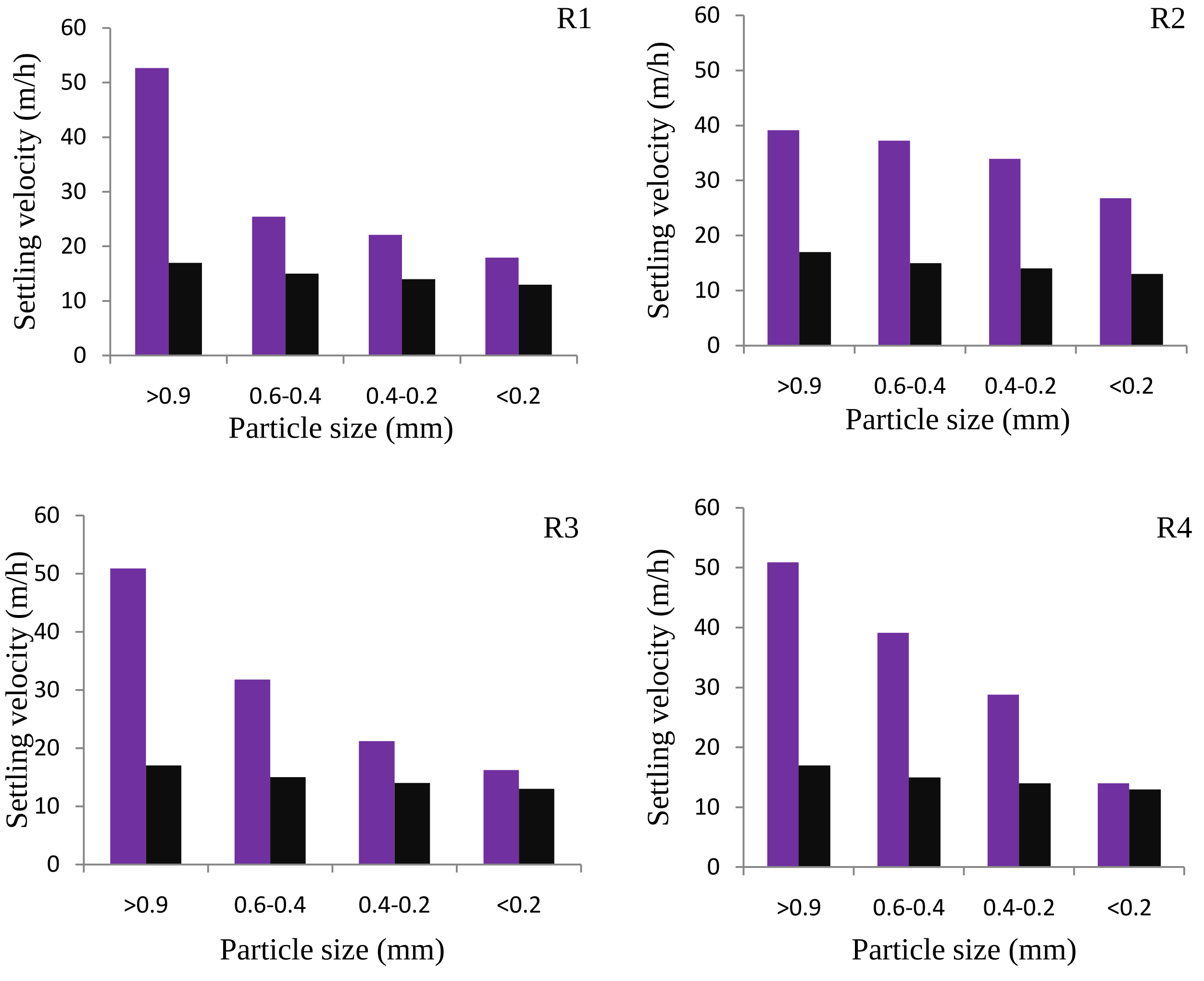


Fig. 5.13 The settling velocity observed in different particle size ranges after 80 days of reactor operation as compared to the seed sludge(R1: control; R2: unburned carbon; R3: commercial activated carbon; R4: zeolite; the sludge of the reactors is purple and seed sludge is black)

The average settling velocity of mature granules is found to vary considerably with size. The highest settling velocity has been attained in the particle of size greater than 0.9 mm. The settling velocity of the aerobic granules has been reported to be associated with granule size and structure [**Liu and Tay, 2004**]. The highest settling velocity was observed to be around 50-60 m/h. The high settling velocity of the developed granules prevents them from being washed out. The particle settling velocity was higher in all the four reactors compared to the seed sludge for all the selected particle size ranges. This indicates the maturation of the particles.

5.8 Integrity coefficient

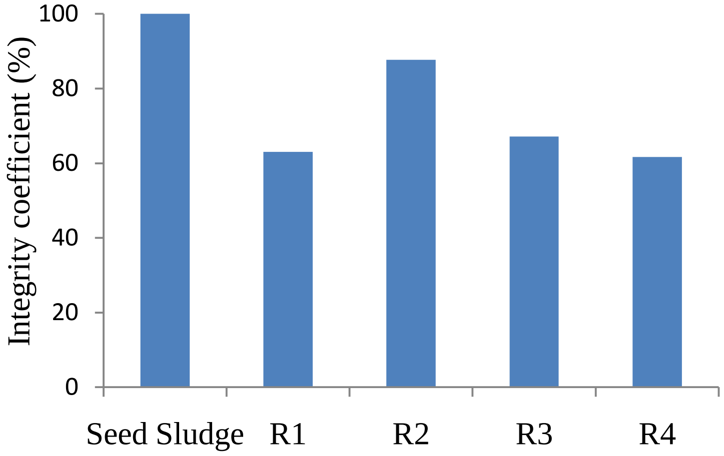


Fig. 5.14 The integrity coefficient of the seed sludge and the sludge in the four reactors after 80 days of reactor operation (R1: control; R2: unburned carbon; R3: commercial activated carbon; R4: zeolite)

The integrity coefficient of the granules decreased from the seed sludge after three months of continuous operation in all the four reactors (Fig. 5.15). The decrease in integrity coefficient indicates the increase in strength of the granules and thereby attribute to its improvement in compactness and strength. R1, R3 and R4 shows higher strength compared to R2. This could be due to the presence of larger particle size in R2, where the interaction between the sludge flocs and the particles was weak.

5.9 Hydrophobicity

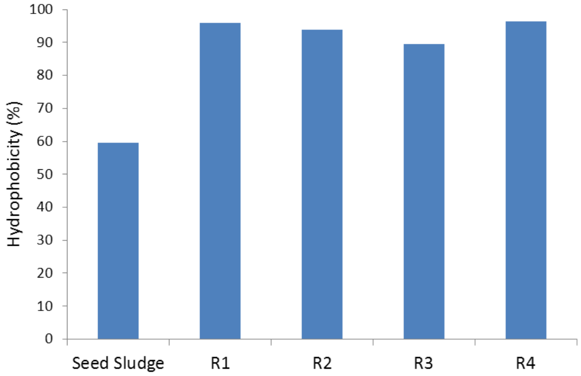


Fig. 5.15 Comparison of cell surface hydrophobicity of the seed sludge and sludge in all the four reactors at the end of the study (R1: control; R2: unburned carbon; R3: commercial activated carbon; R4: zeolite)

The seed sludge used in this study had a surface hydrophobicity of 50%. After the formation of the aerobic granules (3 months of operation), it was found the cell surface hydrophobicity increased to more than 90% in all the four reactors (Fig. 5.11). Similar results were also obtained with sodium acetate feed wastewater of different composition and synthetic wastewater [**Liu et al., 2003, Chen et al., 2009**]. Increased hydrophobicity accelerates flocs agglomeration, resulting in granules [**Chen et al., 2009**].

6. Conclusions

This work reports the formation of aerobic granules in four SBRs fed with a mixture of domestic wastewater and leachate from a two-phase anaerobic digester treating food waste. The conclusions are as follows:

1. The inert nuclei did not seem to enhance the granulation process. So, it is not essential to add inert nuclei to speed up the formation of the granules.
2. After around 60 days of operation, no significant changes in property of the granules were observed across different nuclei.
3. The granules formed are noticed to have compact structure, good settleability, hydrophobicity and integrity coefficient.

7. References

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