THE ROLE OF CALCIUM ION ON ACTIVATED SLUDGE BIOCHEMICAL AND PHYSICAL PROPERTIES IN PHOSPHORUS DEFICIENT GROWTH MEDIUM

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ABSTRACT

THE ROLE OF CALCIUM ION ON ACTIVATED SLUDGE BIOCHEMICAL AND PHYSICAL PROPERTIES IN PHOSPHORUS DEFICIENT GROWTH MEDIUM

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Nutrients and cations have significant effect on activated sludge characteristics and therefore effect the efficiency of whole processes. To determine the properties in phosphorus deficient medium and the effect of calcium ions two reactor sets with two different phosphorus concentration (C/N/P=100/5/0.05 and C/N/P=100/5/1), three different concentrations of calcium (0.5, 5, 15 meq/L) were operated with 8 days of sludge residence time and an effective volume of 2 L. Results showed amount and composition of EPS was dependent on calcium and phosphorus concentrations. Except for the highest calcium concentration, increase in phosphorus concentration resulted in increase in total EPS production. Under phosphorus deficient conditions, calcium ions stimulated the production of carbohydrate type polymers and viscous bulking was observed. However, the increase in phosphorus concentration led to increase in protein type polymer production and bulking condition was cured. Addition of calcium ions increased conductivity in both cases, but increase in phosphorus concentration caused decrease in conductivity. Increase in phosphorus concentration had improved settleability, dewaterability and rheology of sludge. Moreover, effluent turbidity was decreased and COD removal efficiency was recorded as greater than 95 % for all calcium concentrations under phosphorus sufficient conditions. Microscopic analyses showed that under phosphorus deficient conditions flocs were weak,

dispersed and nonresistant. Increase in phosphorus concentration resulted in improvement of floc structure. Same *Enterobacter* and *Citrobacter* species were identified at all calcium concentrations under phosphorus deficient conditions. Yet, under phosphorus sufficient conditions different species were identified in control reactor as compared to 5 meq/L and 15 meq/L concentrations.

Key words: Activated sludge, calcium, phosphorus deficiency, sludge bulking, settleability

ÖZ

KALSİYUM İYONUNUN FOSFOR EKSİK BESİ ORTAMINDA AKTİF ÇAMURUN BİYOKİMYASAL VE FİZİKSEL ÖZELLİKLERİNDEKİ ROLÜ

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Besiyeri ve katyonların aktif çamur karekteristiği üzerinde önemli etkisi vardır ve bu nedenle prosesin tüm verimliliğine etki etmektedir. Fosforun yetersiz olduğu ortamda çamurun özelliklerinin ve kalsiyum iyonunun etkisinin belirlenmesi amacıyla iki farklı fosfor konsantrasyonunda (KOİ/N/P=100/5/0.05 ve KOİ/N/P=100/5/1), üç farklı kalsiyum konsantrasyonunda (0.5, 5, 15 meq/L) 8 günlük çamur yaşında ve 2 L'lik hacimde reaktörler çalıştırılmıştır. Sonuçlar göstermektedir ki, hücre dışı polimerik maddelerin (HDP) miktarı ve kompozisyonu kalsiyum ve fosfor konsantrasyonuna bağlıdır. En yüksek kalsiyum konsantrasyonu hariç, fosfor konsantrasyonundaki artış sonucu toplam HDP üretiminde de artış olmuştur. Fosfor yetersiz durumda, kalsiyum iyonları karbonhidrat tipi polimer üretimini teşvik etmiştir ve filamentli olmayan çamur şişmesi gözlenmiştir. Ancak fosfor konsantrasyonundaki artış protein tipi polimer üretimini teşvik etmiş ve çamur şişmesi problemi giderilmiştir. Kalsiyum iyonun artırılması her iki durumda da iletkenliği artırmıştır fakat fosfor konsantrasyonundaki artış, kalsiyum iyonlarının yumak yapısına daha çok katılması sebebiyle iletkenliğin düşmesine neden olmuştur. Fosfor konsantrasyonundaki artış, çamurun çökelebilirliğini, susuzlaştırılabilirliğini ve reolojisini kolaylaştırmıştır. Ayrıca, fosfor yeterli durumda çıkış suyunun bulanıklığı azalmıştır ve KOİ giderim verimleri bütün kalsiyum konsantrasyonları için % 95'in üzerinde tespit edilmiştir. Mikroskopik analizler fosfor yetersiz durumda yumakların zayıf, dağınık ve dirençsiz olduğunu göstermiştir. Fosfor konsantrasyonundaki artış yumak yapısında iyileşmeye neden

olmuştur. Fosfor yetersiz durumda bütün kalsiyum konsantrasyonlarında aynı *Enterobacter* ve *Citrobacter* türleri tespit edilmiştir. Fakat fosfor yeterli ortamda kontrol reaktöründe 5 meq/L ve 15 meq/L konsantrasyonundakilere göre farklı türler tespit edilmiştir.

Anahtar kelimeler: Aktif çamur, kalsiyum, fosfor yetersizliği, çamur şişmesi, çökelebilirlik

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LIST OF ABBREVIATIONS

CER	: Cation exchange resin
CGY	: Casitone-glycerol-yeast extract
C/N	: Carbon to nitrogen ratio
COD	: Chemical oxygen demand
CST	: Capillary suction time
DNA	: Deoxyribo nucleic acid
DO	: Dissolved oxygen
EDTA	: Ethylenediaminetetraacetic acid
EGTA	: Ethylene glycol tetraacetic acid
EPS	: Extracellular polymeric substances
EPSc	: Carbohydrate constituent of EPS
EPSp	: Protein constituent of EPS
MLSS	: Mixed liquor suspended solids
MLVSS	: Mixed liquor volatile suspended solids
NTU	: Nephelometric turbidity unit
PBS	: Phosphate buffer saline
SRF	: Specific resistance to filtration
SRT	: Sludge residence time
SVI	: Sludge volume index
TKN	: Total kjeldahl nitrogen
VSS	: Volatile suspended solids
ZSV	: Zone settling velocity

CHAPTER 1

INTRODUCTION

Activated sludge system is the most widely used method for biological wastewater treatment system. In activated sludge system, organic matter is biologically oxidized to carbondioxide, water and new microorganisms. The efficiency of the whole system is highly dependent on the solid-liquid separation. Settleable solids and suspended biomass are separated from wastewater by bioflocculation and gravity settling in the sedimentation tank.

Activated sludge flocs are made up of microorganisms that are embedded in a polymeric network including polysaccharides, proteins, lipids and nucleic acids. Bacteria, fungi, protozoa and viruses are the main biological groups of activated sludge (Gray, 2004). However, heterotrophic, with a lesser extent, autotrophic bacteria are the most common and effective group of microorganisms in activated sludge (Ganczarczyk, 1983). By providing backbone for the flocs, filamentous microorganisms support the floc structure and flocs become larger (Sezgin, 1977; Sezgin *et al.*, 1978). On the other hand, excessive amount of filamentous organisms lead to the formation of very loose and large flocs. As a result settling and compaction of sludge deteriorates and sludge bulking is observed (Jenkins *et al.*, 2004).

The extracellular polymeric substances (EPS) are accepted as the one of the most common component of the floc structure which gives functional and structural integrity to flocs (Li and Ganczarczyk, 1990; Pavoni *et al.*, 1972; Harris and Mitchell, 1973; Horan and Eccles, 1986; Urbain *et al.*, 1993). A great number of studies show that EPS comprise many different types of macromolecules and biopolymers including proteins, humic compounds, lipids, acidic and neutral polysaccharides, and nucleic acids (DNA and RNA) (Pavoni *et al.*, 1972; Kiff, 1978; Sakka and Takahashi,

1982; Goodwin and Forster, 1985; Urbain, *et al.*, 1993; Frolund *et al.*, 1996; Palmgren and Nielsen, 1998). These studies were mainly focused on the determination of amount and composition of EPS so as to identify the effect of EPS on sludge characteristics.

Yet, there are many factors that influence the EPS production and bioflocculation process including physical, chemical and biological factors and therefore in literature there are conflicting results about the relative amounts and composition of EPS (Brown and Lester, 1979; Frolund *et al.*, 1996; Higgins and Novak, 1997a,c).

Nutrient amount in the feed medium has a critical role in bioflocculation. Hoa *et al.* (2003) reported that protein concentration was unaffected by phosphorus but there was a negative proportionality between protein concentration and nitrogen. Moreover, phosphorus depletion led to increase in carbohydrate concentration and settling and dewatering properties of sludge deteriorated. Turtin *et al.* (2006) found that under phosphorus deficient growth conditions, excessive amount of carbohydrate type polymers were produced and viscous bulking was observed at high calcium ion concentrations.

McKinney (1952) found that the probability of bacteria sticking together was improved by the adsorption of cations, since adsorbed cations reduce bacterial surface charge. Tezuka (1969) reported the role of divalent cations in flocculation and calcium and magnesium were recorded as important cations for bioflocculation process. McKinney (1952) and Tezuka (1969) first developed the divalent cation bridging theory (DCB). According to this theory, a bridge is formed between divalent cations and negatively charged functional groups within EPS and this bridge formation enhances bioflocculation (Sobeck and Higgins, 2002). The displacement of divalent cations from binding sites resulted in deterioration in the floc properties (Higgins and Novak, 1997a). Sodium, potassium, calcium, magnesium, ammonium, iron and aluminum are the major cations found in activated sludge and flocculation ability of divalent and trivalent cations are generally recorded as better than monovalent ones (Park, 2002).

In literature there are many studies about the role of cations in activated sludge properties. However, due to the variation in the operational conditions and feed medium the results are inconsistent with each other. Moreover, most of the studies were conducted as batch experiments with short duration and mono culture bacteria were used in those experiments. In addition this, the effect of cations on sludge properties in nutrient deficient conditions and microbial properties of sludge under these conditions has not been thoroughly understood yet.

In the light of these findings, the aim of this study is to determine the physical, chemical and microbiological properties of activated sludge grown in the phosphorus deficient medium and the effect of calcium ions. In order to achieve this aim, reactors were operated under phosphorus sufficient and deficient conditions with three different calcium concentrations. As the reactors reached steady state conditions; physical analyses related to settleability, dewaterability and rheology; chemical analyses including chemical oxygen demand (COD), extracellular polymeric substances (EPS), electrical conductivity and microbiological analyses were conducted.

CHAPTER 2

LITERATURE REVIEW

2.1. History and Principles of Activated Sludge System

Activated sludge system is the most widely used continuous or semicontinuous (sequencing batch reactors) aerobic method for biological wastewater treatment system. The working principle of the activated sludge system is based on biological oxidation of dissolved organics to carbondioxide, water and new microorganisms. Furthermore, colloidal biodegradable organics can be sorbed and used by the microorganisms as well (Ganczarczyk, 1983).

The activated sludge process dates back to early 1880s by investigation of aeration of wastewater. However, the invention of activated sludge process dated to 1912 from the experiments of Clark and Gage. During their experiments they realized that by aeration of the wastewater, sludge was produced which facilitates treatment of wastewater. Ardern and Lockett proposed a fill-and-draw system in 1914 that included recycle of suspension formed during aeration. The process was called as *activated sludge* since active group of microorganisms were involved in the treatment of wastewater (Ganczarczyk, 1983; Tchobanoglous *et al.*, 2003).

The activated sludge process, as shown in Figure 2.1, is made up with two basic components. The first one is a reactor (aeration tank) and the second component is a sedimentation tank. In the aeration tank microorganisms are kept in suspension and aerated. While mixing, microorganisms collide with each other and stick together to form larger particles which are called flocs. By colliding with suspended and colloidal materials, flocs grow larger and they can settle easier than individual cells. After aeration process, flocculant settleable solids and suspended biomass are separated from wastewater by bioflocculation and gravity settling in the

sedimentation tank. In order to sustain adequate amount of biomass for adsorption of organic materials in the aeration tank, a portion of settled microorganisms in the sedimentation tank is recycled back to the aeration tank and excess sludge is transferred to separate sludge handling processes. The effluent from sedimentation tank is transferred to facilities for further treatment depending on the properties of receiving body (Tchobanoglous *et al.*, 2003).

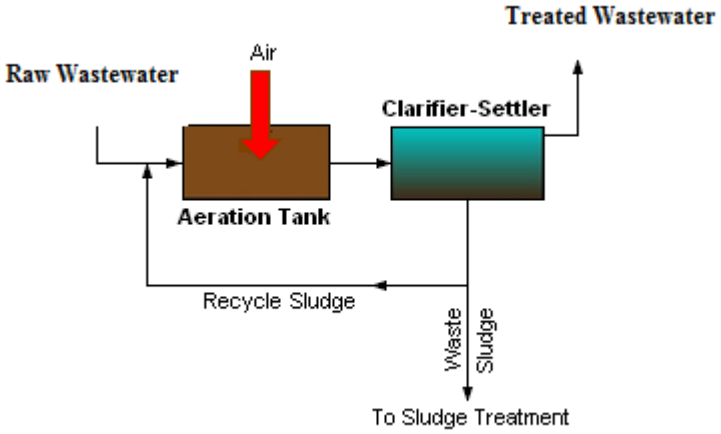

Figure 2.1. Schematic representation of activated sludge process

2.2. Microbiology of Activated Sludge Systems

As being a biological treatment system, the basic principle behind activated sludge system is the oxidation of organics by a mixed culture of microorganisms, thus forming a complete ecosystem with different trophic levels. Main biological groups of activated sludge system are bacteria, fungi, protozoa, and viruses. Algae are also present in the mixed liquor but rarely become established (Gray, 2004).

Despite consisting wide variety of microorganisms, heterotrophic, with a lesser extent, autotrophic bacteria are the most common microorganisms in activated sludge. Heterotrophic bacteria utilize organics as a source of both carbon and energy including genera such as *Pseudomonas*, *Achromobacter*, *Flovabacterium*,

Bacillus, *Alcaligenes*, *Arthrobacter*, *Micrococcus*, *Citromonas*, and *Zooglea*. On the other hand authotrophic bacteria oxidizes mineral compounds for energy requirements and utilize carbondioxide as a carbon source, including genera such as *Nitrosomonas* and *Nitrobacter* (Ganczarczyk, 1983). The major genera in the activated sludge flocs are *Zooglea*, *Pseudomonas*, *Flavobacterium*, *Alcaligenes*, *Achromobacter*, *Corynebacterium*, *Comomonas*, *Brevibacterium*, *Acinetobacter* and *Bacillus* spp. (Bitton, 2005).

Regarding to the reaction to gram stain prokaryotic cells is divided into two groups, Gram-positive and Gram-negative. Majority of activated sludge bacteria are belong to Gram-negative group. The difference originates from the structure of cell wall. Both Gram-positive and Gram-negative bacteria have cell walls that contain peptidoglycan. Fifty to ninety percent of the dry weight of the Gram-positive wall is composed of peptidoglycan. This thick peptidoglycan layer is responsible for the retention of the crystal violet iodine complex. However, for gram negative bacteria 10 percent or less of the weight of cell wall is made up of peptidoglycan. In addition to this, the Gram-negative cell wall contains protein as well as a lipopolysaccharide and lipoprotein layer (Gaudy and Gaudy,1980).

The flocs will be roughly spherical in shape when they are built up by relatively spherical microorganisms through biopolymer bridging. Filamentous organisms provide networks or backbones for the flocs. By this way, irregularly shaped flocs can be seen in activated sludge and these backbones allow the floc to grow larger (Sezgin, 1977; Sezgin *et al.*, 1978). While providing a backbone for the flocs, an overabundance of filamentous organisms can cause activated sludge bulking. There will be deterioration in the settling and compaction of the activated sludge due to the development of very loose and large flocs (Jenkins *et al.*, 2004). The most common filamentous microorganisms associated with activated sludge bulking are *Sphaerotilus natans*, *Geotrichum*, *Beggiatoa*, and *Thiotrix* (Ganczarczyk, 1983).

Fungi are multicellular, nonphotosynthetic, heterotrophic eukaryotes and rarely represented in activated sludge. They become predominant as the bacteria are

inhibited when the pH is lowered to below 6.0 during the treatment of certain acidic wastewaters. *Geotrichum candidum* and *Trichosporon* sp. are the most abundant types (Gray, 2004).

Protozoa are motile, microscopic eukaryotes that are usually single cells and most of them are aerobic heterotrophs. They are commonly found microorganisms in activated sludge which can represent as much as 5 to 12 percent of the dry weight of mixed liquor (Gray, 2004). The major groups of protozoa are flagellates, motile ciliates, stalked ciliate, amoebae, rotifers and nematodes (Ganczarczyk, 1983). As being bacterial feeders, protozoa have an important role in the purification process like bacteria. This property causes two important effects. Firstly, for both dispersed and floc forming, bacteria it regulates bacterial density; in other words prevent bacteria to reach self-limiting numbers which causes increase in bacterial activity when there is no food limitation as well as reducing the overall bacterial biomass. Secondly, the effluent is clarified from suspended material by protozoa. Curds and Fey (1969) demonstrated in their studies by using *Escherichia coli* that protozoa feed on pathogenic bacteria. Floc forming bacteria and dispersed bacteria may be in competition for the soluble substrate under certain food-limiting conditions. The protozoans will reduce the competition by feeding on dispersed bacteria and enhance to produce larger flocs that will improve settleability and reduce suspended material in the effluent (Downing and Wheatland, 1962; Curds *et al.*, 1968). Besides, because of being strict aerobes, protozoa are the indicator of aerobic environments (Water Environment Association, 1987).

Nematodes and rotifers represent a minor part of microbiology of activated sludge. As there is no suitable niche in aeration tank and the population doubling time is so long, nematodes are not commonly found in mixed liquor. However, rotifers are far more common than nematodes (Gray, 2004).

Viruses are made up of a nucleic acid (either DNA or RNA) and a surrounded outer shell of protein (i.e. capsid). A wide variety of viruses are excreted by humans and they can be found in activated sludge. The most important ones are: enteroviruses,

Norwalk viruses, rotaviruses, reoviruses, caliciviruses, adenoviruses and hepatitis A virus (Tchobanoglous *et al.*, 2003).

2.3. History of Bioflocculation

Bioflocculation can be taken as an intermediate step between the microbial growth in the bioreactor and settling. In this context, bioflocculation carries its inevitable importance for the efficiency of treatment process.

Early investigations related to bioflocculation were mainly based on the microscopic observations and isolation of the predominating species (Gaudy and Gaudy, 1980). After such studies Butterfield (1935) stated that floc forming bacteria *Zooglea ramigera*, a microorganism that is highly present in activated sludge, was the cause of flocculation. However, McKinney and Horwood (1952) demonstrated that various species other than *Zooglea ramigera* in sewage were able to form floc and therefore flocculation was not directly related to the presence of *Zooglea ramigera*.

McKinney (1952) proposed a theory related to floc formation. Theory was stated that capsulated bacteria are flocculated by direct chemical interactions between adjacent cells. The net surface charge is reduced through adsorption of cations by negatively charged bacteria that facilitates the cells to come closer. This approach to bioflocculation was rejected by Pavoni *et al.* (1972) by demonstrating floc forming abilities of non-capsulated bacteria. However, Tezuka (1969) supported the approach of McKinney. Despite working with non-capsulated bacteria, Flavobacterium, Tezuka (1969) noticed that metallic cations (calcium and magnesium) interacts with negatively charged surfaces of cells resulted as charge neutralization and form bridges between cells for bioflocculation.

The predominant mechanism of bioflocculation has been explained as neutralization of cell surface charge regardless of the cells inorganic and organic nature. However, charge neutralization cannot be considered as the main mechanism of bioflocculation since in activated sludge processes extracellular polymers secreted

by cells play a major role in bioflocculation by charge neutralization and/or act as a bridge (Busch and Stumm, 1968; Harris and Mitchell, 1973; Treweek and Morgan, 1977).

It is now accepted that activated sludge flocs are made up of microbial colonies embedded in a cloud of extracellular polymers (Wilen *et al.*, 2008).

2.4. Extracellular Polymeric Substances

It is accepted that extracellular polymeric substances (EPS) is the third most common components of sludge flocs following microorganisms and water (Li and Ganczarczyk, 1990) and the flocs' structural and functional integrity is given by EPS (Pavoni *et al.*, 1972; Harris and Mitchell, 1973; Horan and Eccles, 1986; Urbain *et al.*, 1993). There are three sources that contribute to EPS namely; metabolic synthesis, cell lysis and adsorption of pollutants from wastewater. EPS contribution originated from metabolic synthesis is related to the environmental, operational and microbial conditions. Contribution from cell lysis is associated with the physiological conditions and extraction method. In reference to the composition and properties of wastewater, adsorption of pollutants and therefore EPS content and contribution may change (Liao, 2000). Products from metabolic synthesis are polysaccharides and proteins while the lysed products can be listed as proteins, polysaccharides, lipids, and nucleic acids. Polymeric substances from wastewater are humic acids and other introduced synthetic and organic polymers (Murthy, 1998).

EPS produced from metabolic synthesis can be classified in two groups; tightly bound (capsular) and loosely adhered (slime type) or free dissolved matter (Bhaskar and Bhosle 2005). Slime type is unattached or loosely attached to sludge surfaces. However, capsule layer is directly and tightly attached to the exterior of cell wall (Liao, 2000) either by linkages between carboxyl groups of EPS and hydroxyl groups of lipopolysaccharides (Sutherland, 1977) or by a covalent bonding between phospholipids (Roberts, 1996) and glycoproteins (Chester and Murray, 1978). Its polymeric structure is more organized and densely packed as compared to slime

type (Bhaskar and Bhosle, 2005). Slime type does not take part in sludge bioflocculation as they are almost free from the cell. However, capsular material plays an important role in bioflocculation (Gehr and Henry, 1983).

Three dimensional, gel-like, highly hydrated and often charged biofilm matrix is formed by EPS. Within this biofilm, the percentage of EPS varies from 50 to 90 of the total organic matter (Flemming and Wingender, 2001). EPS produced by bacteria provides cells to compete in a variety of natural environments (Neidhardt *et al.*, 1990) and provide protection against phagocytosis, amoebic attack, bacteriophages, dessication, biocides; aid dispersal of cells in aquatic environments and aid in the uptake of ions (Wilkinson, 1958; Liu and Fang, 2003).

2.4.1. Composition of EPS

A great number of studies show that EPS comprise many different types of biopolymers including proteins, humic compounds, lipids, acidic and neutral polysaccharides, and nucleic acids (DNA and RNA) (Pavoni *et al.*, 1972; Kiff, 1978; Sakka and Takahashi, 1982; Goodwin and Forster, 1985; Urbain, *et al.*, 1993; Frolund *et al.*, 1996; Palmgren and Nielsen, 1998). Polysaccharides have often been regarded as the major component of EPS (approximately 60% of dry solids content) (Wingender *et al.*, 1999) and therefore most of the research has focused on the extracellular polysaccharides (Forster, 1971; Horan and Eccles, 1986; Bejar *et al.*, 1998). However, many studies have also shown that extracellular proteins were in significant amounts even greater than extracellular polysaccharides (Tenney and Verhoff, 1973; Brown and Lester, 1980; Barber and Veenstra, 1986; Eriksson and Alm, 1991; Urbain *et al.*, 1993; Higgins and Novak, 1997a, b; Jorand *et al.*, 1998).
Proteins can be found as several classes in bacterial exocellular environment including extracellular enzymes, proteinaceous S-layers, lectins, intracellular protein from cell-lysis or cell wall turnover or polypeptide capsular material. The exocellular protein extracted from an activated sludge sample can be a combination of these sources. Among these sources of proteins, lectins are one of the most possible types that take part in bioflocculation (Higgins and Novak, 1997c). Lectins are

carbohydrate-targeting proteins. They take part in bacterial agglutination and attachment and many of them are bound with a carbohydrate component in glycoproteins and (lipo)polysaccharides by the aid of divalent cations (Imberty *et al.*, 2004). Besides, lectins can be found in appendages (e.g. pili and fimbriae) of bacteria (Higgins and Novak, 1997c).

Aminoacid sequencing analysis of Higgins and Novak (1997c) reveals that the extracellular protein exhibits lectin like activity and includes carboxyl containing groups like aspartate (asx) and glutamate (glx) that may be involved in the binding of divalent cations. Moreover, extracted protein consists of a high proportion of aminoacids with hydrophobic groups like glycine (gly) and alanine (ala). This hydrophobic property results in the involvement of the formation of hydrophobic bonds which are in relation with electrostatic bonds in floc structure (Urbain *et al.*, 1993).

Proteins were found to be the major constituent of EPS by the pyrolysis/GC/MS analysis in the study of Dignac *et al.* (1998). Main fragments gathered from analysis were characteristic of proteins such as pyridine, methylpridine, styrene, pyrrole, methylprrole, benzonitrile, indole and methylindole. Furaldehyde and methylfurfural presence were related to neutral sugars. The presence of furfuryl alcohol was related to presence of nucleic acids. The acetamide peak represents the presence of aminosugars in EPS. Same study determined that carboxyl containing groups of aminoacids (e.g. aspartic acid) comprises a great portion of aminoacids.

Wilen *et al.* (2003) extracted EPS from seven wastewater treatment plants. Protein was found as the major component of EPS in most of the samples changing between 19-45% and carbohydrates follow the protein percentage (7-32%). 1-3% of EPS are made up of uronic acid.

Guibaud *et al.* (2005) study related to the metal complexation potential of EPS extracted from activated sludge and from eight pure cultures of bacteria isolated from same activated sludge reported that protein was found as the major component of EPS from sludge and pure cultures of bacteria.

Park *et al.* (2008) found that protein concentrations in five different activated sludge extracts obtained by cation exchange resin procedure, base extraction and sulfide treatment are greater than polysaccharide concentrations. For all samples and extraction procedures, protein to polysaccharide ratio is greater than or equal to two.

Tago and Aida (1977) revealed that mucopolysaccharide which was made up of glucosamine, glucose, mannose, galactose and rhamnose and comprised 10% of the total polysaccharide isolated contributed to the floc formation. The remaining 90% of the polysaccharide did not take part in floc formation.

Horan and Eccles (1986) characterized and purified exopolysaccharide fractions from five different effluent treatment works. Monomer composition and molecular weight distribution of the samples showed many similarities. Only five monomers were detected by them namely; glucose, galactose, mannose, gluconic acid and galactronic acid and all the polysaccharide fractions were of high molecular weight changing from 300000 to 2000000.

Hejzlar and Chudoba (1986) isolated polymers that were formed during growth, starvation and decomposition stages of microorganisms. All isolated polymers contain sugars, amino sugars, uronic acids and aminoacids that indicates their heteropolysaccharidic character.

Exopolysaccharide are rarely used as a source of carbon and energy by microorganisms. Curdlan, a high molecular weight polymer of glucose, producing bacteria *Cellulomonas flavigena*, is one of the rare microorganism that produces an extracellular enzyme capable of degrading the EPS to utilizable products (Voepel and Buller, 1990).

Bejar *et al.* (1998) characterized the exopolysaccharides produced by *Halomonas eurihalina* species. It was found that, EPS from all strains include uronic acids and hexoamines. Moreover, study revealed that the EPS from *H. eurihalina* strains show similar results regarding to the neutral sugar composition. Carbohydrates are the major constituent of EPS by comprising 31-44% of total dry weight of EPS.

Uronic acids present in polysaccharides benefit bioflocculation through charge bridging with divalent cation by providing polyanionic nature to polysaccharides (Murthy, 1998).

Hung *et al.* (2005) isolated extracellular polysaccharides from *Pseudomonas fluorescens* Biovar II. Up to 70% of total carbohydrates were detected as uronic acids. The composition of EPS in both particulate and dissolved fractions includes rhamnose, fucose, arabinose, ribose, xylose, mannose, galactose and glucose. Galactose, arabinose and mannose are three major neutral sugars.

Subramanian *et al.* (2010) extracted and purified slime EPS from six selected pure bacterial strains belong to *Pseudomonas serratia*, *Bacillus*, *Microbacterium*, *Enterobacter* sp. and consortium of these microorganisms. Except the bacterial consortium, in all cases total carbohydrate of EPS is higher than total protein of EPS.

2.4.2. Factors Affecting Production of EPS

There are many factors that influence the EPS production and bioflocculation process including physical, chemical and biological factors. Nutrient in the feed wastewater is an important factor that affects the EPS, both composition and concentration during biodegradation of wastes (Bura *et al.*, 1998). C/N ratio of a typical municipal wastewater varies between 25/1 and 20/1 where carbon represented by COD and N represented by ammonium ion (Gaudy and Gaudy, 1980). Durmaz and Sanin (2001) revealed that when C/N ratio increases microorganism concentration and EPS produced increases too. Furthermore, high

C/N ratios favor production of carbohydrate type EPS rather than proteins. On the other hand, in literature there are contradictory results about the impact of mean cell residence time on EPS production. At high MCRTs (i.e. when endogenous metabolism predominates) amount of EPS extracted would be higher (Pavoni *et al.*, 1972; Cha *et al.*, 1979). Sanin *et al.* (2006) stated that total amount of EPS increases with increase in MCRT and protein becomes the predominant type of polymer. However, Liao *et al.* (2001) found that total amount of EPS is independent of MCRT.

EPS production and composition is highly affected by the type of microorganisms. Brown and Lester (1980) reported that hexose sugar concentrations were much higher in the *Klebsiella aerogenes* culture than in the activated sludge samples. In another study, Subramanian *et al.* (2010) extracted EPS directly from sludge, bacterial consortium and individual bacterial strains. EPS produced by bacterial consortium was found to be greater than EPS produced by individual bacterial strains. When they grow in consortium, they failed to produce an equivalent quantity of EPS which is related to the individual cell metabolism. Bejar *et al.* (1998) indicated that chemical composition of EPS produced by *Halomonas eurihalina* differs from strain to strain. Garnier *et al.* (2006) identified, isolated and cultured *Enterobacter* and *Klebsiella* originated from the paper mill sludge. Study revealed that EPS produced by pure *Enterobacter* and *Klebsiella* strains were higher than the combination and *Enterobacter* was predominant in terms of EPS produced.

Growth phase of bacteria is another factor that affects the production of EPS. Pavoni *et al.* (1972) noted that biological flocculation does not occur until the microorganism entered the endogenous phase. Flocculation predominates in the endogenous phase related to a decrease in cell dry weight accompanied by the release of polymers (Vallom and McLoughlin, 1984).

In contrast to these observations, the flocculant production of *Zooglea MP6* began in the middle exponential phase and the maximum flocculating activity of *Alcaligenes*

latus occur in the exponential phase and start to decrease at the end of stationary phase (Unz and Farrah, 1976; Tago and Aida, 1977).

As being composed of protein, lipid and polysaccharides; temperature changes affect EPS. Changes in temperature alter the structure of proteins and lipids which cause changes in cell membrane, EPS structure and functioning. Furthermore viscosity of EPS decreases at high temperatures that may lead to deterioration of bioflocculation (Çetin and Sürücü, 1990).

In wastewater treatment processes the pH range for growth is given between 4 and 9 and the typical growth pH for heterotrophic bacteria is 7 (Benefield and Randall, 1980; Brock and Madigan, 1991). Increasing pH above the isoelectric point of bacteria results in an increase in the number of available reactive sites on cell surfaces and on EPS which affects the bioflocculation ability positively (Pavoni *et al.*, 1972; Çetin and Sürücü, 1990).

DO concentration and cation concentration in the wastewater are the other factors that influence the production of EPS. Starkey and Karr (1984) reported that during low DO concentration EPS concentration is approximately 32% lower. Moreover, the carbohydrate to protein ratio of EPS increases with the increase in DO which results in deterioration in the settleability of the activated sludge (Hoa, 2002). Various studies revealed that metal ions enhance bioflocculation by binding EPS (Higgins and Novak, 1997a,b,c; Murthy and Novak, 1998; Sanin and Vesilind, 2000). Turtin *et al.* (2006) reported that calcium ions had some stimulating effects on the production of carbohydrate type polymers.

The relationship between solids retention time (SRT) and EPS and sludge characteristics have been studied heavily. Molecular weights of the polymers produced are closely related to the SRT. At low SRTs low molecular weight polymers are produced. By increasing SRT, bacterial cells produce high molecular weight polymers which are more favorable for flocculation. Ericsson *et al.* (1992) stated that as the sludge age increases, EPS produced and surrounded bacterial

cells increases too. Murthy (1998) operated reactors at SRTs ranging from 5 to 50 days. Solution polysaccharide concentration increased with an increase in SRT and solution protein concentration increased in the effluent when SRT increased to above 10 days. In contrast to these findings, there are also contradictory results in relation to the effect of SRT on EPS production. The results indicated that changes in SRT have little effect on EPS production. However, composition of EPS changes with different SRT operations (Liao *et al.*, 2001; Sesay and Sanin, 2004).

Quantification of EPS is also strongly dependent upon the extraction methods (Wingender *et al.*, 1999). Physical extraction methods include centrifugation (Brown and Lester, 1980), blending (Gehr and Henry, 1983), sonication (Urbain *et al.*, 1993; Dignac *et al.*, 1998) or steaming (Rudd *et al.*, 1983). Chemical extraction methods include uses of aminoacids and bases (Brown and Lester, 1980; Rudd *et al.*, 1983), chealating agents such as EDTA (Brown and Lester, 1980; Eriksson and Alm, 1991), EGTA (Bruus *et al.*, 1992; Sanin and Vesilind, 2000), cation exchange resin (CER) (Frolund *et al.*, 1996; Liao *et al.*, 2001; Durmaz and Sanin, 2001) and combination of some of these methods.

Brown and Lester (1980) observed that high-speed centrifugation was the most effective extraction method for the *Klebsiela aerogenes* culture and steaming treatment was noted as the most effective EPS extraction method for activated sludge due to less cellular disruption than EDTA and NaOH treatments.

Frolund *et al.* (1998) applied CER procedure for the extraction of EPS. Regarding to the yield of EPS and minimal disruption of exopolymers, CER was considered as more efficient method than thermal heating and sodium hydroxide extraction methods. In addition to this, enzyme extraction method yields lower amount of polymers as compared to CER method (Sesay *et al.*, 2006).

Liu and Fang (2002) compared the effectiveness of six extraction procedures for EPS. For each gram of volatile solids formaldehyde-NaOH process extracted the highest yield of EPS which was about two to three times more than CER process.

EPS are generally characterized by measuring proteins, carbohydrates, lipids and nucleic acids using colorimetric methods and characterization of EPS extracted varies from one measurement method to another due to the interferences. Colorimetric protein measurement methods Lowry, Bicinchononic acid and Bradford (Lowry *et al.*, 1951; Bradford, 1976; Smith *et al.*, 1985) can be subjected to interferences with other organic compounds in the sludge (Ras *et al.*, 2008).

Studies of Frolund *et al.* (1996) and Durmaz and Sanin (2001) showed that Bradford method gives lower EPS protein content. Moreover, standards used in colorimetric methods may not be representative of the complexity of the organic matrix (Dignac *et al.*, 1998).

2.5. Mechanism of Bioflocculation

Activated sludge flocs are made up of microorganisms that are embedded in a polymeric network including polysaccharides, proteins, lipids and nucleic acids. As being one of the most critical prerequisite of solid/liquid separation, several researches have been carried out about the mechanism of bioflocculation.

The mechanisms of floc formation in literature can be listed as *Zooglea ramigera* theory (Butterfield, 1935; Heukelekian and Litman, 1939), filament backbone model (Parker *et al.*, 1971; Sezgin *et al.*, 1978), polymer bridging model (Tenney and Stumm, 1965; Busch and Stumm, 1968), double layer compression theory (DLVO theory) (Zita and Hermansson, 1994), metal ion bridging theory (McKinney, 1952; Tezuka, 1969; Kakii *et al.*, 1985; Eriksson and Alm, 1991; Bruus *et al.*, 1992), gel formation theory (Bruus *et al.*, 1992; Sanin and Vesilind, 1996), and hydrophobic interactions (Urbain *et al.*, 1993; Jorand *et al.*, 1994).

In the *Zooglea ramigera* theory, this group of bacteria, which produces a gelatinous matrix, was accepted as the predominant bacteria in activated sludge and responsible for flocculation (Butterfield, 1935; Heukelekian and Litman, 1939). However McKinney and Horwood (1952) and McKinney and Weichlein (1953)

isolated further floc-forming bacteria other than *Zooglea ramigera* and stated that flocculation is not solely dependent on the presence of that bacteria.

Parker *et al.* (1971) stated that filamentous bacteria provide a backbone for the attachment of floc forming microorganisms. The filamentous backbone theory (Sezgin *et al.*, 1978) suggests that the structure of activated sludge floc is formed at two levels, microstructure and macrostructure. Microstructure is associated with bioflocculation of floc forming bacteria and these flocs are small, spherical, compact but mechanically weak. In contrast to microstructure, formation of macrostructure is provided by filamentous microorganisms through forming a backbone within the floc where floc formers are attached to this backbone by extracellular polymers (In Chio Lou, 2006).

The polymer bridging model (Tenney and Stumm, 1965; Busch and Stumm, 1968) was based on the idea of electrostatic surface charge reduction to zero. Polymers of high molecular weight may absorb on surfaces from solutions and through this way parts of the polymer chain extend into the solution which gives the possibility that parts of the same polymer chain can absorb on different particles, leading to polymer bridging (Mara and Horan, 2003). The adsorption on solid surfaces is triggered by the close contact of free ends of polymer segments with cell. As a result of the physical interaction of these segments, relatively strong chemical or electrochemical bonds can be formed (Ganczarczyk, 1983).

Another theory in relation with bioflocculation is DLVO theory named after its developers Derjaguin, Landau, Verwey and Overbeek and known as double layer model. This theory is a classical colloidal theory in which the surface charge of the particles and the counter-ion charge in the solution form a double layer surrounding the particle. Due to the attraction of the counter-ions in solution by colloid, a firm layer around the surface of the colloid is formed which is known as *Stern* layer. And the formation of second layer (called as diffuse layer) is the result of repulsive forces. Near the surface of the particle, the concentration of the counter-ions are at maximum level and starts to decrease with distance from the particle surface until

the concentrations of positive and negative ions become equal. As a result, an electrical potential develops around the particle. Due to the repulsion of adjacent particles, aggregation is inhibited. Aggregation can be promoted by increasing ionic strength. By this way the repulsion between particles decrease and short range Van der Waals attractive forces become effective. Therefore addition of cations result compression of the double layer and improve bioflocculation (Nguyen *et al.*, 2007). Cousin and Ganczarczyk (1999) reported that addition of sodium increased floc size. Zita and Hermansson (1994) indicated that solution ionic strength is increased by addition of any ions and reported that potassium and calcium have positive effects on particle stability. However, Sobeck and Higgins (2002) showed that the monovalent cation addition resulted deterioration in settling and dewatering properties of sludge due to negative effect of monovalent cations on double layer compression.

McKinney (1952) reported that the adsorption of cations resulted in a reduced bacterial surface charge and enhanced the probability of bacteria sticking together. Sooner, Tezuka (1969) demonstrated the role of divalent cations in floc formation and reported that calcium and magnesium were important cations for bioflocculation processes. Under the light of these studies, McKinney (1952) and Tezuka (1969) were the first researchers that proposed divalent cation bridging (DCB) theory. DCB theory is based on the bridge that is formed between divalent cations and negatively charged functional groups within EPS as shown in Figure 2.2. Such a bridge formation provides aggregation and stabilization of matrix of biopolymer and enhances bioflocculation (Sobeck and Higgins, 2002). The study of Higgins and Novak (1997a) also supported the DCB theory by demonstrating the deterioration in the floc properties because of displacement of divalent cations from binding sites within the floc.

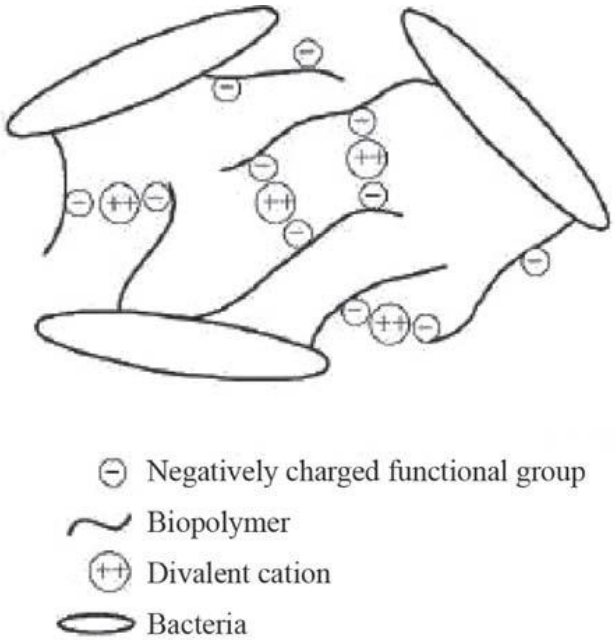

Figure 2.2. Representation of divalent cation bridging in the floc matrix (Nguyen *et al.*, 2007)

Alginate theory was first proposed by Bruus *et al.* (1992) and suggested that in the presence of calcium ions alginate gel is formed and this gel is referred as egg-box model. Alginate is a polysaccharide that is made up of repeating mannuronic and gluronic acids and it is produced by several bacteria including *Azotobacter sp.* and *Pseudomonas aeruginosa* which suggests that alginate may be present in activated sludge (Sobeck and Higgins, 2002). Bruus *et al.* (1992) reported that as a result to the addition of high concentration of sodium to activated sludge, floc properties deteriorates due to the displacement of calcium within the floc structure. This finding shows that alginate aggregation is specific for calcium (Figure 2.3).

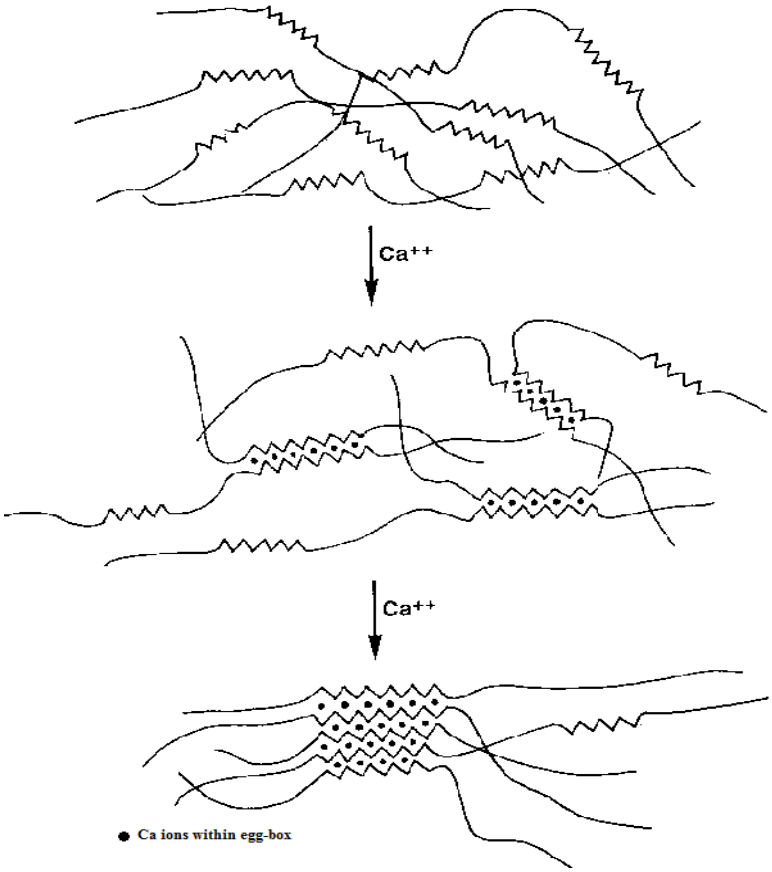

Figure 2.3. Representation of alginate gel formation in the presence of calcium in egg-box model

The shell layer of bound water near the cell surface accounts for hydrophobic/hydrophilic interactions. As two similar surfaces come close to each other, the bound water layers surround the surfaces will overlap and results the displacement of the bound water layer into the bulk water. For the case of hydrophobic surfaces, such displacement promotes a decrease in the free energy. In contrast to the hydrophobic surfaces, for hydrophilic surfaces free energy will increase due to this displacement. As a result, hydrophobic cells favor aggreage formation and hydrophilic cells are found in a dispersed state. Therefore any alteration of the cell surface to increase the surface hydrophobicity promotes the floc formation (Liao, 2000). Urbain *et al.* (1993) indicated that hydrophobic molecules like lipids or proteins can be associated to the floc structure and cell surface shows

hydrophobic areas. It was reported that internal hydrophobicity of sludge particles acts as essential adhesives between the cells. Jorand *et al.* (1998) commented that a great amount of EPS was made up of proteins that possess hydrophobic characteristics because of the presence of aminoacids with hydrophobic groups such as glycine and alanine (Higgins and Novak, 1997c) and stated that the involvement of EPS into the floc cohesion may occur either through the hydrophilic chains provided by polysaccharides creating a matrix where bacteria are attached or through a glue creating bridges or reticular points between polysaccharides provided by hydrophobic heteropolymers.

2.6. Effects of Nutrients and Cations on Activated Sludge Systems

The effective long term biological treatment can be achieved by satisfying the required amount of nutrients to microorganisms. Several studies about the proper evaluation of these requirements indicate that necessities show differences for different wastewaters and for different methods and degrees of treatment (Ganczarczyk, 1983). The major elements that are used by microorganisms can be listed as C, H, N, O, S and P. Carbon is one of the most required element for microbial growth. Hydrogen and oxygen comprises of many organic compounds. Sulphur is needed for biosynthesis of aminoacids and some vitamins. Nitrogen and phosphorus are very important nutrients by taking part in biosynthesis and energy transfer. Phosphorus is essential for the synthesis of nucleic acids and adenosine triphosphate as well as a component of various membrane phospholipids (Mara and Horan, 2003). Sufficient macronutrient in the wastewater feed is commonly accepted as C/N/P ratio equals to 100/5/1 which is based on the assumption that the net sludge yield is 0.5 gVSS when 1 g of organic matter is removed and the sludge includes 10% N and 2% P on a VSS basis. Nutrient deficiency in activated sludge processes is generally caused by deficient amount of nitrogen or phosphorus on the feed to the system (Jenkins *et al.*, 2004).

Poor settling characteristics of activated sludge can be defined as “bulking” and nutrient deficiency in the feed medium is one of the main reasons of activated

sludge bulking. Nitrogen and phosphorus deficiencies can encourage excessive production of filamentous microorganisms, due to their high resistance to starvation and they become predominant over floc formers. Filamentous microorganisms produce a diffuse floc structure, as a result settling and compaction of activated sludge become very difficult. Under low nitrogen conditions, type 021N and *Thiotrix* spp. may grow while *Sphaerotilus natans*, *Haliscomenobacter hydrossis* and *Nostocoida limicola* may be encountered at low phosphorus conditions (Jenkins *et al.*, 2004; Bitton, 2005).

Nutrients are also holding an important role in the composition and amount of EPS production. Hoa *et al.* (2003) found that protein concentration was inversely proportional to nitrogen while unaffected by phosphorus. Furthermore, both nitrogen and phosphorus were inversely proportional to carbohydrate concentration but phosphorus had a more pronounced affect on carbohydrate than nitrogen. Phosphorus depletion caused an increase in carbohydrate concentration which resulted deterioration in settling and dewatering properties of sludge. Viscous (or Zoogleal) bulking is caused by excessive amounts of EPS production that may be related to zoogleal growth or not. Dispersed and flocculent microbial cells are surrounded by extracellular polymers and poor settling and compaction of activated sludge occurs (Jenkins *et al.*, 2004). Similar findings were reported by Turtin *et al.* (2006). It was shown that phosphorus deficiency resulted in an increase in the carbohydrate concentration and viscous bulking was observed.

In activated sludge systems amount and type of cations in wastewater can affect the performance of the treatment process because of physicochemical interactions between bacteria and cations, ion bridging and ion exchange mechanisms (Ganczarczyk, 1983). Cations become significant structural components since biopolymers are negatively charged; cations provide binding between negatively charged biopolymers where microorganisms can be embedded (Bruus *et al.*, 1992; Urbain *et al.*, 1993; Higgins and Novak, 1997a). Major cations found in activated sludge can be listed as sodium, potassium, calcium, magnesium, ammonium, iron

and aluminum. In literature it is generally accepted that divalent and trivalent cations are better flocculants than monovalent ones (Park, 2002).

Ionic charge and the size of the hydration shield of the cations strongly affect different cations to form cation bridges. Hydration shell can be explained as the water molecules that surround the ion and hydration shell radius is inversely proportional to ionic radius. Cations with high valence and thin hydration shell can easily approach charged surfaces for bond formation through multiple sites of negative charge (Piirtola, 1999).

Table 2.1 below represents the radii and relative flocculation power of sodium, potassium, magnesium and calcium. As it can be observed from this table, for monovalent cations, potassium has lower hydrated radius than sodium and holds higher flocculation ability than sodium. When divalent cations are compared, it can be stated that calcium established stronger bond with negatively charged sites than magnesium since calcium has lower hydrated radius that can be easily losed during bond formation. From this table it can be concluded that calcium is the best flocculator among these cations and sodium is the poorest one.

Table 2.1. Radii and relative flocculation power of cations

Cation	Valence	Ionic Radius (nm)	Hydrated Radius (nm)	Relative Flocculation Power
Sodium	1	0.095	0.79	1.0
Potassium	1	0.133	0.53	1.7
Magnesium	2	0.065	1.08	27.0
Calcium	2	0.099	0.96	43.0

It was reported that when calcium is removed from floc structure using CER, EDTA or EGTA deflocculation occurs and settling and dewatering properties of sludge

deteriorates (Keiding and Nielsen, 1997; Kakii *et al.*, 1985; Bruus *et al.*, 1992). Although representing similar properties, findings indicate that interactions of calcium ion and sludge reduced bound water was remarkable as compared to magnesium ion. When calcium and magnesium solutions were passed through a column of precipitated polymer packed with sand, it was observed that there was a significant removal of calcium ions by the column whereas magnesium ions were not removed (Forster and Lewin, 1972).

Steiner *et al.* (1976) revealed that there were differences between the adsorption of calcium to polymers depending on the form of the polymer. Calcium adsorption by soluble polymers was achieved through salt formation with carboxyl groups. On the other hand, adsorption by solid polymers was explained by an attachment via electrostatic forces to hydroxyl groups present in the hexose or pentose molecules. There are several studies about the interactions between specific EPS and divalent cations. Bruus *et al.* (1992) stated that polysaccharides in biopolymers are alginates and they bind calcium ion in order to form a gel-like floc structure.

Urbain *et al.* (1993) and Dignac *et al.* (1998) reported that proteins have higher affinity for calcium and magnesium than polysaccharides. In addition to this, Sanin and Vesilind (2000) proposed a sludge floc model. In this model, microbial colonies interact with each other through polymer bridges. The role of calcium ion in this model is to take part in flocculation by charge neutralization through bridging of the polymers and specific interactions of polymers for gel-like formation.

Trivalent cations are often found at high concentrations in activated sludge and they have lower solubility than divalent cations (Kakii *et al.*, 1985). Kakii *et al.* (1985) reported that iron and aluminum were not affected by acid treatment of activated sludge as compared to calcium and magnesium, and resulted that iron and aluminum were more strongly associated with sludge matrix than divalent cations. Murthy (1998) proposed that iron has an affinity to biopolymers and enhance dewaterability of the sludge.

Several studies showed that high concentrations of monovalent cations have detrimental effects on floc stability (Bruus *et al.*, 1992; Higgins and Novak, 1997a; Sobeck and Higgins, 2002). Murthy (1998) reported that when sodium hydroxide is used for pH control wastewater treatment, resulting sludge has poor settling and dewatering properties. According to Murthy *et al.* (1998) potassium improved the floc strength and settling property of sludge. However, it caused deterioration in dewatering property and effluent quality.

Higgins and Novak (1997b) reported that the ratio of monovalent to divalent cations have an important effect on sludge characteristics. Several investigations of full and lab scale activated sludges showed that the monovalent to divalent ratio was positively correlated with sludge filterability. The idea behind this ratio is that the displacement of divalent cations in the cation bridge floc structure by monovalent cations through ion exchange and deterioration in floc properties.

2.7. Activated Sludge Properties Related to Bioflocculation

2.7.1. Dewaterability

The efficiency of biological wastewater treatment system depends on the performance of substrate utilization and sludge synthesis, biological flocculation and sedimentation, and sludge dewatering. Substrate utilization and microbial synthesis is related to the characteristics of wastewater and operational conditions. Yet flocculation, and sedimentation of sludge depend on the physiological and biochemical properties of sludge organisms (Wu *et al.*, 1982). It is generally accepted that sludge dewatering is a critical step in sludge handling due to the high sludge water content and huge volumes of daily sludge production (Vesilind, 1988).

Vesilind (1994) defined four water fractions associated with activated sludge floc as: free (bulk) water, interstitial water, vicinal water and water of hydration. Free (bulk) water is independent of suspended solid particles. Interstitial water is the water trapped in the interstitial spaces of flocs and microorganisms. Vicinal water can be

defined as the multiple layers of water molecules held tightly to particle surface through hydrogen bonding. And vicinal water is not free to move when physical confinement is removed as compared to interstitial water. Water of hydration is chemically bound to the particles and cannot be removed unless the consumption of thermal energy occurs.

Dewatering characteristics of the sludge is affected by many factors such as pH and particle charge, organic content, bound water content, filtrate viscosity, alkalinity, solids concentration, nitrogen content, grease content, conditioning, type of sludge, compressibility coefficient, mechanical strength of particles, mixing, particle size and so on (Karr and Keinath, 1978).

Capillary Suction Time (CST), Specific Resistance to Filtration (SRT) and Floc Strength are the methods used for determining dewaterability of sludge.

CST, developed by Baskerville and Gale (1968), is a quick and easy method for determining sludge dewaterability. CST device is made up of 2 plastic blocks, 3 electrical contacts that are located in the upper plastic block, a stainless steel collar, filter paper and an electrical timer. Capillary suction time is the time that pass between flow of water from first two contacts and the second contact. Permeation of water through filter paper depends on condition of sludge and filterability of sludge cake that is formed on the filter paper (Vesilind, 1988).

SRF is another sludge characterization technique. SRF test is based on an analysis of pressure drop for flow through a porous medium using Darcy equation. The parameter resulted from the test is related to permeability (Vesilind, 1988). SRF had been the widely used test in the past, however more complicated nature of it compared to CST test made it as a less preffered method lately.
Floc strength is an important parameter in terms of sludge dewatering properties. Floc strength can be explained as the energy required breaking flocs under tension, compression or shearing. And it is generally measured by shear tests and determining changes in CST over time (Zhang *et al.*, 1999; Jarvis *et al.*, 2005).

Karr and Keinath (1978) determined that dewaterability of activated sludge improved when pH decreased. This is due to the reduction of primary charge of colloidal particles with pH and as a result aggregation is favored and total surface area is reduced.

When small scale solids are captured in the filter cake and/or the filter medium, due to the deterioration of the release of water in the sludge cake, dewatering deteriorates. This phenomenon is called “blinding” (Sorensen *et al.*, 1997). Sludge of low floc density related to high bound water content represent poor dewatering properties (Murthy and Novak, 2001).

EPS is highly hydrated and this water retention affects dewaterability of sludge. Therefore for the efficiency of dewaterability water need to be removed (Neyens *et al.*, 2004). Kang *et al.* (1989) reported that increase in EPS of activated sludge resulted in deterioration of the dewaterability of activated sludge. However, Houghton *et al.* (2001) found that there is a level of EPS and above this level dewatering property of sludge is lowered. Moreover, Higgins and Novak (1997a) concluded that the production of protein type polymers improved dewaterability of sludge. It was indicated that removal of EPS from activated sludge leads to the release of small particles that have the ability to clog the filtration pores of filter paper and sludge cake (Sanin and Vesilind, 1994).

Dewaterability of activated sludge can be affected by cations in several ways such as stimulation of synthesis of EPS, changing the amount of sludge bound water content, neutralization of negative surface charges, bridge formation between floc components and building up a floc network (Sanin *et al.*, 2006). Bruus *et al.* (1992) reported an increase in turbidity and a decrease in dewaterability due to the removal of calcium ions from activated sludge. When the calcium and magnesium concentration increases, cake solids, floc density, bound biopolymer concentration and floc strength increases while bound water content and polymer required for conditioning decreases. Therefore, divalent cations form tighter bound network of biopolymer that are more resistant to shear (Higgins and Novak, 1997a; Sobeck and

Higgins, 2002) however monovalent ions have negative impacts on the dewaterability of activated sludge by replacing divalent cations (Sanin *et al.*, 2006).

2.7.2. Settleability

Settling plays a prominent role in the effectiveness of the whole activated sludge process and it primarily depends on the performance of sludge floc formation (Wilen and Balmer, 1999). Settleability of activated sludge is determined by Zone Settling Velocity (ZSV) and Sludge Volume Index (SVI).

ZSV test is performed by measuring settling velocities over biomass concentrations. The determined settling velocities are then used for calibrating different models. The Vesilind Model (Vesilind, 1968) is the most commonly used one for these measurements (Schuler and Jang, 2007). At different solids concentrations, serial settling tests are done and the change in solid-liquid interface with time is noted. Settling velocity is recorded as a function of time (Vesilind *et al.*, 1994).

By holding the advantage of simplicity and convenience, SVI is commonly used measurement for an indicator of settleability. SVI is the volume in milliliters occupied by 1 g of suspension after 30 min. settling. SVI value greater than 150 represents bulking sludge and below 120 is generally considered as acceptable (Jenkins *et al.*, 2004).

The settling property of activated sludge is highly dependent on physical, chemical and biological properties of flocs. The major factors affecting the floc properties include influent and process conditions, microbial community and activity, and chemical composition (Jin e*t al.*, 2003).

In literature it was shown that solids retention time has an important effect on settleability of sludge. Liao *et al.* (2001) reported that at lower SRTs, SVI takes higher values. The study of Sesay and Sanin (2004) related to the effects of SRT on

physical properties of activated sludge revealed that at higher SRTs, sludge settles at a faster rate due to decrease in carbohydrate content of the EPS produced.

Jenkins *et al.* (2004) revealed that deficiency in nitrogen and phosphorus in the feed stimulated the growth of filamentous microorganisms which led to sludge bulking. Bura *et al.* (1998) reported that when C/P ratio increased from 100 to 500, protein content of EPS increased. At higher C/N ratio, production of carbohydrate type polymers and SVI increases. This leads to viscous bulking of sludge (Durmaz and Sanin, 2003). A study of Turtin *et al.* (2006) showed lack of phosphorus in the feed medium led to excessive production of carbohydrate type polymers. As a result, due to overproduction of carbohydrates settling deteriorated severely where viscous bulking was observed.

Dissolved oxygen (DO) and temperature also affects the settling property of sludge. The size distribution of activated sludge flocs is highly dependent on the availability of DO (Li and Ganczarczyk, 1993). Starkey and Karr (1984) showed that at low DO concentrations, flocculation of sludge was very weak and effluent was more turbid. Similar findings were reported by Wilen and Balmer (1999). Poor settling and thickening properties were observed at low DO concentrations because of overproduction of filamentous bacteria. On the other hand, Çetin and Sürücü (1990) recorded low settling velocities at high temperatures. At temperatures lower than 35^0C, sludge settled well, however at 35^0C tendency of bulking was observed. Krishna and Loosdrecht (1999) reported similar results, settleability decreases with increasing temperature.

Several studies showed that at lower EPS, settleability of sludge is better and linear correlation between SVI and EPS was determined in most of the studies (Forster, 1971; Kiff, 1978; Eriksson and Alm, 1991; Urbain *et al.*, 1993; Liao *et al.*, 2001). However in some studies negative correlation (Yun *et al.*, 2000; Goodwin and Forster, 1985) or no correlation (Chao and Keinath, 1979; Jorand *et al.*, 1998) between SVI and EPS were recorded. Yet more than amount, composition of EPS affects the settleability of sludge. Sponza (2004) reported that settleability of sludge

is positively affected due to the presence of large amount of protein. Supporting to these findings, Higgins and Novak (1997a, c) found that increase in bound protein led to decrease in SVI and therefore settleability of sludge was improved. In addition to this, overproduction of carbohydrates under phosphorus deficient conditions resulted to increase in SVI and viscous bulking was observed (Turtin *et al.*, 2006).

Settleability is also affected by floc size and filament content. Jin *et al.* (2003) reported that presence of large flocs and high quantity of filaments had a negative impact on settleability. Because of having lower density and larger surface area, large flocs can not compact well (Andreadakis, 1993). Sezgin (1982) reported an increase in SVI with increase in filament length when the filament length was over 10^7 μm/mg MLSS. Below this value, no relation was determined between filament length and SVI.

Type and concentration of cations in the feed medium affect the settleability of sludge (Forster, 1985b; Bruus *et al.*, 1992; Higgins and Novak, 1997 a, b, c; Murthy *et al.*, 1998). In cation-bridging model (Tezuka 1969; Forster and Lewin, 1972; Bruus, 1992; Higgins and Novak, 1997a) cations act as a bridge between EPS and cells, and the removal of cations from the bridge lead to deterioration of settleability. Higgins and Novak (1997a) found that at least 0.7-2 meq/L calcium and magnesium was required for acceptable settling of sludge. Further increase in concentrations resulted in increase of bound protein content and improve the settleability of sludge. As the monovalent to divalent cation ratio exceeded approximately 2 to 1, settleability of sludge deteriorated due to replacement of monovalent cations with divalent ones in the floc structure.

2.7.3. Rheology

Rheological measurements posses a critical role for mixing, chemical conditioning, dewatering or pumping processes of wastewater treatment (Dentel, 1997). Yet, it is very difficult to characterize sludges because of the complex composition of them which include long-chain polymers, bacteria, colloids or large flocculant structure (Jorand *et al.*, 1995).

Viscosity, as a most commonly used representative measure of rheology, is defined as the deformation of a body under shear stress. The sludge behavior can be characterized by determining the shear stress as a function of shear rate (Guibaud *et al.*, 2004). Depending on the flow behavior, fluids are classified as Newtonian and non-Newtonian.

Including pure and single phase liquids (Dick and Ewing, 1967), most of the liquids exhibit Newtonian behavior in which shear stress is linearly proportional to shear rate. Viscosity of Newtonian liquids is dependent of flow rate at a given temperature and pressure. The viscosity of Newtonian fluids is as follows:

$$\tau = \eta\,(dv/dy) \qquad \text{.....(2.1)}$$

where;
τ = shear stress,
η = viscosity,
dv/dy = shear rate.

There are vast amount of studies related to rheological properties of sludges in literature. According to these studies, sludge exhibits non-Newtonian flow behavior (Behn, 1962; Dick and Ewing, 1967; Moeller and Torres, 1997; Dentel, 1997; Forster, 2002; Sanin, 2002) since there is not a linear proportion between the shear stress exerted on sludge and shear rate. In Figure 2.4 types of different rheological behavior of Newtonian and non-Newtonian fluids are exhibited.

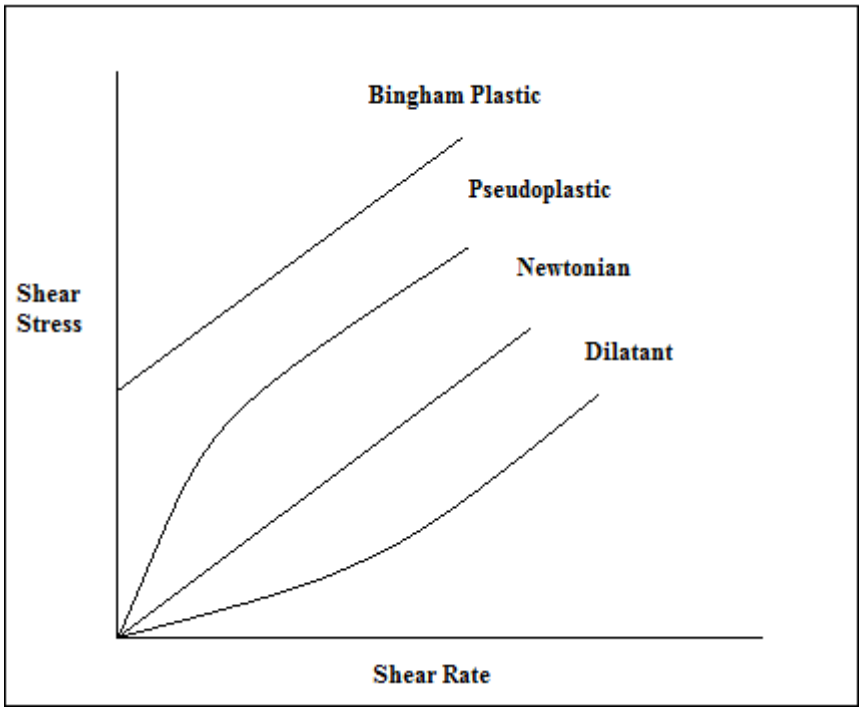

Figure 2.4. Rheograms of Newtonian and non-Newtonian fluids

Bingham plastic behavior can be observed when the solid phase is in sufficient concentration to form continuous structure. This type of fluids act like an elastic solid until the applied stress becomes greater than the yield stress. After this point, liquid starts to flow viscously (Dick an Ewing, 1967). The relationship between shear stress and shear rate in Bingham plastic fluids are:

$$\tau = \tau_y + \eta\,(dv/dy) \quad \text{.....(2.2)}$$

where,

τ = shear stress,

τ_y = yield stress,

η = Bingham viscosity,

dv/dy = shear rate.

In pseudoplastic behavior, which is also called as shear thinning, increase in shear stress leads to decrease in viscosity. The reason is the breakdown of flocs into smaller units with increase in shear stress. This type of fluids are represented by a power law equation as in Equation 2.3,

$$\tau = K (dv/dy)^n \quad \text{.....(2.3)}$$

where,
τ = shear stress,
K = fluid consistency index,
dv/dy = shear rate,
n = flow behavior index.

n is a number of which value is smaller than 1 for pseudoplastic liquids and the more it gets smaller than 1, the flow behavior deviates more from Newtonian flow behavior (Sanin, 2002).

Dilatance is the opposite of pseudoplastic behavior. The increase in shear rate results increase in viscosity. This flow behavior is called as shear thickening. The same equation (Equation 2.3) used by pseudoplastic flow represents dilatance but at this time flow behavior index (n) is greater than 1.

Moreover, activated sludge exhibits thixotrophic rheological properties. The viscosity of sludge does not stay constant at a given shear rate; rather it is a function of time (Tixier *et al.*, 2003).

In case of suspensions, the rheological characteristics depend on their suspended solids concentration. However, in activated sludge there are particle-particle interactions that cause the measured viscosity being greater. In this sense, the relationship between viscosity and solids concentration is represented by Einstein equation (Forster, 2002):

$\eta = \eta_0 (1 + 2.5\emptyset)$……...(2.4)

where;

η = suspension viscosity,

η_0 = solvent viscosity,

$\emptyset$ = volume fraction of the suspension occupied by particles.

Viscosity of sludge is dependent on many factors like pH, ionic strength, solids concentration, flocculation, particle size and shape. Furthermore, Sanin and Vesilind (1994) reported that the removal of polymers by centrifugation resulted decrease in viscosity. Sanin (2002) reported that increase in solids concentration resulted to decrease in flow behavior index and increase in pseudoplastic viscosity. A positive correlation between pH and viscosity was recorded by Sanin (2002) and Tixier (2003). Viscosity was increased with increase in pH. The reason is that sludge particles start to carry more negative charges on their surfaces as the pH increased. The increase in negative charges led to the repulsion between particles and floc structure become expanded. Moreover, Forster (1983) found that addition of cations to the system caused a decrease in viscosity. Cation addition decreased the bound water content meanwhile the surface charge of the particles decreased. As a result interparticle interactions decreased and lower viscosity values were observed.

CHAPTER 3

MATERIALS AND METHODS

3.1. Reactor Operation

Two liters semi-continuous activated sludge reactors were operated. The reactors were seeded with mixed culture bacteria obtained from the primary sedimentation tank effluent of Ankara Central Wastewater Treatment Plant. The operational conditions were kept constant until the reactors reached steady-state and during the experiments as well.

In order to examine the difference in activated sludge characteristics, two reactor sets were operated. In each set six lab-scale semi-continuous reactors were operated that are shown in Figure 3.1 with three different calcium concentrations and the reactors were operated as duplicates. The calcium concentrations in reactors were 0.5, 5 and 15 meq/L. The 0.5 meq/L reactor was used as a control.

Figure 3.1. Representation of reactors

3.1.1. Phosphorus Deficient Reactors

The reactors were fed with synthetic medium that contains nutrients and minerals for the microorganisms. The composition of the synthetic feed medium for phosphorus deficient condition for control reactor is given in Table 3.1.

Table 3.4. The composition of the synthetic feed medium for phosphorus deficient condition for control reactor

Constituent	Concentration (mg/L)
Glucose	935
NH_4Cl	225
$FeSO_4.7H_2O$	3.75
$ZnSO_4.7H_2O$	3.75
$MnSO_4.H_2O$	2.287
$NaHCO_3$	42
Peptone	60
KCl	37.25
$MgSO_4.H_2O$	123
$CaCl_2.2H_2O$	36.75
Tris buffer	36.3

In this reactor set, the C/N ratio of the feed to the reactors (in terms of COD/TKN ratio) was 20.95. Biosate peptone ,which is a special type phosphorus rich peptone, was the only phosphorus source for the reactors. The reactors received 0.624 mg/L phosphorus that corresponds approximately to 1/20 of the stoichiometrically required value from the addition of peptone to the feed medium. In order to avoid the formation of calcium-phosphate salt precipitation, phosphate buffer was not used to adjust the pH in the reactors. Air pumps were used to supply oxygen to the reactors. By this way, the reactors were mixed completely and the dissolved oxygen (DO) concentration was kept as minimum 3 mg/L in the reactors. The pH was maintained at 6.8±0.2 by using tris buffer. The reactors were placed in water bath so as to keep

the temperature of the reactors constant at 25°C. The reactors were operated with 8 days mean cell residence time that corresponds to wasting 1/8 (250 mL) of sludge from each reactor every day. The alimentation of the reactors was started with the complete mixing of the reactors and then 250 mL of sludge was wasted from each reactor. After wasting of sludge, reactors were left for settling for 2 hours. Then the supernatant part of the reactors was siphoned out. Finally, feed medium was added to the reactors and by using distilled water the volume of the reactors were completed to 2 L.

3.1.2. Phosphorus Sufficient Reactors

The calcium concentrations of the reactors were kept same as in the phosphorus deficient reactors. Yet, the composition of the synthetic feed medium was changed and it is given in Table 3.2.

Table 3.5. The composition of the synthetic feed medium for phosphorus sufficient condition for control reactor

Constituent	Concentration (mg/L)
Glucose	24
NH_4Cl	75
$FeSO_4.7H_2O$	3.75
$ZnSO_4.7H_2O$	3.75
$MnSO_4.H_2O$	2.287
$NaHCO_3$	21
Peptone	1100
KCl	37.25
$MgSO_4.H_2O$	123
$CaCl_2.2H_2O$	36.75
Tris buffer	18.15

The C/N ratio of the feed to the reactors (in terms of COD/TKN ratio) was 18.3 and C/N/P ratio was 125/6.82/1 which is very close to stoichiometrically required value. Biosate peptone was also used in this set and the reactors received 11.44 mg/L phosphorus. Phosphate buffer was not used to adjust the pH in the reactors and the pH was maintained at 7.8±0.3 by using tris buffer. Other operational conditions were same as the phosphorus deficient reactors.

3.1.3. Steady-State Determination

Steady state determination is very crucial for conducting the analyses. The operational conditions for both phosphorus deficient and phosphorus sufficient reactor sets were kept constant and the semi-continuous reactors were operated until they reach the steady state. For determining the steady state, mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations (Appendix A) were taken into consideration. Measurements were conducted at least 3-day-intervals and obtaining identical results from 3-5 consecutive measurements indicated that the semi-continuous reactors reached the steady state.

3.2. Analyses Conducted Under Steady State Conditions

After reaching steady state, chemical, physical and microbiological analyses were conducted for two reactor sets so as to determine the effect of calcium ion on activated sludge properties.

3.2.1. Chemical Analyses

The chemical analyses conducted under steady state conditions include conductivity measurement and extracellular polymer extraction by using cation exchange resin (CER) followed by carbohydrate and protein analyses.

3.2.1.1. Conductivity

The electrical current is transported by the ions in solution so dissolved inorganic solids in water affects the conductivity. The conductivity measurements were conducted in order to observe the effect of ion concentrations in activated sludge reactors. The measurements were conducted by using CyberScan PC 510 pH/conductivity meter. Measurements were started with well mixing of the reactors. Then the reactors were left for settling for 2 minutes. After 2 minutes, conductivity was measured by putting the probe into the reactors at 25°C. The results were expressed as milisiemens per centimeter.

3.2.1.2. Polymer Extraction

Polymer extraction plays an important role for determining the amount and composition of EPS in activated sludge. The polymer extraction by using the cation exchange resin (CER) method, which results in less cell lysis and less disruption of EPS, was first recommended by Frolund *et al.* (1996). From the studies of Park and Novak (2007), it was found that the CER process is very selective for calcium bound EPS. Consequently, in this study CER extraction method developed by Durmaz and Sanin (2001) in the light of the method developed by Frolund *et al.* (1996) was performed.

During the CER extraction process, DOWEX 50X80, 20-50 mesh Na^+ form strongly acidic cation exchange resin supplied from Fluka was used. The CER dose required for the extraction procedure was selected as 100 g CER/ g VSS which was recommended in the previous study of Durmaz and Sanin (2001). From the MLVSS value of each reactor before the extraction procedure, the required CER according to the chosen dose were weighed and put into jar test beakers and washed with phosphate buffer saline (PBS) by stirring for 1 hour at 120 rpm to prevent contamination from the CER or any other interferences during chemical analysis. The composition of PBS is given in Table 3.3. After washing, CER was filtered using 0.45 µm Millipore filter and dried at room temperature until the next day.

Table 3.6. Composition of the phosphate buffer saline (PBS) solution

Constituent	Concentration (mg/L)
NaCl	4
KCl	0.1
KH_2PO_4	0.06
Na_2HPO_4	0.455

Polymer extraction procedure was performed by using daily wasted 250 mL sludge of each reactor. 50 mL of the sludge was used during MLVSS measurements. The remaining 200 mL of the sludge was centrifuged at 3500 rpm for 15 minutes. Centrate was discarded and the pellet was resuspended by PBS to 200 mL in a jar test beaker. After resuspension, the washed and dried CERs for each reactor were added to the beakers.

In order to determine the effect of sludge and CER to the analyses, two control samples were conducted. The first control sample was called as “sludge control” that contains only sludge sample without CER. Result of this control sample indicated that whether there was a contribution by stirring or not. Second control sample was called as “CER control” that contains only CER and PBS without sludge. This control sample was used to identify the contribution of CER itself. “Sludge control” was performed for each reactor however only one “CER control” was used because the contribution of CER itself was negligible. For each reactor and control samples the same procedure was applied. The beakers were stirred for 5 hours at 120 rpm at a standard jar test apparatus. The stirring time and speed were selected from the previous study of Durmaz and Sanin (2003).

At the end of 5 hours stirring, the beakers were left for settling for 30 minutes. Supernatent part was centrifuged for 15 minutes at 3500 rpm and the centrate was used for carbohydrate and protein analyses.

3.2.1.2.1. Carbohydrate Analysis

Carbohydrate type polymer analysis was conducted using the phenol-sulphuric acid method of Dubois *et al.* (1956) and alginate was used as a standard.

First, 2 mL of centrate for each reactor were put into seperate test tubes as triplicates. Then, 50 µL phenol from 80% (w/w) and 5 mL sulphuric acid from 98% were added to the tubes respectively. The samples were allowed to stand for 10 minutes at room temperature. After 10 minutes, the tubes were vortexed and put into the incubator at 30°C for 15 minutes. The characteristic color for this method is yellow-orange and the absorbance of this characteristic color for each sample was measured by using Pharmacia LKB Novaspec II Spectrophotometer at 480 nm. The carbohydrate concentration of each sample was calculated by using the standard calibration curve (Appendix B) that was prepared before the experiment by using alginate. The contribution of CER to the results was negligible and did not considered in calculations, however “sludge control” was contributed and therefore results of “sludge control” was subtracted from the results during calculations.

3.2.1.2.2. Protein Analysis

Protein content of the EPS was determined using folin-ciocalteu phenol reagent method of Lowry *et al.* (1951) and bovine serum albumin was used as a standard.

Analysis was started with the preparation of four reagents which were named as reagent A, B, C, and D. Reagent A included 2% w/v sodium carbonate in 0.1 N NaOH. Reagent B contained 1% w/v sodium potassium tartarate dissolved in 0.5% w/v cupric sulphate. Reagent C was prepared using 1 mL of Reagent B and 49 mL of Reagent A. Reagent D was composed of the folin-ciocalteu’s phenol reagent diluted with deionized water by the ratio of 10:9.

The test tubes were prepared as triplicates for each sample and 600 µL centrate from CER extraction procedure was added to the tubes. 3 mL Reagent C was added

to the tubes and allowed to stand for 10 minutes at room temperature. After 10 minutes, 300 µL Reagent D was added and the tubes were vortexed immediately. Lastly, vortexed samples were left to stand for 30 minutes at room temperature. The absorbance of the characteristic blue color was measured by using Pharmacia LKB Novaspec II Spectrophotometer at 750 nm. The protein concentrations were calculated by using the standard calibration curve (Appendix B) prepared before the experiment in which bovine serum albumin was used as a standard. During calculations “CER control” results were not considered because of being negligible but “sludge control” results were subtracted from the values calculated for samples.

3.2.2. Physical Analyses

Viscosity, capillary suction time (CST), sludge volume index (SVI), and turbidity analyses were conducted for the determination of the effect of calcium ion concentration on the settling and dewatering characteristics of the activated sludge under both phosphorus deficient and sufficient conditions.

3.2.2.1. Viscosity

Viscosity measurements were carried out of 6 different shear rates; 1.83, 3.67, 7.34, 14.7, 36.7, 73.4 sec^{-1} because of the non-Newtonian behavior of the sludge. At a constant shear rate, the calculated ratio of shear stress to shear rate gives the apparent viscosities of the activated sludge. So as to determine the relationship between the apparent viscosity and suspended solids concentration (MLSS), sludge samples were diluted with phosphate buffer saline (PBS) solution and viscosity measurements were performed at 4 different suspended solids concentration.

The measurements of sludge rheological properties were carried out with the rotational viscometer Brookfield $LVDVII^{+}$ with ultra low viscosity adapter and the time required for measurement was 1 min and it was taken as constant for all viscosity measurements. The temperature values were also recorded before measurements. The flow characteristics of the samples were identified by plotting the shear stress

(dyne/sq.cm) versus shear rate (sec^{-1}) graphs. Furthermore, for a constant MLSS value, apparent viscosity (cP) versus MLSS (mg/L) graphs were plotted to compare the viscosity values of the samples.

3.2.2.2. Capillary Suction Time

Capillary suction time (CST) measurements were conducted for determining the dewatering property of the sludge. Measurements were performed by using the Method 2710G (APHA 2000). Type 304 M Triton Electronics Capillary Suction Timer as shown in Figure 3.2 was used for measurements. First, Whatman 17 chromotographic paper was placed into the test block and the cylinder called as a sludge resevoir was put into this test block. Then the well-mixed sludge sample was added in the sludge reservoir.

Test block contained two electrical points; inner and outer electrical points. As the sludge reach the inner electrical point, timer started and when it reached the outer electrical point the timer stopped. Finally, the measured value was read from display as seconds.

Figure 3.2. CST apparatus

3.2.2.3. Sludge Volume Index

Sludge volume index (SVI) is a measure of the setteability of the sludge. The mixed sludge sample was placed into the 1 L graduated cylinder and allowed to settle for 30 minutes. After 30 minutes, settled volume of the sludge was recorded. For determining the SVI values, suspended solids concentration was measured as MLSS and the SVI value was calculated using the following formula:

$$SVI = \frac{\text{Settled sludge volume after 30 minutes (mL/L)} * 1000}{\text{Suspended Solids Concentration (MLSS) (mg/L)}} \quad \text{.....(3.1)}$$

3.2.2.4. Turbidity

The sludge sample was placed into the 1 L graduated cylinder and left for settling for an hour. At the end of an hour, supernatent part was taken and put into the turbidity measurement cell and analysis was carried out by Hach Turbidimeter 2100N. The values were recorded as Nephelometric Turbidity Units (NTU).

3.2.3 Microbiological Analyses

In order to determine microbial populations in two reactor sets, microbiological analyses were conducted. These analyses were performed using API identification system and two types of kits were used during the experiments; API 20E and API Coryne. Moreover, the samples taken from the reactors were observed under light microscope to identify the floc structure.

3.2.3.1. Bacterial Identification by using API Kit System

3.2.3.1.1. Sampling and Culturing of Activated Sludge Microorganisms

The sludge samples taken from the reactors were decimal diluted to acquire 30-300 colonies on the petri plates. Dilutions were done under asceptic conditions by using 0.1% w/v peptone dissolved in distilled water. 0.5 ml sample for each reactor was taken from the last three decimal dilutions and spread on CGY agar for the application of API 20E kit. Pike *et al.* (1972) stated that CGY agar gave the highest counts for enumeration of activated sludge bacteria. CGY agar includes 5 g/L casitone, 5 g/L glycerol, 13 g/L agar, and 1 g/L yeast extract and supports the growth of most heterotrophic organisms. After inoculation process, the petri plates were incubated at 35°C until the colonies became observable on the plates. As the colonies were chosen on the plates, they were re-streaked on to the new CGY agar plates and re-incubated to obtain discrete colonies. Then, the selected colonies were taken and applied to API kits for identification.

As given in the API manual for the application of API Coryne kit, decimal diluted samples were spread on Blood Agar (5% sheep blood). After incubation period, colonies were chosen on plates and then re-streaked and re-incubated. These discrete colonies were applied to API kits to identify the microorganisms.

3.2.3.1.2. Description of API Kit System

API identification system is a microbial identification tool and it is a standardized and miniaturized version of the present identification techniques. After application procedure of a selected discrete colony to API kit, the system provides results within certain probability limits for the identification of the microorganisms.

There are many different API kits for the identification of several microorganisms which are API 20E, API 20NE, API Coryne, API 10S, RapiD20E, API Staph, API 20

Strep, API 20A, API Listeria, API NH, API Campy. During this study, API 20E and API Coryne kits were used.

API 20E is an identification system for *Enterobacteriaceae* and other non-fastidious Gram-negative rods including *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Kluyvera*, *Pasteurella*, *Proteus*, *Providencia*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella*, *Vibrio* and *Yersinia* species. Those genera of bacteria are very commonly found in activated sludge. In addition to this, *Alcaligenes*, *Escherichia* and *Pseudomonas* are belonging to the group of significant floc-formers. Thus, API 20E was selected as the most proper identification system.

API 20E identification system includes 25 biochemical tests and a database. The strip contains 20 microtubes which include dehydrated substrates. These microtubes are inoculated with bacterial suspension and addition of suspension rehydrates the substrates. During incubation period, metabolic products are produced and results in color changes in microtubes spontaneously or by addition of further reagents. The reactions that can be followed by the API 20E kit are; beta-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S production, urease, tryptophane deaminase, indole production, acetoin production, gelatinase, fermentation or oxidation of glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygladin, arabinose. The reactions are evaluated by using Reading Table and identification is obtained by the help of API 20E Software.

API Coryne system provides 24-hour identification of coryneform bacteria including *Actinomyces, Arcanobacterium, Arthrobacter, Brevibacterium, Cellulomonas, Corynebacterium, Listeria, Microbacterium, Nocardia, Propionibacterium and Rhodococcus species.* These microorganisms are belonging to Gram-positive, non-spore forming, facultatively areo-anaerobic rods. *Arthrobacter*, *Brevibacterium* and *Corynebacterium* are generally found in activated sludge flocs. *Nocardia* is one of the filamentous microorganism and excessive presence of *Nocardia* resulted in poor

settling of sludge. Under light of these, API Coryne identification system was selected apart from API 20E identification system.

The API Coryne strip consists of 20 microtubes that contains dehydrated substrates and by adding inoculated bacterial suspension, enzymatic activity or the fermentation of carbohydrates will take place. Due to the formation of metabolic end products, color changes are observed either spontaneously or by addition of reagents. Nitrate reduction, pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase, beta-glucoronidase, beta-galactosidase, alpha-glucosidase, N-acetyl-β-glucosaminidase, β-glucosidase, urease, gelatine (hydrolysis), fermentation of glucose, ribose, xylose, mannitol, maltose, lactose, sucrose, and glycogen are the enzymatic and fermentative reactions that can occur during the incubation period. According to the Reading Table, the reactions are noted as negative or positive and by using API Coryne Software the identification is obtained.

3.2.3.1.3. Preliminary Tests

Gram staining, oxidase and catalase tests were performed to choose the most proper API identification system.

Gram staining: A drop of distilled water is placed on a slide. A loopful of discrete culture is transferred to the slide and spreaded on the slide. Then it is left to dry in air. After drying, slide is passed over a bunsen flame as if cutting the flame across thus the material on the slide is fixed. The fixed material is flooded with crystal violet for 1 minute and washed with tap water. The gently washed slide is flooded with lugolls iodine solution for 1 minute and then decolorized with acetone/alcohol mixture for 30 seconds. Lastly, the fixed material is counterstrained with safranin for 30 seconds and washed with tap water. As the staining procedure completed, the slide is observed under microscope. Gram positive cells are blue in color and the negative ones will appear pink.

Oxidase test: Oxidase test defines the presence or absence of the enzyme cytochrome oxidase where in the presence of this enzyme bacteria can utilize oxygen for energy production. The test starts with the saturation of a filter paper with 2-3 drops of oxidase reagent. Then the discrete colony is placed to the reagent saturated filter paper. Color change to dark purple within 30 seconds represents an oxidase positive culture.

Catalase test: Catalase enzyme catalyzes the decomposition of hydrogen peroxide to water and oxygen. The test is performed by placing a drop of 3% w/v hydrogen peroxide solution on the selected culture. The release of bubbles of oxygen in seconds shows that the reaction is catalase positive.

3.2.3.1.4. Inoculation of API Kit System

The inoculation of API 20E kit was started with the preparation of a homogenous bacterial suspension by mixing 0.85% NaCl medium with the selected colony. The tubes and cupules for citrate utilization, gelatinase activity and acetoin production were filled with the prepared bacterial suspension medium. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and urease activity test tubes were filled with the suspension medium and the cupules were overlayed with mineral oil so as to sustain anaerobic conditions. The other test tubes were also filled with the suspension medium. Strips were incubated at 35-37°C for 18-24 hours. After incubation period, the results were noted as negative or positive according to color changes given in the Reading Table of the kit. The negative and positive test results according to color change on API 20E kit are given in Figure 3.3. When 3 or more tests were recorded as positive for a strip, additional reagents required for identification were added and the results were noted as negative or positive in reference to the Reading Table of the kit again. Then, identification was obtained by using Analytical Profile Index and API Software, respectively. After incubation period for some strips less than 3 positive results were recorded. In such cases, these strips were re-incubated for 24-hours and after re-incubation additional reagents for identification were added and the same procedure was applied for identification.

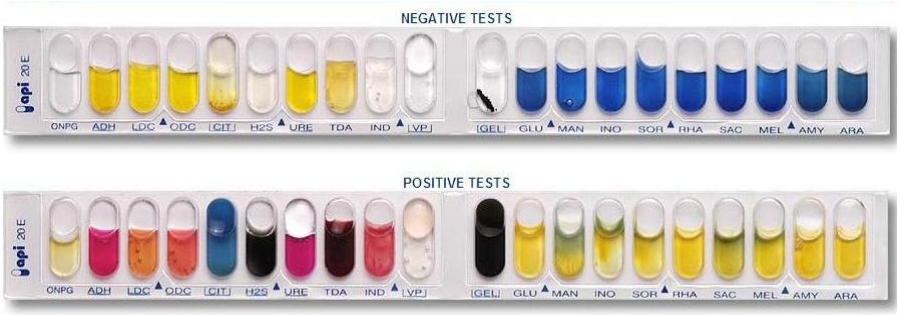

Figure 3.3. Negative and positive test results on API 20E

The API Coryne kits were inoculated with bacterial suspension medium with a turbidity greater than 6 McFarland. Nitrate reduction, pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase, beta-glucoronidase, beta-galactosidase, alpha-glucosidase, N-acetyl-β-glucosaminidase and β-glucosidase activity tubes and cupules were filled with bacterial suspension. Urease activity, glucose, ribose, xylose, mannitol, maltose, lactose, sucrose, and glycogen fermentation tubes were filled with suspension medium and the cupules were filled with mineral oil for anaerobisis. Both tube and cupule of gelatine hydrolysis was filled with the suspension medium. Strips were incubated at 35-37°C for 24 hours. After incubation period, additional reagents were added and negative and positive results in reference to color changes given in the Reading Table were noted (Figure 3.4). Regarding to the negative and positive results, identification was performed by using Analytical Profile Index and API Software.

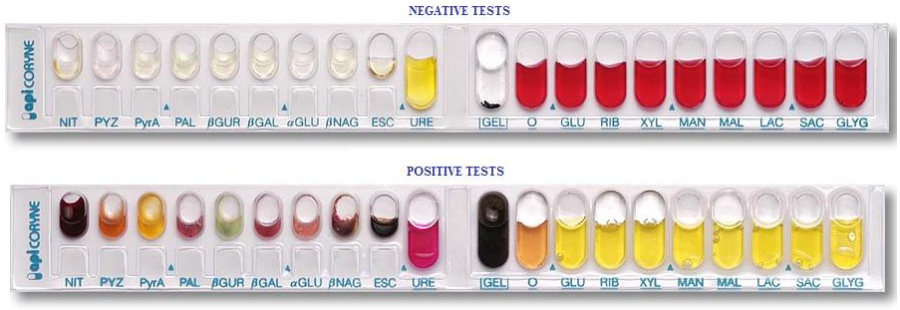

Figure 3.4. Negative and positive test results on API Coryne

3.2.3.2. Micro-photographs

The micro-photographs of the samples were taken using light microscope with necessary magnifications. These microphotographs were used to determine the floc structure of the sludge samples.

3.2.4. Other Measurements

MLSS and MLVSS: Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations were used as control parameters for the activated sludge reactors. MLSS is the dry solids concentration in mixed liquor in the aeration tank and MLVSS is the microbiological suspension in an aeration tank. Measurements were conducted at least 3-day-intervals by using the Method 2540D and 2540E (APHA, 2005). Steady state determination was based on the similar results of the measurements.

Chemical Oxygen Demand (COD): COD can be defined as the oxygen required to chemically oxidize the organic matter in wastewater in mg/L. The COD measurements were performed using the closed reflux colorimetric method (EPA Method 410.4) and Hach DR2000 spectrophotometer was used.

Dissolved Oxygen (DO): Dissolved oxygen was measured by Hach Sension 378 pH/conductivity/DO meter to ensure that the DO concentration as minimum 3 mg/L in reactors.

pH: pH was measured with CyberScan PC 510 pH meter/conductivity meter.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Effect of Calcium Ion Concentration in Phosphorus Deficient Reactors

This part of the study was conducted with three different concentrations of calcium ions so as to determine the effect calcium ion concentration on chemical, physical and microbiological properties of activated sludge under phosphorus deficient conditions. The analyses were conducted after steady state was reached. During phosphorus deficient conditions six reactors were operated as it was given in Table 4.1. In this stage of study, sludge was severely bulking and the average values of replica reactors were taken except for the control reactors. Physical appearance and experimental results of sludge in C(1) started to deteriorate, its color became darker day by day. Therefore, the results of C(2) was taken into consideration in this set rather than taking average the average.

4.1.1. Steady State Conditions

Phosphorus deficient laboratory scale semi-continuous reactors were operated under three different calcium concentrations. The calcium ion concentrations in the reactors are 0.5 meq/L, 5 meq/L and 15 meq/L. The reactors were operated as duplicates as shown in Table 4.1. The reactors with 0.5 meq/L calcium ion concentration were selected as control reactors in order to observe the effects of increase in ion concentration. The increments in ion concentration were provided with addition of $CaCl_2.2H_2O$ to synthetic feed medium and peptone was the only source of phosphorus. Before conducting analyses, reactors were brought to steady state. Forster and Dallas-Newton (1980) reported that 2-3 times of mean cell residence time is required for adaptation of microorganisms in activated sludge to the new conditions.

Steady state was determined by measuring mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations. Variation of MLSS and MLVSS data less than 15% was taken as an indicator of the achievement of steady state.

Table 4.1. Calcium ion concentrations in reactors under phosphorus deficient conditions

Calcium Ion Concentration (meq/L)	Reactors
0.5	C(1), C(2)
5	5(1), 5(2)
15	15(1), 15(2)

4.1.2. Effect of Calcium Ion on Chemical Characteristics of Activated Sludge under Phosphorus Deficient Conditions

Analyses conducted under phosphorus deficient conditions to determine the effect of calcium ion on chemical characteristics of activated sludge are the determination of effluent soluble chemical oxygen demand (COD), EPS composition and conductivity.

4.1.2.1. Effluent Soluble Chemical Oxygen Demand (COD)

In order to determine the effect of calcium ion concentration on effluent quality effluent soluble COD values were measured. As it can be observed from Figure 4.1, effluent COD concentration decreased as calcium concentration was increased to 5 meq/L from 0.5 meq/L and then to 15 meq/L. There was a sharp decrease in effluent COD concentration when calcium concentration shifted from 0.5 meq/L to 5 meq/L, but further addition of calcium ions did not show a significant decrease in effluent COD concentration as compared to this shift.

COD of the synthetic feed medium was 1308 mg/L which was smaller than effluent soluble COD values found for the entire calcium ion concentrations. Therefore COD was not removed; contrary it was increased in the effluent. This can be explained by the fact that under phosphorus deficient conditions sludge was severely bulking. In addition to difficulty in settling, some finely dispersed flocs were also present and microorganisms contributed to COD because of this floc structure.

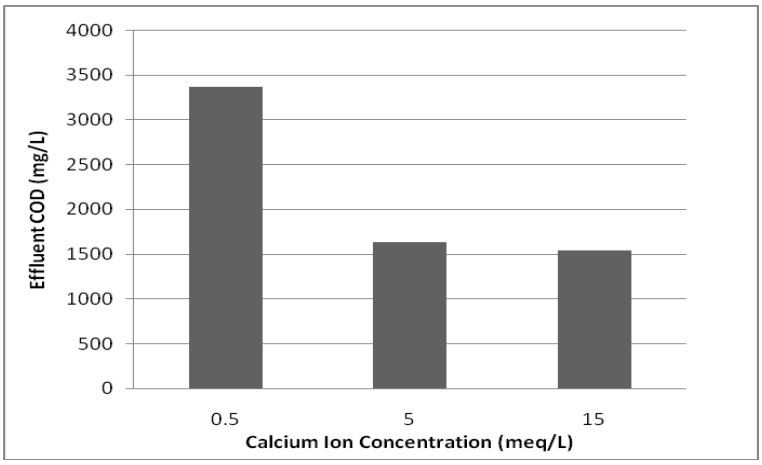

Figure 4.1. Effect of calcium ions on effluent COD concentration under phosphorus deficient condition

4.1.2.2. Production and Composition of Extracellular Polymers

Extracellular polymeric substances are one of the most common components of floc structure (Li and Ganczarczyk, 1990) and comprises different types of biopolymers including proteins, humic compounds, lipids, acidic and neutral polysaccharides, and nucleic acids (DNA and RNA) (Pavoni *et al.*, 1972; Kiff, 1978; Sakka and Takahashi, 1982; Goodwin and Forster, 1985; Urbain, *et al.*, 1993; Frolund *et al.*, 1996; Palmgren and Nielsen, 1998). EPS are important for the physico-chemical properties of activated sludge flocs (Frolund *et al.*, 1996).

Regarding to the yield of EPS and minimal disruption of exopolymers, cation exchange resin (CER) procedure was applied for the extraction of EPS. In this method, cations in the sludge matrix are removed by CER and due to the removal of cations floc structure is broken then EPS will be released (Frolund *et al.*, 1996).

As presented in Figure 4.2, quantity and composition of EPS extracted changes at different concentrations. Total EPS concentration was lowest at 0.5 meq/L calcium concentration and further addition of calcium led to increase in total EPS concentration. Since reactors were operated at phosphorus deficient conditions, C/N/P ratio was disturbed due to excess carbon and nitrogen in the medium. It is known that EPS production increases when carbon in the medium is higher than required for growth and maintenance. In this sense, a binding agent is needed for produced EPS to the microorganisms. In literature it was reported that cations provide binding between negatively charged biopolymers in which microorganisms are embedded (Bruus *et al.*, 1992; Urbain *et al.*, 1993; Higgins and Novak, 1997a). This is why polymer concentration was increased with increment in calcium concentration.

At the same figure carbohydrate and protein concentrations as a function of calcium ion concentration is given. Carbohydrate concentration was greater as compared to protein concentration for 5 meq/L and 15 meq/L calcium concentrations. Figure 4.3 represents the ratio between protein type and carbohydrate type polymers for increasing calcium ion concentrations. As calcium concentration was increased from 0.5 meq/L to 5 meq/L, carbohydrate content of EPS increased significantly but there is not a significant change in EPSp/EPSc ratio for further increase in calcium concentration. Dominancy of carbohydrate type polymers is also supported by the findings of Sanin *et al.* (2006). In this study, it was reported that calcium ions stimulated the production of carbohydrate type polymers and they had a tendency to bind that type of polymers within the EPS matrix.

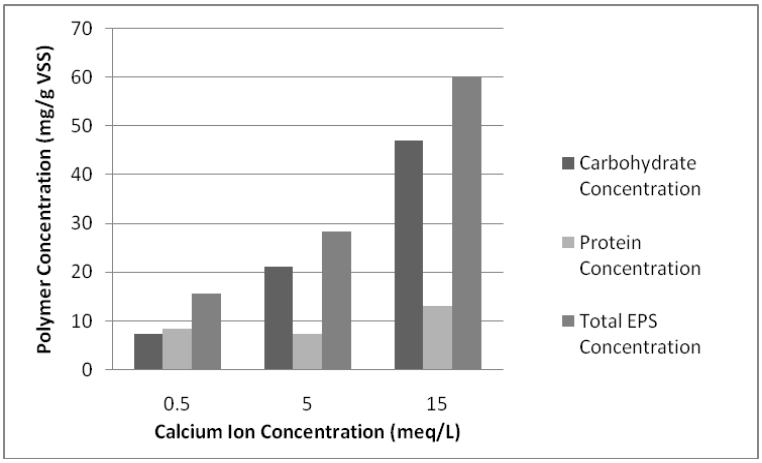

Figure 4.2. Effect of calcium concentration on production and composition of EPS under phosphorus deficient condition

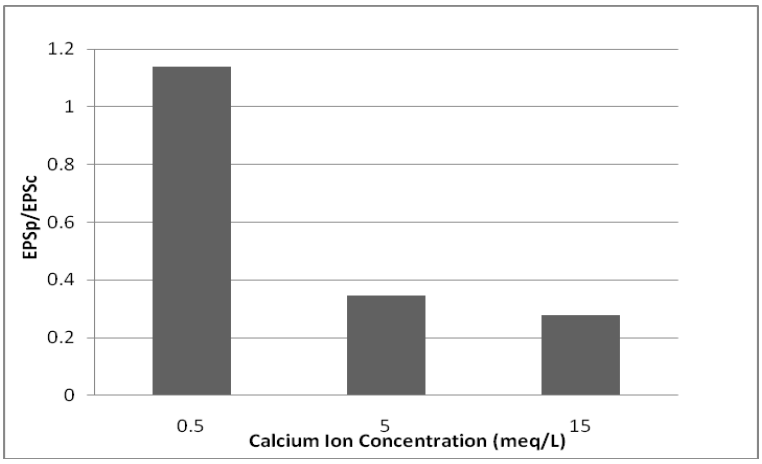

Figure 4.3. EPSp/EPSc ratio with respect to calcium concentrations under phosphorus deficient condition

4.1.2.3. Electrical Conductivity

Electrical conductivity is related to the amount of dissolved ions in water and as the ionic concentration increases, conductivity increases too. Figure 4.4 represents the relation between calcium ion concentration and conductivity. Feeding regime and

constituents were kept constant for all reactors except for the calcium addition in the form of $CaCl_2.2H_2O$. As it was expected, conductivity is highest at 15 meq/L. Thus, conductivity results are directly related to dissolved $CaCl_2.2H_2O$ concentration.

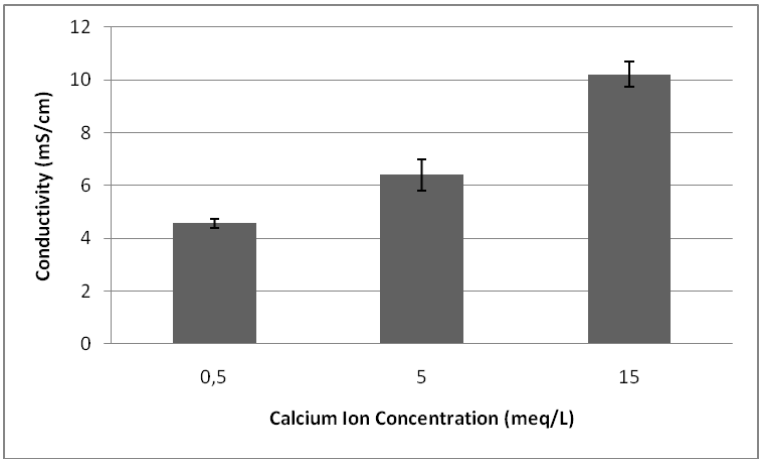

Figure 4.4. Conductivity values with respect to calcium concentrations under phosphorus deficient condition

4.1.3. Effect of Calcium Ion on Physical Characteristics of Activated Sludge under Phosphorus Deficient Conditions

In order to determine the effect of calcium on dewaterability, settleability, rheology and turbidity of activated sludge under phosphorus deficiency capillary suction time (CST), sludge volume index (SVI), viscosity and turbidity analyses were conducted.

4.1.3.1. Dewaterability

Dewaterability was determined by measuring capillary suction time. Bruus *et al.* (1992) found that the removal of calcium ions from sludge resulted in a decrease in dewaterability. Similar findings were reported by Higgins and Novak (1997a). It was reported that increasing concentrations of calcium and magnesium, improved settling and dewatering properties of sludge. However, as it is shown in Figure 4.5.

increase in capillary suction time (CST) was correlated with the increase in calcium concentration which means deterioration in dewaterability of sludge. This inconsistency with literature can be explained by the fact that under phosphorus deficient conditions increment in calcium concentration stimulates the production of EPS, especially carbohydrate fraction of EPS (Figure 4.2) and this leads to deterioration of dewaterability of sludge. As the amount of EPS and EPSc increase, amount of sludge bound water content increases and sludge become harder to dewater.

Hoa *et al.* (2003) found that phosphorus deficient conditions led to increase in carbohydrate concentration which resulted deterioration in settling and dewatering properties of sludge.

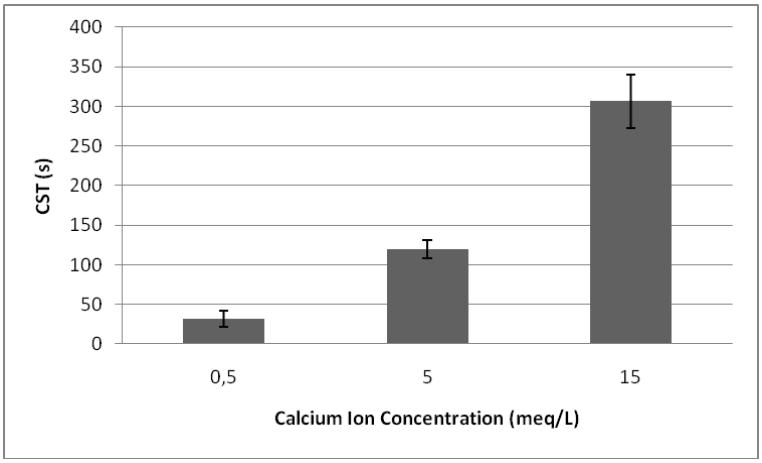

Figure 4.5. CST values with respect to calcium concentrations under phosphorus deficient condition

4.1.3.2. Settleability

Sludge volume index (SVI) was measured to determine settleability of sludge. Good settling sludge represents SVI value less than 120. SVI value greater than 120 means there is a settling problem and above 150 sludge bulking is observed. Under phosphorus deficient conditions for entire concentrations, SVI values were greater than 150 and slightly negatively correlated with calcium concentration (Figure 4.6).

Daily alimentation of the reactors was started with the complete mixing of the reactors and then 250 mL of sludge was wasted from each reactor. After wasting of sludge, reactors were left for settling for 2 hours. Then the supernatant part of the reactors was siphoned out. Yet, under phosphorus deficient operational conditions sludge was severely bulking and after 2 hours of settling period almost no settling was observed and therefore siphoning of the supernatant could not be performed properly. As a result of insufficient siphoning, unconsumed part of feed and some products of microorganisms remained in the solution and affected the efficiency of settling.

Composition of EPS also affects the settleability of sludge (Bura *et al.,* 1998). Durmaz and Sanin (2003) reported that high carbohydrate content of EPS caused settleability problems. Turtin *et al.* (2006) also showed that increase in carbohydrate concentration under phosphorus deficiency, resulted viscous bulking of sludge. In accordance with the findings of Durmaz and Sanin (2003) and Turtin *et al.* (2006), under phosphorus deficient conditions except for 0.5 meq/L calcium concentration excessive production of carbohydrate type polymers was recorded.

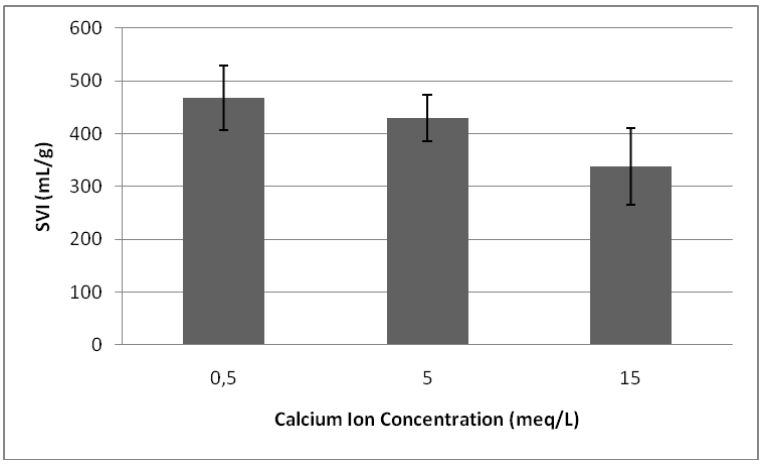

Figure 4.6. SVI values with respect to calcium concentration under phosphorus deficient condition

4.1.3.3. Rheology

Rheological properties of sludge determine the design of pumping, mixing and settling of the sludge. A common rheological measurement, viscosity analyses were performed. Viscosity can be defined as the resistance of fluid to deform under shear stress. In this sense to investigate the rheological property of sludge, shear stress versus shear rate graphs were plotted for each calcium concentration, tested with Newtonian and non-Newtonian rheological models. As it is shown in Figure 4.7, 0.5 meq/L calcium concentration data best fit to the non-Newtonian Bingham plastic flow behavior which is represented by:

$$\tau = \tau_y + \eta\,(dv/dy) \quad \text{.....(4.1)}$$

where,
τ = shear stress,
τ_y = yield stress,
η = Bingham viscosity,
dv/dy = shear rate.

Bingham plastic behaves as a rigid body until a certain stress is applied, yield stress. Beyond this value, sludge starts to flow with increasing shear stress. Further increase in calcium concentration to 5 meq/L and 15 meq/L seemed to a change in flow behavior of sludge. Best fits were obtained to power law model for 5 meq/L and 15 meq/L calcium concentration (Figure 4.7). Rheograms of 5meq/L and 15 meq/L calcium concentrations shows non-Newtonian pseudoplastic flow described by:

$$\tau = K\,(dv/dy)^n \quad \text{.....(4.2)}$$

where,
τ = shear stress,
K = fluid consistency index
dv/dy = shear rate
n = flow behavior index.

As the shear stress increases, flocs start to break down and viscosity decreases. The flow behavior index (n) equals 1 means that flow behavior approximates Newtonian model and n gets smaller values than 1, flow character deviates from Newtonian. The results show that shift in calcium concentration from 5 meq/L to 15 meq/L flow behavior deviates more from Newtonian.

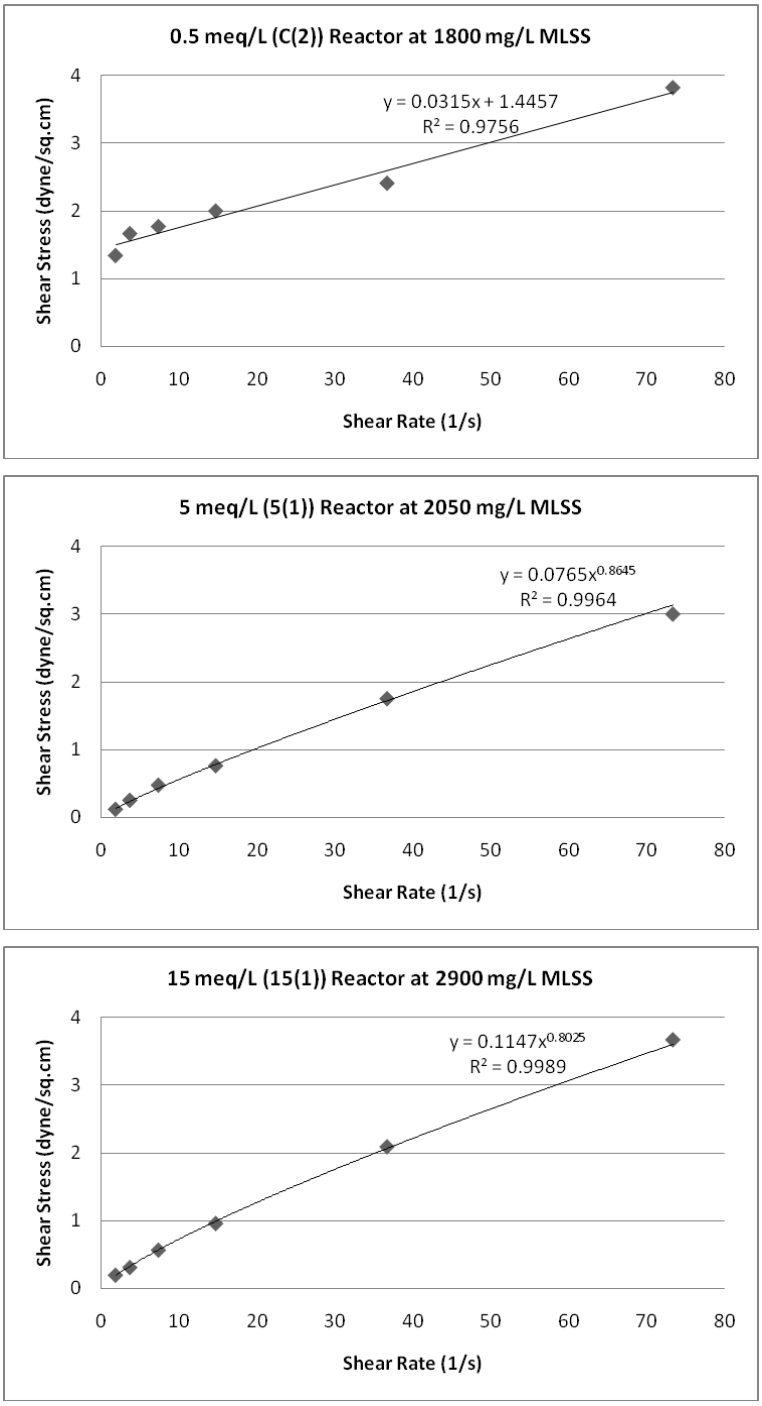

Figure 4.7. Rheograms of phosphorus deficient activated sludge samples at different calcium concentrations

The effect of solids concentration on viscosity is given in Figure 4.8. At 4 different MLSS concentrations, apparent viscosity values were measured at a constant shear rate of 73.4 s^{-1}. The increase in solids concentration was correlated to increase in apparent viscosity at every calcium concentration and this is consistent with literature (Sanin, 2002). At a fixed concentration of 1500 mg/L, apparent viscosity versus calcium concentration graph revealed that the addition of calcium ion resulted in decrease of viscosity. The shift in calcium concentration from 0.5 meq/L to 5 meq/L led to significant decrease in viscosity but further addition of calcium to 15 meq/L resulted a very small decrease in viscosity.

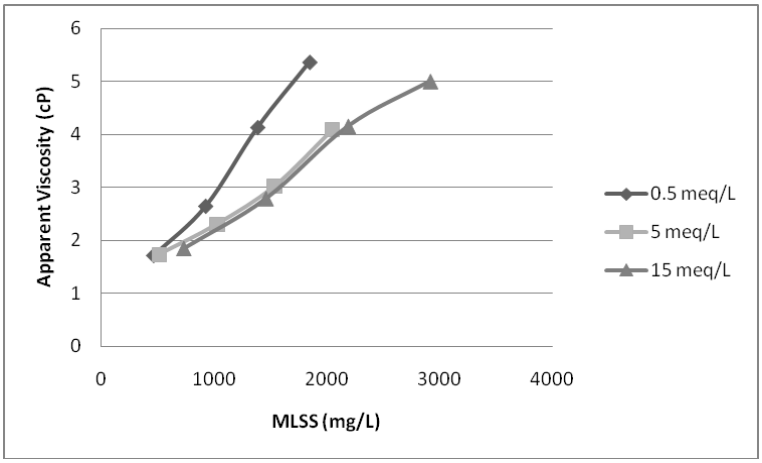

Figure 4.8. Apparent viscosities at different calcium concentrations at a shear rate of 73.4 sec^{-1}

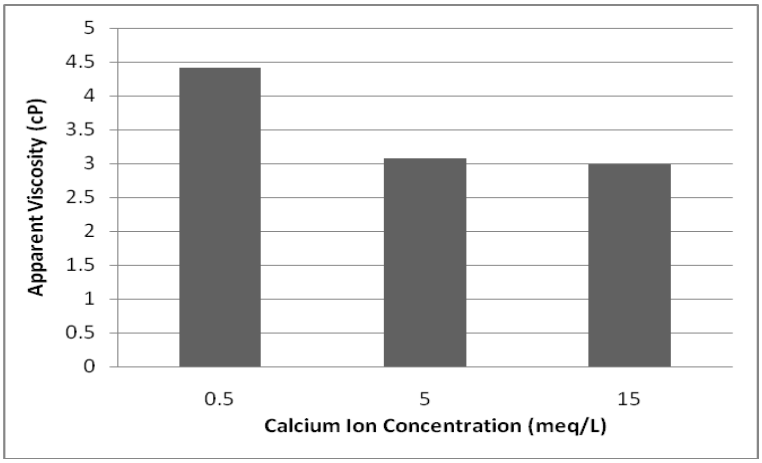

Figure 4.9. Apparent viscosity values with respect to calcium concentration at 1500 mg/L MLSS

In literature it was mentioned that EPS affect the viscosity of sludge (Forster, 1985a). In this sense, it is expected that as the production of EPS increases, viscosity must be increased. However, under phosphorus deficient conditions results showed that rather than the amount, composition of EPS has a more significant effect on viscosity. Decrease in EPSp/EPSc ratio resulted in decrease in viscosity.

Furthermore, Sanin (2002) reported that there is a negative correlation between conductivity and viscosity. The findings indicated that rising addition of calcium led to increase in conductivity and decrease in viscosity (Figure 4.4 and Figure 4.9).

4.1.3.4. Turbidity

Measured turbidity values are shown in Figure 4.10. There is a small decline in turbidity from 5 to 15 meq/L calcium concentration but increasing concentrations of calcium led to increase in turbidity. In other words, effluent total suspended solids increased by rising calcium concentration. All turbidity values measured at all calcium concentrations are excessively higher compared to the desirable turbidity values for activated sludge effluents which are between 20-40 NTU. The results indicates very bad floc structure in phosphorus deficient conditions.

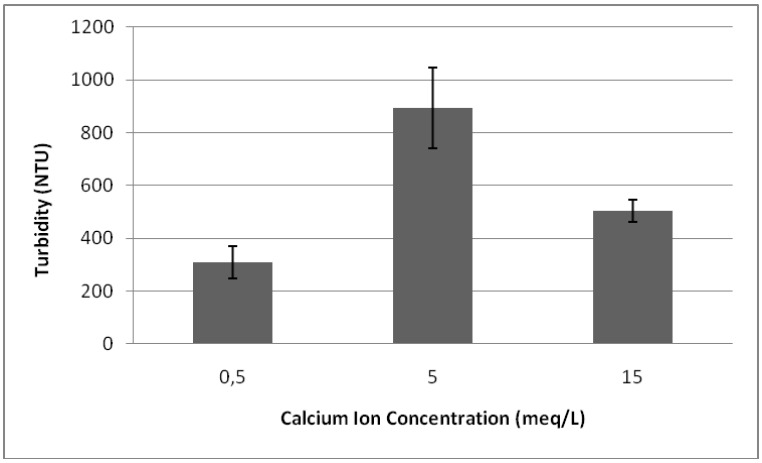

Figure 4.10. Turbidity values with respect to calcium concentration under phosphorus deficient condition

4.1.4. Microbiology of Activated Sludge under Phosphorus Deficient Conditions

Microbial properties of activated sludge were determined by applying API Kit System (API 20E). In addition to this, microphotographs of sludge samples were taken to investigate the floc structure under different calcium concentrations.

After inoculation and incubation, gram staining and oxidase tests were applied for the selected discrete colonies. It was recorded that all colonies from 0.5, 5 and 15 meq/L calcium concentrations were showing oxidase negative results. In literature it was mentioned that a great part of activated sludge bacteria are Gram-negative (Gaudy and Gaudy, 1980). The results of gram staining test, performed before applying API Kit System, were consistent with literature that all groups of bacteria were recorded as Gram-negative.

Output of API 20E software is given in Table 4.2. As it is seen in Table 4.2, *Enterobacter sakazakii* and *Citrobacter freundii* were common in all calcium concentrations.

*Enterobacte*r is a motile, gram negative, facultatively anaerobe and rod-shaped bacteria belonging to the Enterobacteriaceae family. Many strains of this bacterium were recorded as pathogenic (Sanders and Sanders, 1997). *Enterobacter sakazakii* is one most commonly found Enterobacter specie which was first identified by Farmer *et. al* (1980). It is a gram negative and oxidase negative bacteria and has the ability to ferment glucose. Typical property of it is the production of a yellow pigment.

In addition to *Enterobacter sakazakii*, *Citrobacter freundii*, which is a gram negative rod-shaped bacteria uses only citrate as the carbon source (Lipsky *et. al*, 1980), was also recorded as the common bacteria for all calcium concentrations.

Except from the commonly found bacterial species in 5 meq/L and 15 meq/L reactors, *Aeromonas* (*Aeromonas hydrophila*, *Aeromonas caviae* or *Aeromonas sobria*), which is a gram negative facultative anaerobic bacteria, was identified at genus level in the selected colonies from 0.5 meq/L calcium concentration samples.

At this point it should be noted that some outputs of API software are not shown in Table 4.2 since further tests were required for verification.

Table 4.2. API 20E results under phosphorus deficient conditions

Calcium Ion Concentration (meq/L)	Significant Taxa	Identification (%)
0.5	*Enterobacter sakazakii*	93.4
	Citrobacter freundii	99.4
	Aeromonas genus	95.8
5	*Citrobacter freundii*	99.4
	Enterobacter sakazakii	99.5
15	*Citrobacter freundii*	99.4
	Enterobacter sakazakii	99.5

Removal efficiency of the activated sludge system is highly dependent on the C/N/P ratio of wastewater. In general, a ratio of 100/5/1 is required for proper removal and growth of microorganisms is highly dependent on nitrogen and phosphorus. Hoa *et al.* (2003) reported that there is negative correlation between nitrogen and phosphorus concentration and carbohydrate concentration. In accordance with these findings, Turtin *et al.* (2006) found that overproduction of carbohydrates under phosphorus deficient conditions resulted to viscous bulking of sludge.

Microphotographs of calcium reactors given in Figure 4.11a,b,c show that the flocs were weak, dispersed and unstable towards shear. As compared to 0.5 meq/L, further increase in calcium concentration resulted deterioration in floc structure. Sludge is dispersed further as can be seen in Figure 4.11b and c. There were no filamentous microorganisms observed under microscope. So filamentous bulking was not the reason of high SVI values (Figure 4.6). Even though the floc structures do not look too expanded, but rather dispersed, SVI values still indicated severe bulking. Knowing that the carbohydrate composition of EPS is high and observing no filamentous microorganisms under microscope, it is suggested that the type of bulking is of viscous bulking here.

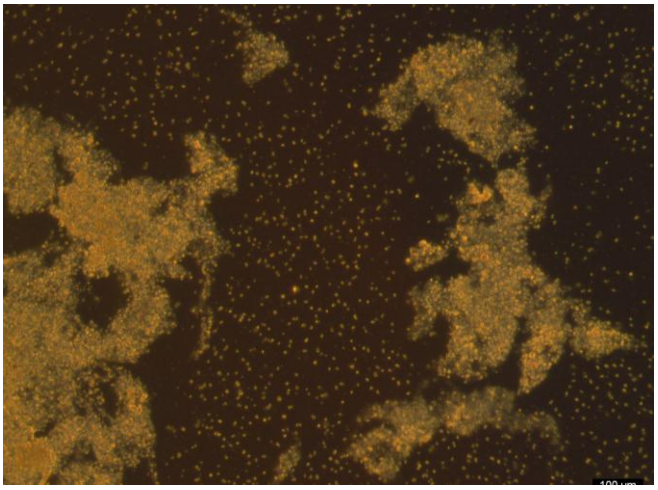

Figure 4.11a Microphotograph of 0.5 meq/L calcium reactor

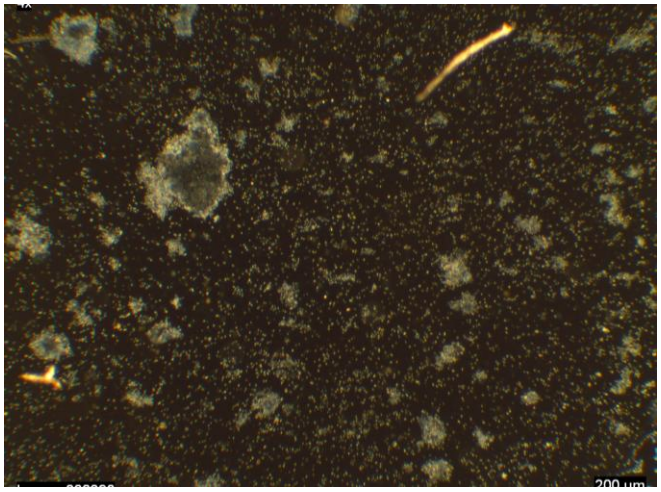

Figure 4.11b Microphotograph of 5 meq/L calcium reactor

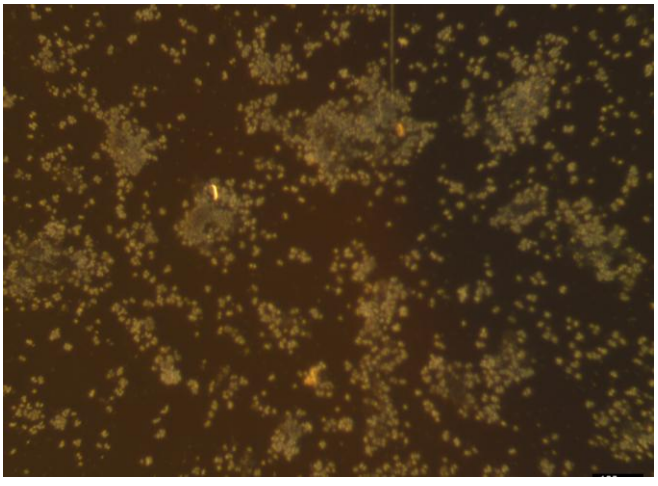

Figure 4.11c Microphotograph of 15 meq/L calcium reactor

4.2. Effect of Calcium Ion Concentration in Phosphorus Sufficient Conditions

The aim of this part is to determine the effect of calcium ion concentrations on chemical, physical and microbiological properties of activated sludge in phosphorus sufficient conditions and make a comparison of results with those in phosphorus deficient conditions. The analyses were conducted after steady state was reached. As in the case of phosphorus deficient conditions, six reactors were operated (Table 4.3). The replication between replica reactors was very good for all reactors. Therefore steady state results were reported by taking the average values of replica reactors C(1), C(2); 5(1), 5(2); 15(1), 15(2) was reported.

4.2.1. Steady State Conditions

At three different calcium concentrations 6 reactors were operated as replicas at this stage (Table 4.3). The reactors with 0.5 meq/L calcium ion concentration were selected as control reactors in order to observe the effects of increase in ion concentration. Required calcium concentrations in the reactors were provided by adding calculated amount of $CaCl_2.2H_2O$ to synthetic feed medium.

In this stage, reactors were operated at different C/N/P ratio from phosphorus deficient conditions and the increase in phosphorus concentration was achieved with the addition of precalculated amount of peptone. As in the phosphorus deficient case, all conditions were kept constant until steady state was reached, then the analyses on the reactors were conducted.

Table 4.3. Calcium ion concentrations in reactors under phosphorus sufficient conditions

Calcium Ion Concentration (meq/L)	Reactors
0.5	C(1), C(2)
5	5(1), 5(2)
15	15(1), 15(2)

As it was mentioned in literature, sludge requires at least 3 SRTs to reach steady state and before performing analyses by measuring mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations steady state was determined as explained in the first set.

4.2.2. Effect of Calcium Ion Concentration on Chemical Characteristics of Activated Sludge under Phosphorus Sufficient Conditions

As parallel to the first stage, effluent soluble chemical oxygen demand (COD), EPS composition and conductivity analyses were performed so as to determine the effect of calcium ion on chemical characteristics of sludge.

4.2.2.1. Effluent Soluble Chemical Oxygen Demand (COD)

Figure 4.12 represents effluent soluble COD concentrations. Effluent COD concentration indicated that all of the reactors were performing well when enough phosphorus was provided. Moreover, COD removal efficiency was greater at highest calcium ion concentration and the removal efficiency was greater than 95% for the entire calcium concentrations (Figure 4.13). This slight improvement in COD removal with increasing calcium concentration can be explained by the flocculation power of calcium ion. Calcium ion strengthened the floc structure by acting as a bridge by leaving much less dispersed floc fragments and soluble COD behind (Sanin and Vesilind, 2000).

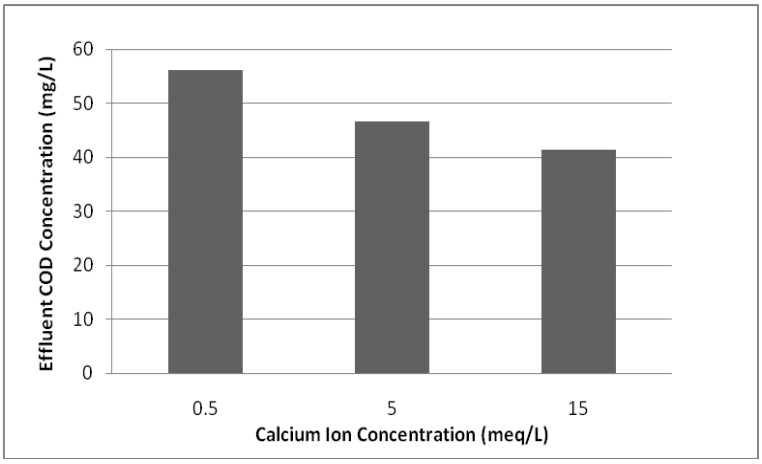

Figure 4.12. Effect of calcium ions on effluent COD concentration under phosphorus sufficient condition

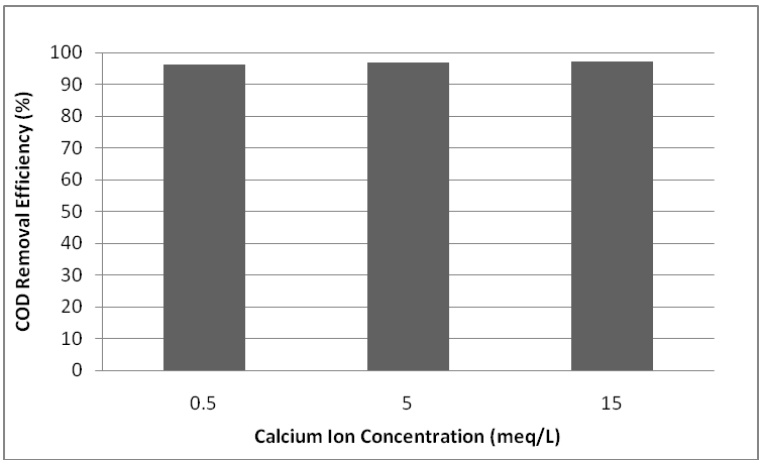

Figure 4.13. Effect of calcium ions on COD removal efficiency under phosphorus sufficient condition

4.2.2.2. Production and Composition of Extracellular Polymers

CER extraction method was performed for determining EPS as in the case of phosphorus deficiency condition. Park and Novak (2007) reported that this method is highly selective for calcium bound EPS.

Figure 4.14 represents the amount and composition of EPS at different calcium concentrations. Total EPS concentration increased when calcium concentration increased from 0.5 meq/L to 5 meq/L. Further increase to 15 meq/L led to a smaller decrease in total EPS concentration. However, total EPS concentration can not be taken as the only consideration to determine the effect of operational conditions on EPS, composition of EPS should be considered as well. According to Figure 4.15, protein content of EPS increased with increment in calcium concentration. For 5 meq/L and 15 meq/L calcium concentrations, EPSp/EPSc ratio was greater than 1 and almost 2 for 15 meq/L. Many studies in literature reported that exocellular protein concentration was greater than carbohydrate concentration (Tenney and Verhoff, 1973; Brown and Lester, 1980; Barber and Veenstra, 1986; Eriksson and Alm, 1991; Urbain *et al.*, 1993; Higgins *et al.*, 1997a, b; Jorand *et al.*, 1998). In this sense, it can be said that under phosphorus sufficient conditions, as the calcium concentration increased, protein type polymers become predominant.

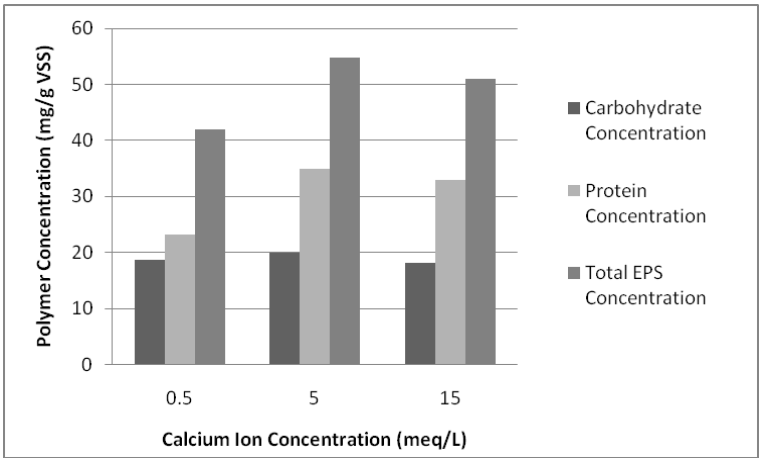

Figure 4.14. Effect of calcium concentration on production and composition of EPS under phosphorus sufficient condition

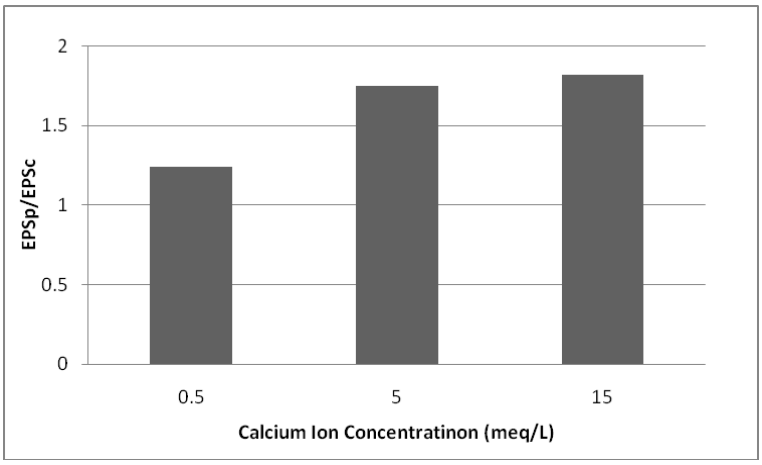

Figure 4.15. EPSp/EPSc ratio with respect to calcium concentrations under phosphorus sufficient condition

4.2.2.3. Electrical Conductivity

Figure 4.16 represents the electrical conductivity results. As it was expected electrical conductivity increased with increase in calcium concentration in solution and gets the highest value at 15 meq/L calcium concentration.

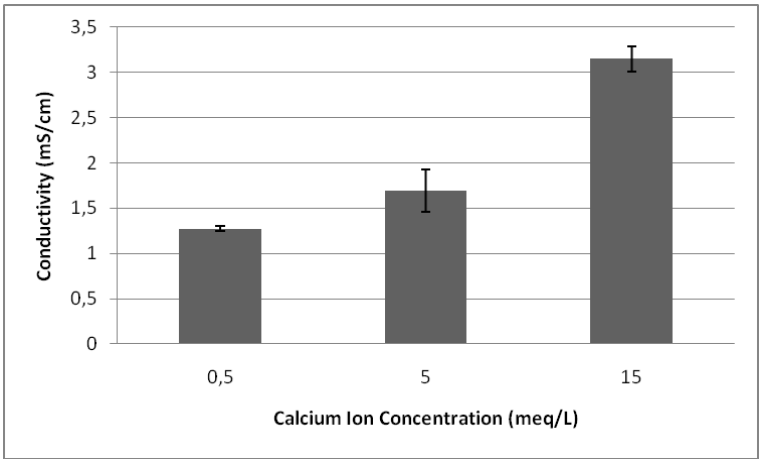

Figure 4.16. Conductivity values with respect to calcium concentrations under phosphorus sufficient condition

4.2.3. Effect of Calcium Ion on Physical Characteristics of Activated Sludge under Phosphorus Sufficient Conditions

Capillary suction time (CST), sludge volume index (SVI), viscosity and turbidity analyses were conducted to determine the effect of calcium on dewaterability, settleability and rheology of activated sludge.

4.2.3.1. Dewaterability

Dewaterability analyses were conducted through measuring capillary suction time (CST). In literature it was mentioned that removal of calcium ions from sludge leads to deterioration in dewaterability of sludge. And settling and dewatering properties of sludge are improved by increasing calcium concentration (Bruus *et al.*, 1992; Higgins and Novak 1997a). As it is seen from Figure 4.17, CST value for control reactor is 9.1 s and it decreases to 7.6 s for 5 meq/L and further increases to 8.6 s for 15 meq/L. Although there is not a decreasing trend consistent with literature, CST values for all concentrations are so small and it can be said that dewatering property of sludge was quite good when phosphorus was sufficiently provided to the reactors.

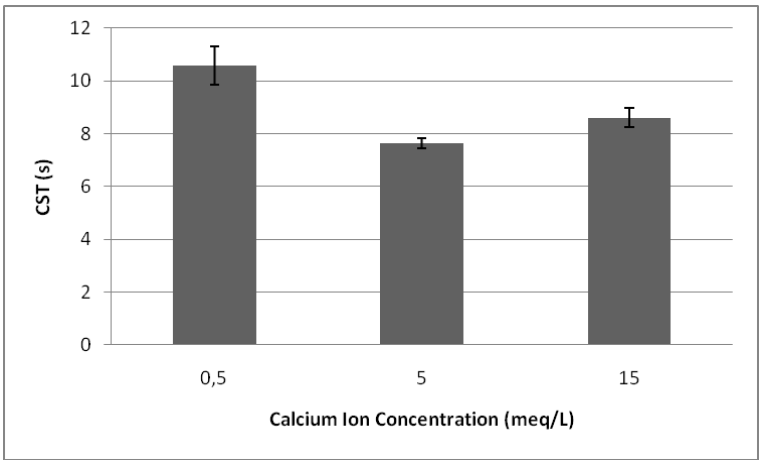

Figure 4.17. CST values with respect to calcium concentrations under phosphorus sufficient condition

4.2.3.2. Settleability

Sludge volume index (SVI) values are shown in Figure 4.18. Rising calcium concentrations resulted decrease in SVI and therefore improved settleability of sludge. For the entire calcium concentrations, SVI value was recorded as lower than 150 and none of the sludges were bulking in this set. The results indicate that there is a relation between carbohydrate content of EPS and SVI. SVI value increases with the increase of carbohydrate type EPS which is also supported by the findings of Durmaz and Sanin (2003). This previous study indicated that increase in carbohydrate concentration led to deterioration in settleability of sludge, which is also the trend observed in this work.

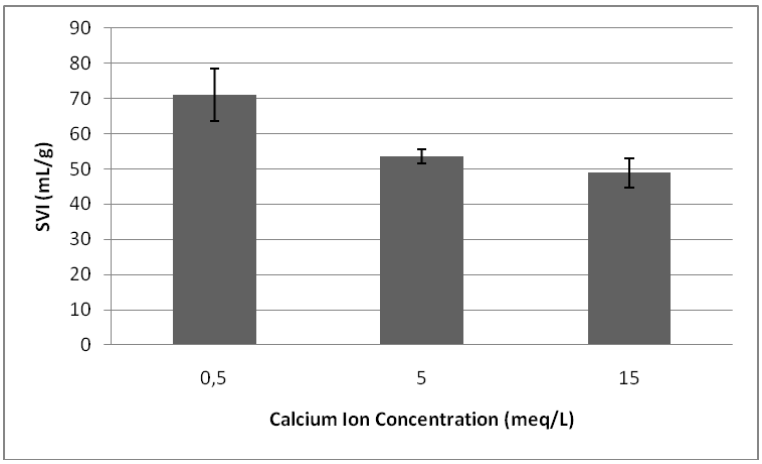

Figure 4.18. SVI values with respect to calcium concentration under phosphorus sufficient condition

4.2.3.3. Rheology

Shear stress versus shear rate graphs were plotted so as to determine the flow behavior of sludge samples. After testing Newtonian and non-Newtonian flow models, as it can be seen in Figure 4.19, that the curves exhibited highest fit to non-Newtonian pseudoplastic flow (Equation 4.2). When the flow behavior index (n) of pseudoplastic flow deviates more from 1, the flow behavior deviates more from Newtonian. Increase in calcium concentration from 0.5 meq/L to 5 meq/L was resulted reduction in n. However, further increase in calcium concentration caused increase in n value.

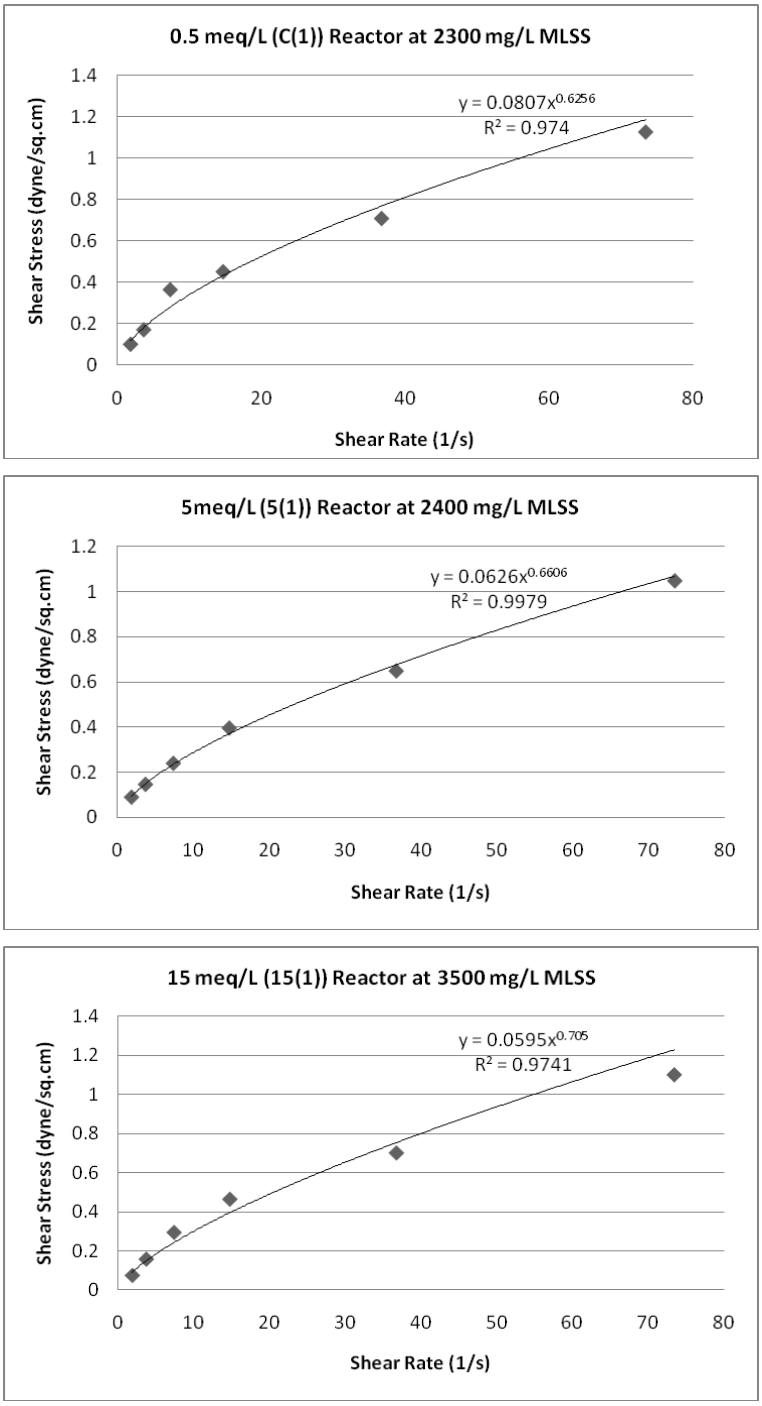

Figure 4.19. Rheograms of phosphorus sufficient activated sludge samples at different calcium concentrations

The effect of solids concentration on viscosity is shown in Figure 4.20. The apparent viscosities were plotted for 4 different solids concentrations at a constant shear rate of 73.4 sec^{-1}. As it can be observed from this figure, apparent viscosity values of 5 meq/L and 15 meq/L calcium concentrations slightly change as the MLSS concentration increases but there is an increasing trend for 0.5 meq/L concentration.

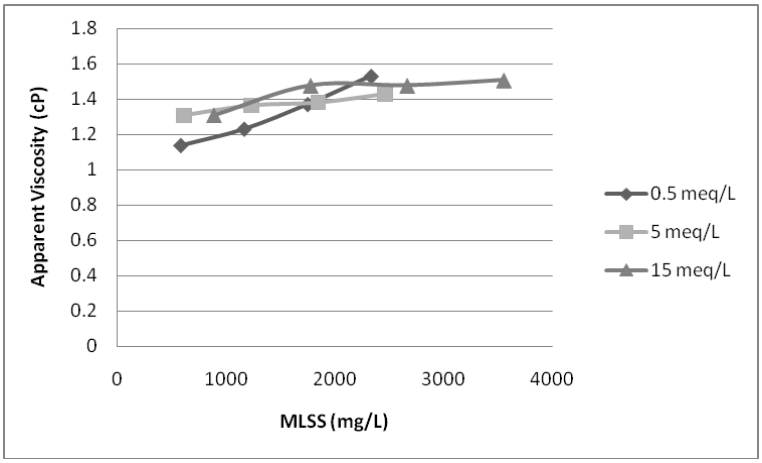

Figure 4.20. Apparent viscosities at different calcium concentrations at a shear rate of 73.4 sec^{-1}

In order to determine the relation between calcium ion concentration and viscosity, apparent viscosity versus calcium ion concentration graph was plotted at a constant MLSS value of 1500 mg/L. Figure 4.21 showed that, even if increments are so slight, there is a positive correlation between calcium ion concentration and viscosity. In literature it was mentioned that EPS affect the viscosity of sludge (Forster, 1985a). Moreover, Sanin (2002) reported that polymeric substances have high molecular mass and high viscosity. In this sense, it can be said that increase in EPS production led to an increase in viscosity.

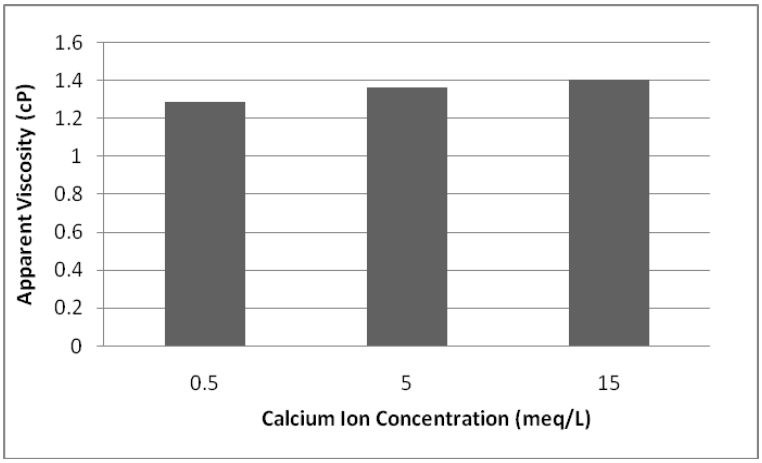

Figure 4.21. Apparent viscosity values with respect to calcium concentration at 1500 mg/L MLSS

4.2.3.4. Turbidity

Turbidity measurements were performed after 1 hour settlement of samples. According to the Figure 4.22, there is a slight decrease in turbidity from 0.5 meq/L to 5 meq/L, but further addition of calcium to 15 meq/L led to an in increase in turbidity. However, in all the reactors turbidities measured were very close to each other and near the desirable values (20-40 NTU) for activated sludge effluents.

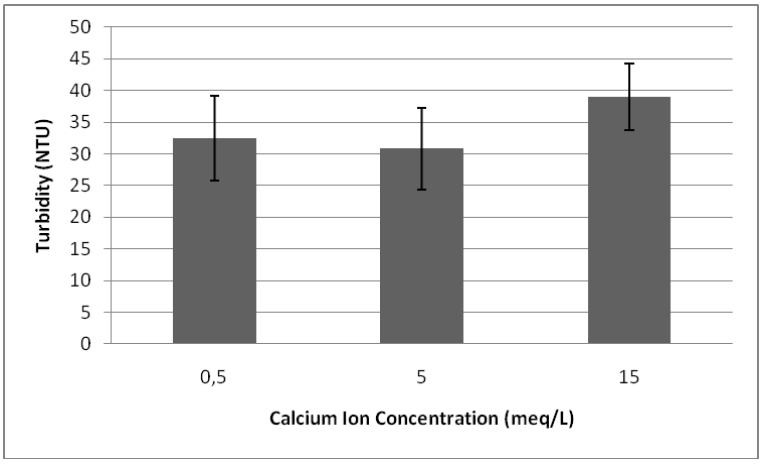

Figure 4.22. Turbidity values with respect to calcium concentration under phosphorus sufficient condition

4.2.4. Microbiology of Activated Sludge under Phosphorus Sufficient Conditions

API 20E and API Coryne Kit Systems were determined as the most suitable microbial identification tests right after gram staining, oxidase and catalase tests applied to the discrete colonies selected from 0.5 meq/L, 5 meq/L and 15 meq/L calcium concentrations. In contrast to common findings related to gram staining of activated sludge microorganisms, some colonies were noted as Gram-positive.

Enterobacter cloacae and *Enterobacter sakazakii* were determined as the common microorganisms for 5 meq/L and 15 meq/L calcium concentrations. Disparately, *Corynebacterium propinquum*, *Brevibacterium* spp. and *Arthrobacter* spp. were identified at lower calcium concentration (Table 4.4).

Enterobacter sakazakii and *Enterobacter cloacae* are motile, Gram-negative, oxidase negative, catalase positive, facultatively anaerobe and rod-shaped bacteria belonging to the Enterobacteriaceae family. *Enterobacter sakazakii* can ferment glucose and produce a yellow pigment while *Enterobacter cloacae* can ferment D-sorbitol (Sanders and Sanders, 1997).

Brevibacterium spp. belonging to Brevibacteriaea family and *Corynebacterium propinquum* belonging to Corynebacteriaea family are both Gram-positive bacteria. *Brevibacteria* is a short, nonbranched, obligately aerobic, catalase positive, and non-fermenting bacteria (Gruner *et. al*, 1993). *Corynebacterium propinquum* is a rod-shaped, non-fermenting corynebacteria and contrary to the other non-fermenting corynebacteria, it degrades tyrosine (Funke *et. al*, 1997).

Arthrobacter spp. are Gram-positive, strict aerobes that can oxidize a wide variety of organic compounds. As the culture ages, the morphology of this bacteria changes and this is the distinguishing characteristic of it. The young cells are irregularly shaped rods usually in short chains. As the culture ages, the rods divide into cocci and they often stay attached in the original configuration and resulted in angular, rigid chains (Gaudy and Gaudy, 1980).

As in the case of phosphorus deficiency, some outputs of API software was not shown in Table 4.4 due to the requirements of further tests for verification.

Table 4.4. API 20E and API Coryne results under phosphorus sufficient conditions

Calcium Ion Concentration (meq/L)	Significant Taxa	Identification (%)
0.5	*Brevibacterium* spp.	94.9
	Corynebacterium propinquum	91.2
	Arthrobacter spp.	99.9
5	*Enterobacter sakazakii*	91.1
	Enterobacter cloacae	95.1
15	*Enterobacter sakazakii*	91.1
	Enterobacter cloacae	95.1

Microphotographs of sludge samples under phosphorus sufficient conditions are shown in Figure 4.23a, b, c. As it can be seen from this figure, increase in calcium concentration from 0.5 meq/L to 5 meq/L resulted in improve of floc structure. Flocs were compact and firm. Moreover, filamentous microorganisms that are crucial for healthy floc structure were also observed during microscopic examinations.

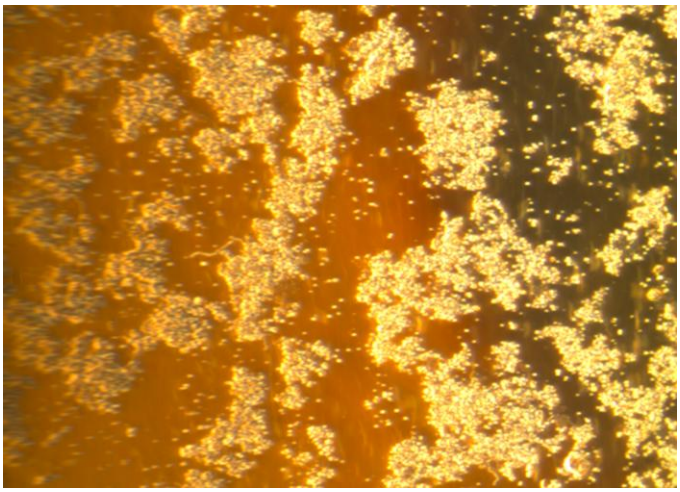

Figure 4.23a Microphotograph of 0.5 meq/L calcium reactor

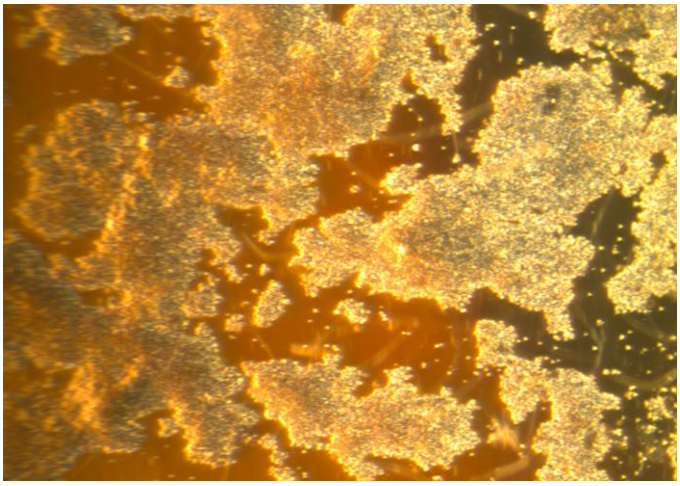

Figure 4.23b Microphotograph of 5 meq/L calcium reactor

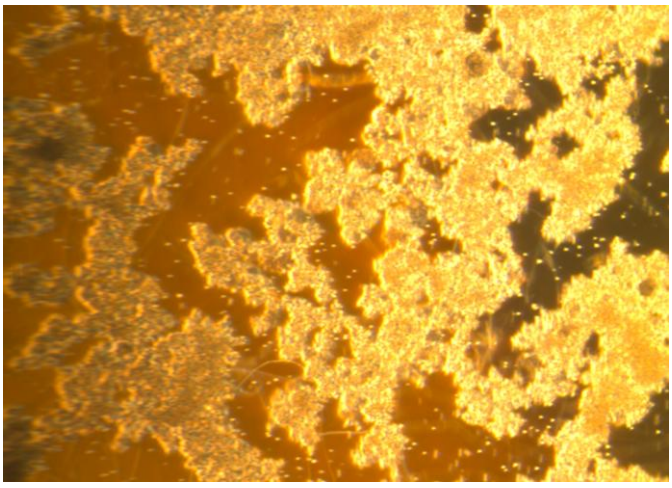

Figure 4.23c Microphotograph of 15 meq/L calcium reactor

4.3. Comparison of Results of Phosphorus Deficient and Phosphorus Sufficient Reactors

After performing analyses, it was observed that calcium ion and phosphorus concentration affects sludge characteristic differently. This section includes comparison and discussion about the presence of calcium ion under phosphorus deficient and phosphorus sufficient conditions.

4.3.1. Comparison of Chemical Characteristics

4.3.1.1 Effluent Soluble Chemical Oxygen Demand (COD)

The effluent COD concentrations represented similar tendency of calcium concentration for phosphorus deficient and phosphorus sufficient reactors. As the calcium concentration increased, effluent COD concentration decreases in each case (Table 4.5). However, it should be noted that under phosphorus deficiency conditions effluent COD concentration was greater than influent (1308 mg/L) and therefore rather than removal, COD was contributed to the effluent. On the other hand, effluent COD removal efficiency of the reactors operated under phosphorus sufficient conditions was greater than 95% for each calcium concentration. This indicated that all of the reactors were performing well when enough phosphorus was provided. This clearly indicated the importance of phosphorus in the feed to the activated sludge system.

Table 4.5. Effluent COD concentration in phosphorus deficient and phosphorus sufficient conditions

Calcium Ion Concentration (meq/L)	Effluent COD Concentration (mg/L)	
	P – Deficient Condition	P – Sufficient Condition
0.5	3365	56
5	1636	46
15	1544	41

4.3.1.2. Production and Composition of Extracellular Polymers

As it can be seen from Table 4.6, the increase in phosphorus concentration was positively correlated with increase in total EPS concentration for all calcium concentrations except for 15 meq/L. Under phosphorus deficient conditions, highest amount of EPS was produced by 15 meq/L reactor but further increase in phosphorus concentration to sufficient levels led to decrease in total EPS production at 15 meq/L calcium concentration. However this decrease was not considered to be a very significant one.

In addition to the amount produced, composition of EPS changed significantly by rising phosphorus concentration. Under phosphorus deficient conditions, except for the control reactor, greater than 70% of total EPS produced was composed of carbohydrates. Under this operational condition, sludges exhibited viscous bulking. This is also supported by the findings of Turtin *et al.* (2006). Contary to the decrease in 0.5 meq/L, switching from phosphorus deficient to sufficient resulted in increase in EPSp/EPSc ratio of 5 meq/L and 15 meq/L calcium concentrations. Under phosphorus sufficient conditions for all calcium concentrations protein composition of EPS was greater than 50%. Higgins and Novak (1997a) reported that increase in divalent cations in the feed resulted increase in bound protein content.

In this sense, it can be stated that under phosphorus deficiency, calcium ion stimulates the production of carbohydrate type polymers. And it is evident that carbohydrate dominancy resulted in viscous bulking of sludge. As the phosphorus concentration increased to stoichiometrically required value, sludge properties improved significantly.

Table 4.6. Production and composition of EPS under phosphorus deficient and phosphorus sufficient conditions

	Calcium Ion Concentration (meq/L)	EPSp (mg/g VSS)	EPSc (mg/g VSS)	Total EPS Concentration (mg/g VSS)	EPSp/EPSc
P Deficient Condition	0.5	8.3	7.3	15.6	1.14
	5	7.2	21	28.2	0.34
	15	13.1	47	60.2	0.28
P Sufficient Condition	0.5	23.2	18.7	42	1.24
	5	34.8	19.9	54.7	1.75
	15	32.9	18	51	1.82

4.3.1.3. Electrical Conductivity

As it was expected for each condition, the increase in conductivity was associated with an increase in calcium ion concentration added to the reactors. However, when the individual values of conductivity under phosphorus deficient condition was compared with phosphorus sufficient condition, a remarkable decrease was observed when sufficient phosphorus was added to the reactors (Table 4.7). This result suggests that under phosphorus sufficient condition, calcium ions tend to be associated within the floc structure rather than staying in the solution.

Table 4.7. Conductivity values with respect to calcium concentration under phosphorus deficient and phosphorus sufficient conditions

Calcium Ion Concentration (meq/L)	Conductivity (mS/cm)	
	P – Deficient Condition	P – Sufficient Condition
0.5	4.5	1.2
5	6.4	1.7
15	10.2	3.1

4.3.2. Comparison of Physical Characteristics

4.3.2.1. Dewaterability

CST analyses were conducted to determine the dewaterability of activated sludge under phosphorus deficient and phosphorus sufficient conditions. During the phosphorus deficient set, the CST values were ranging between 32 to 306 sec indicating that phosphorus deficient sludges are definitely very difficult to dewater. This clearly shows that the sludge floc structure is not properly developed when there is insufficient phosphorus in the system. The CST results in Table 4.8 show that increase in phosphorus concentration in the feed from deficient to sufficient led to decrease in CST for all calcium ion concentrations.

As a result, dewaterability of sludge was greatly improved when enough phosphorus was provided in the feed which means that under sufficient phosphorus condition, sludge became easier to release its water and easier to dewater.

When the EPSp/EPSc ratios for phosphorus deficient and phosphorus sufficient conditions are compared, it is recorded that increase in EPSp concentration are correlated with increase in phosphorus concentration. In this sense, it can be concluded that EPSp content also has a positive impact on the improvement of dewatering properties of sludge that is also supported by the findings of Higgins and Novak (1997a).

Table 4.8. CST values with respect to calcium concentration under phosphorus deficient and phosphorus sufficient conditions

Calcium Ion Concentration (meq/L)	CST (s)	
	P – Deficient Condition	P – Sufficient Condition
0.5	32	10.5
5	120	7.6
15	306	8.6

4.3.2.2. Settleability

SVI values of phosphorus deficient and phosphorus sufficient reactors are given in Table 4.9. Results indicate that phosphorus concentration has significant effect on settleability of sludge; settleability improves by increasing phosphorus concentration in the feed. Under phosphorus deficient operational conditions all of the sludges were severely bulking and SVI values are greater than 150. On the other hand, none of the sludges were bulking under phosphorus sufficient conditions and this indicates that phosphorus, when it is provided in sufficient amounts, cures the sludge bulking problem. Improvement in settling properties of sludge is associated with the decrease in carbohydrate content of EPS. Durmaz and Sanin (2003) reported that deterioration in settleability of sludge is correlated to high carbohydrate concentration which is also observed in this study.

Table 4.9. SVI values with respect to calcium concentration under phosphorus deficient and phosphorus sufficient conditions

Calcium Ion Concentration (meq/L)	SVI (mL/g)	
	P – Deficient Condition	P – Sufficient Condition
0.5	468	71
5	429	53
15	408	49

4.3.2.3. Rheology

As it can be seen in Table 4.10, apparent viscosity values under phosphorus deficient conditions is higher than the viscosities of phosphorus sufficient conditions. This is probably related to the bulking property of sludge observed in phosphorus deficient case. After addition of sufficient amount of phosphorus to the system, viscosity was improved which was correlated to pumpability of sludge.

Rheological analyses showed that, except for Bingham plastic behavior of 0.5 meq/L calcium concentration reactor operated under phosphorus deficient conditions, the flow behavior of sludge in each case of phosphorus concentration exhibited non-Newtonian pseudoplastic behavior. This is in accordance with the findings of literature that activated sludge has been reported as either Bingham plastic (Dick and Ewing, 1967; Unno and Akehata, 1985; Dick, 1986) or pseudoplastic (Behn, 1962; Moeller and Torres, 1997). However, the rheograms observed in phosphorus sufficient condition are more curved (n values are much smaller than 1) compared to those ones observed in the phosphorus deficient condition. This indicates different development in floc structure between two operational conditions.

Table 4.10. Apparent viscosities under phosphorus deficient and phosphorus sufficient conditions at 1500 mg/L MLSS

Calcium Ion Concentration (meq/L)	Apparent Viscosity (cP)	
	P – Deficient Condition	P – Sufficient Condition
0.5	4.41	1.28
5	3.08	1.36
15	2.99	1.40

4.3.2.4. Turbidity

Turbidity values under different phosphorus concentrations did not exhibit a trend with respect to calcium concentration. However, in all reactors turbidities measured under phosphorus sufficient conditions were very close to each other (Table 4.11) and near the desirable values for activated sludge effluents. On the other hand these turbidity values measured under phosphorus sufficient conditions are remarkably smaller than those ones measured under phosphorus deficient conditions. Moreover, the positive effect of increase in phosphorus concentration on turbidity is also in perfect agreement with settleability improvement with sufficient phosphorus in the system.

Table 4.11. Turbidity values with respect to calcium concentration under phosphorus deficient and phosphorus sufficient conditions

Calcium Ion Concentration (meq/L)	Turbidity (NTU)	
	P – Deficient Condition	P – Sufficient Condition
0.5	308	32
5	894	30
15	854	39

4.3.3. Comparison of Microbiological Characteristics

The results of the microbiological analyses showed that phosphorus amount in feed medium affects microbiology of sludge substantially. Under phosphorus deficient operating conditions, all selected colonies were recorded as Gram-negative. When the phosphorus concentration was increased, Gram-positive bacteria were also identified beyond Gram-negative ones in the samples of least calcium concentration by API Coryne tests. The identification of Gram-positive bacteria indicates the presence of filamentous microorganisms in the activated sludge system under phosphorus sufficient operational conditions.

Another remarkable result of microbiological analyses can be noted as the species identified by API 20E. Same *Enterobacter* and *Citrobacter* species were identified at all calcium concentrations under phosphorus deficiency and *Enterobacter* species were common at 5 meq/L and 15 meq/L concentrations in sufficient phosphorus conditions.

In addition to bacteriological identifications, microphotographs of activated sludge samples revealed that there is a strong correlation between flocculation and concentration of phosphorus and calcium in the feed. Increase in calcium concentration at phosphorus deficient conditions resulted deterioration in flocculation, flocs were weak and dispersed. On the other hand when phosphorus was sufficient in feed medium, rising calcium concentration led to the improvement of flocculation (Figure 4.11a, b,c and Figure 4.23a, b, c).

CHAPTER 5

CONCLUSIONS

Nutrients and cations have significant effect on activated sludge characteristics. This study determined the effect of calcium on chemical, physical and microbiological characteristics of activated sludge under phosphorus deficient operation conditions.

The specific conclusions drawn from this study are as follows:

- Under phosphorus deficient conditions, effluent COD concentrations were greater than influent COD for all calcium concentrations which mean that COD was contributed to the system by the on settling particulates and floc fragments. However, when the phosphorus concentration in the feed was increased to sufficient level, increase in calcium concentration was correlated to decrease in effluent COD and greater than 95 % removal efficiency for all calcium concentration was recorded.

- Results indicated that amount and composition of EPS changed at different concentrations of calcium in the presence and absence of phosphorus. In phosphorus deficient case, except for the control reactor carbohydrate type polymers were recorded as dominant type of EPS. Furthermore, increase in calcium concentration led to an increase in production of carbohydrate type polymers which can be concluded that calcium ion favors the production of carbohydrate type polymers and viscous bulking was observed. Contrary to these findings, as the phosphorus level adjusted to the stoichiometrically required value, sludge became rich in terms of EPSp except for the control reactor. Moreover, increase in phosphorus concentration resulted in an increase in total EPS produced except for 15 meq/L calcium concentration.

- The increase in calcium concentration led to increase in electrical conductivity. However the conductivity values were much smaller under phosphorus sufficient conditions as compared to deficient case. This could be the reason of association of calcium ion into the floc structure rather than stay in the solution when enough phosphorus was provided to the system.

- Settleability and dewaterability of sludge improved as the phosphorus concentration increased, sludge turned from bulking sludge into a well-settling non-bulking sludge when enough phosphorus was provided. At sufficient level of phosphorus, protein content of EPS increased and both CST and SVI values at all calcium concentration decreased.

- Rheological analyses conducted under phosphorus sufficient and deficient conditions showed that sludge in each case exhibited pseudoplastic behavior except for the control reactor operated under phosphorus deficient conditions. Moreover, apparent viscosity values sharply decreased with increase in phosphorus concentration and therefore pumpability of sludge improved.

- Although there was not a trend with respect to calcium concentration, turbidity measurements indicated that increase in phosphorus concentration in the feed led to the significant improvement in effluent turbidity.

- Results of microbiological analyses indicated that phosphorus and calcium concentrations affect the microbial characteristics of sludge. Under phosphorus deficient conditions, all selected colonies were recorded as Gram-negative and same *Enterobacter* and *Citrobacter* species were identified. On the other hand, with increase in phosphorus concentration types of microorganisms were changed. However, same *Enterobacter* species were identified in 5 meq/L and 15 meq/L reactors. Contrary to the findings of phosphorus deficient conditions, Gram-positive colonies were recorded at 0.5 meq/L calcium concentration when there was sufficient phosphorus in the system.

- In addition to this, microphotographs of sludge samples indicated that under phosphorus deficiency, flocs were weak, dispersed, nonresistant and flocculation deteriorated as calcium concentration increased. In this operating condition, viscous bulking was observed. Yet, rise in phosphorus and calcium concentration led to the improvement of flocculation.

CHAPTER 6

RECOMMENDATIONS FOR FUTURE WORK

When the results of this study are considered, it is observed that for future works there are some points needed to be investigated. These are as follows:

- The results of the study showed that increase in calcium concentration in phosphorus sufficient conditions led to improve sludge properties. Therefore the impact of further addition of calcium concentration on sludge properties can be investigated.

- This study includes the effect of calcium ion on physical, chemical and microbiological characteristics of sludge. And further researches can be conducted about the effect of calcium ion on surface chemical characteristics of sludge including hydrophobicity and zeta potential.

- Finally, determining the microbiological properties of activated sludge under these operational conditions might be more extensively evaluated by applying molecular biology techniques such as 16S rRNA gene sequencing for identification of bacterial strains.

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APPENDIX A

SOLIDS CONCENTRATIONS OF REACTORS UNDER PHOSPHORUS DEFICIENT AND SUFFICIENT CONDITIONS

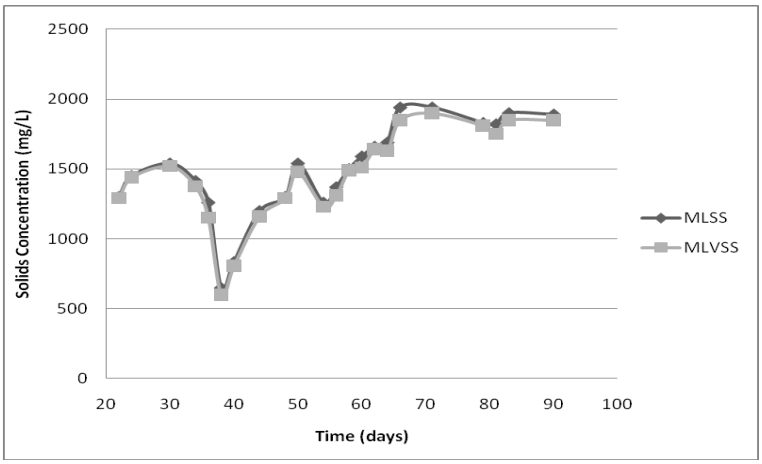

Figure A.1. Solids concentration versus time graph for C(1) reactor under phosphorus deficient condition (Ca = 0.5 meq/L)

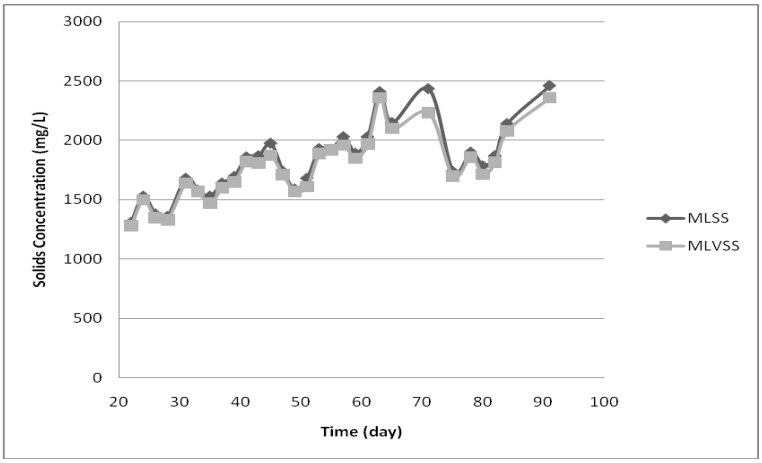

Figure A.2. Solids concentration versus time graph for C(2) reactor under phosphorus deficient condition (Ca = 0.5 meq/L)

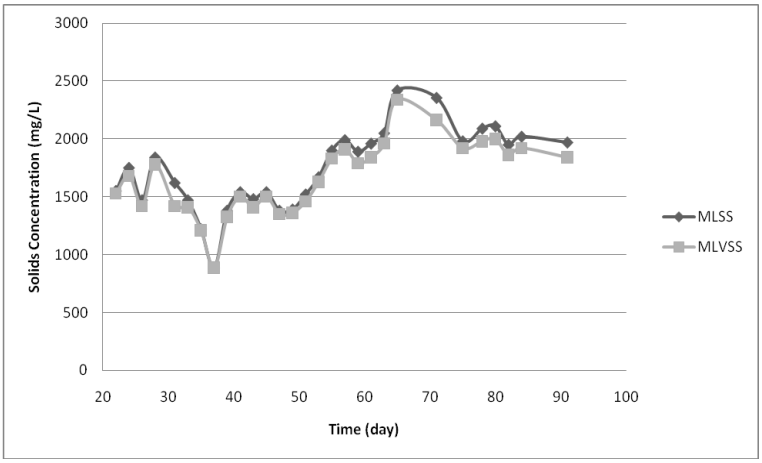

Figure A.3. Solids concentration versus time graph for 5(1) reactor under phosphorus deficient condition (Ca = 5 meq/L)

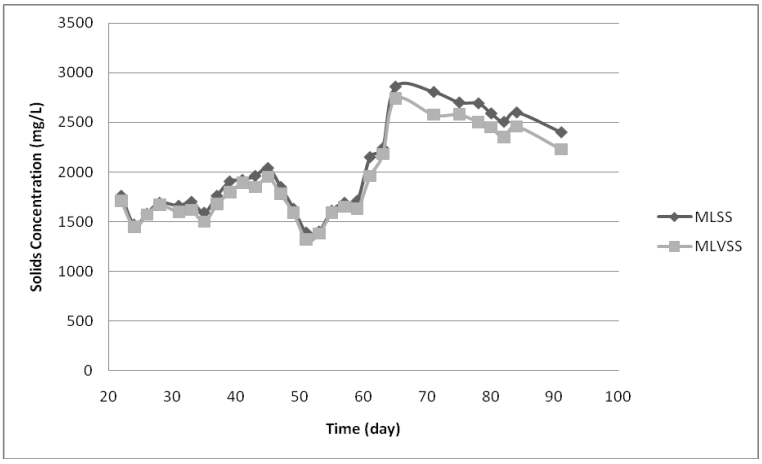

Figure A.4. Solids concentration versus time graph for 5(2) reactor under phosphorus deficient condition (Ca = 5 meq/L)

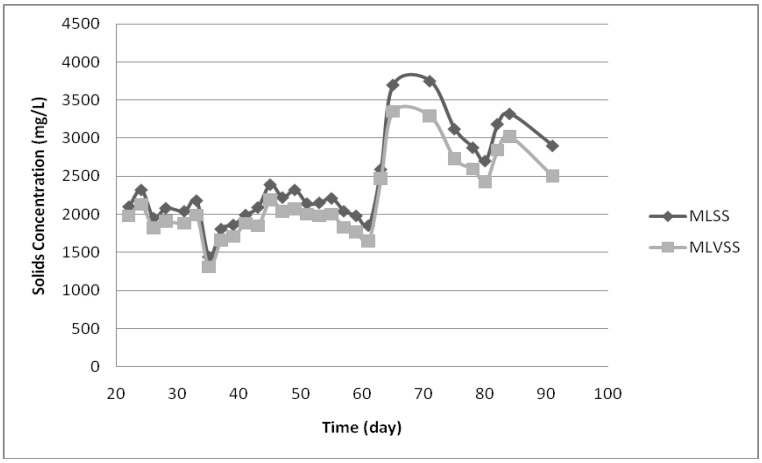

Figure A.5. Solids concentration versus time graph for 15(1) reactor under phosphorus deficient condition (Ca = 15 meq/L)

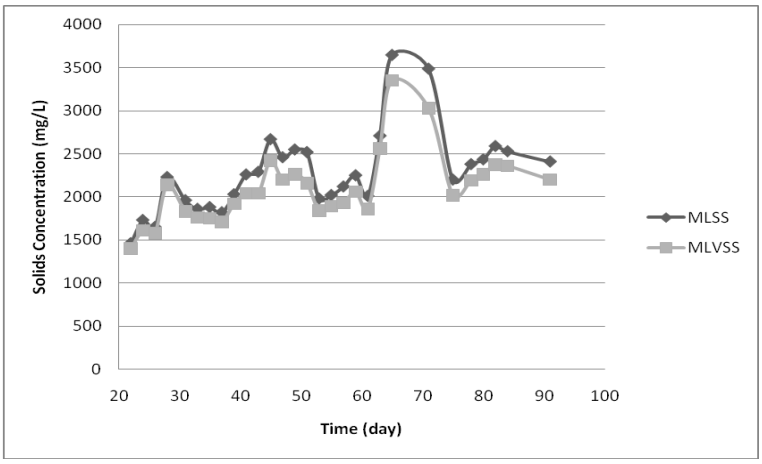

Figure A.6. Solids concentration versus time graph for 15(2) reactor under phosphorus deficient condition (Ca = 15 meq/L)

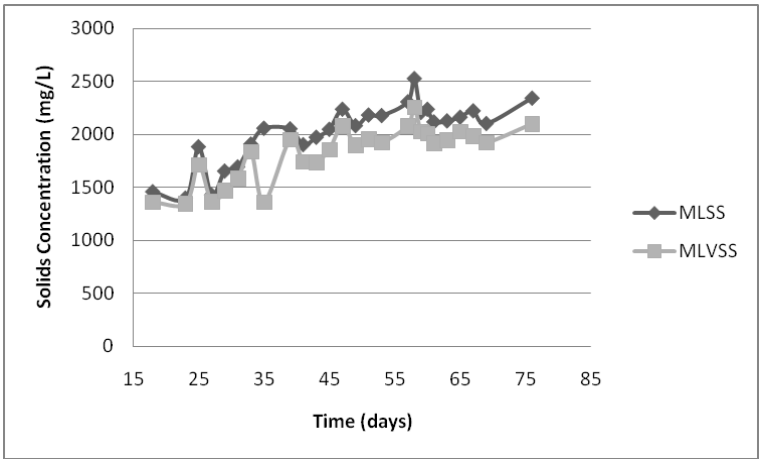

Figure A.7. Solids concentration versus time graph for C(1) reactor under phosphorus sufficient condition (Ca = 0.5 meq/L)

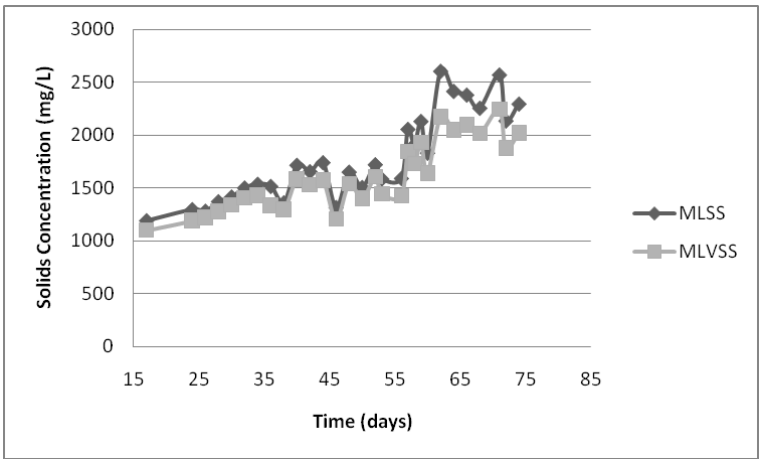

Figure A.8. Solids concentration versus time graph for C(2) reactor under phosphorus sufficient condition (Ca = 0.5 meq/L)

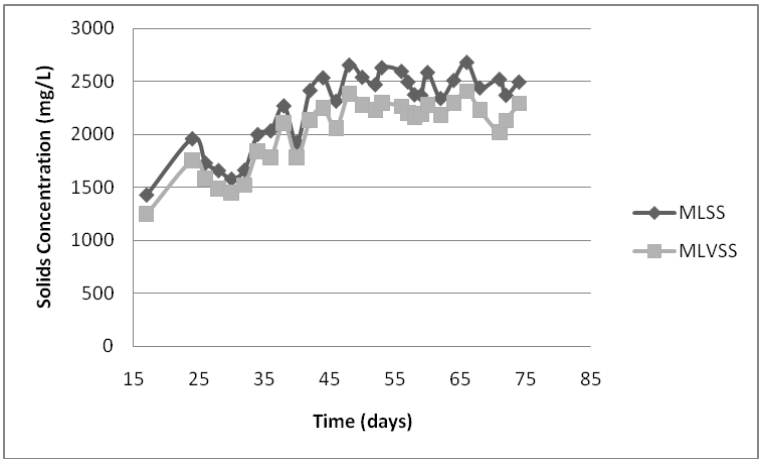

Figure A.9. Solids concentration versus time graph for 5(1) reactor under phosphorus sufficient condition (Ca = 5 meq/L)

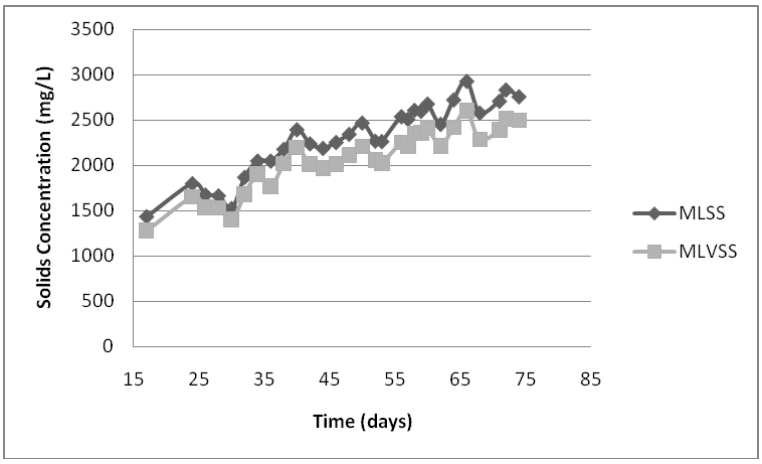

Figure A.10. Solids concentration versus time graph for 5(2) reactor under phosphorus sufficient condition (Ca = 5 meq/L)

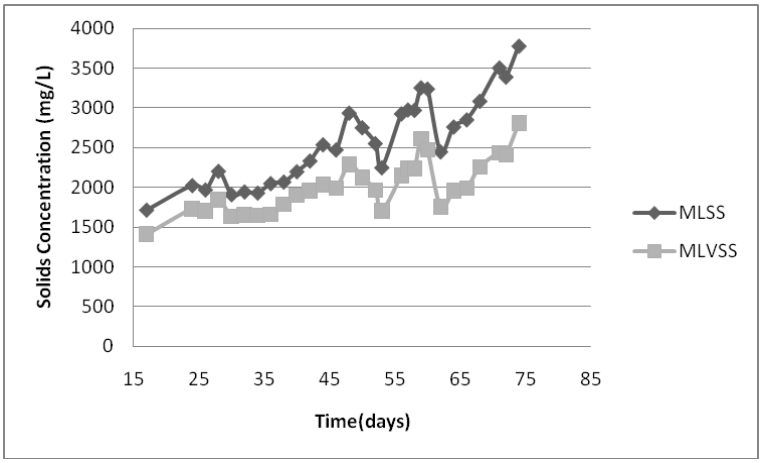

Figure A.11. Solids concentration versus time graph for 15(1) reactor under phosphorus sufficient condition (Ca = 15 meq/L)

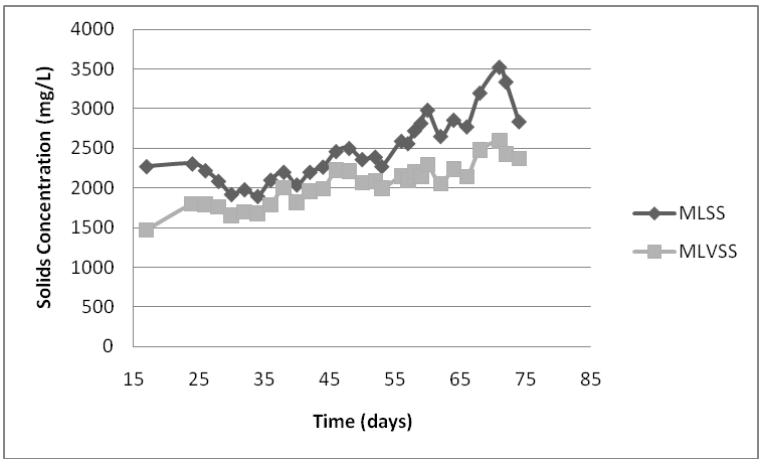

Figure A.12. Solids concentration versus time graph for 15(2) reactor under phosphorus sufficient condition (Ca = 15 meq/L)

APPENDIX B

CALIBRATION CURVES FOR EPS CHARACTERIZATION

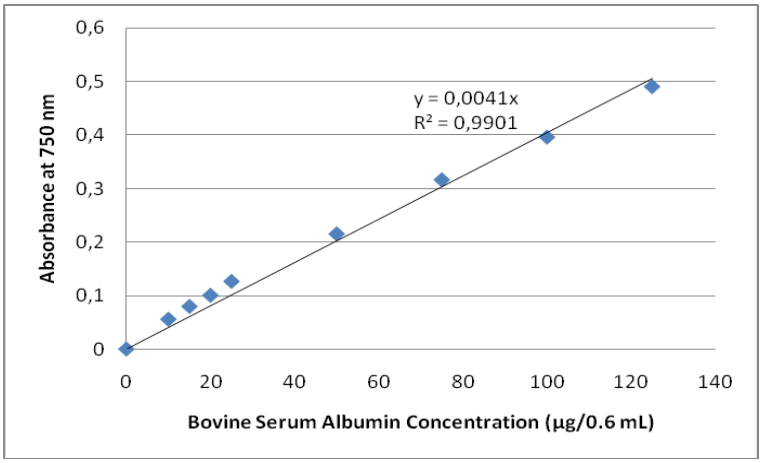

Figure B.1. Calibration curve for protein by using Lowry Method under phosphorus deficient conditions

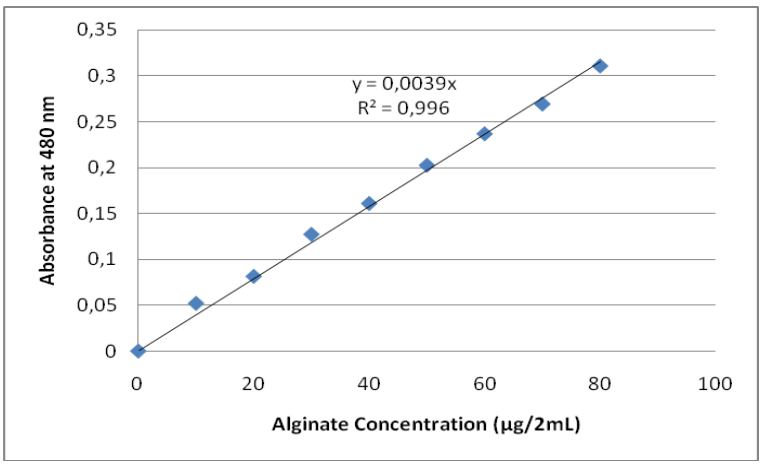

Figure B.2. Calibration curve for carbohydrate by using Dubois Method under phosphorus deficient conditions

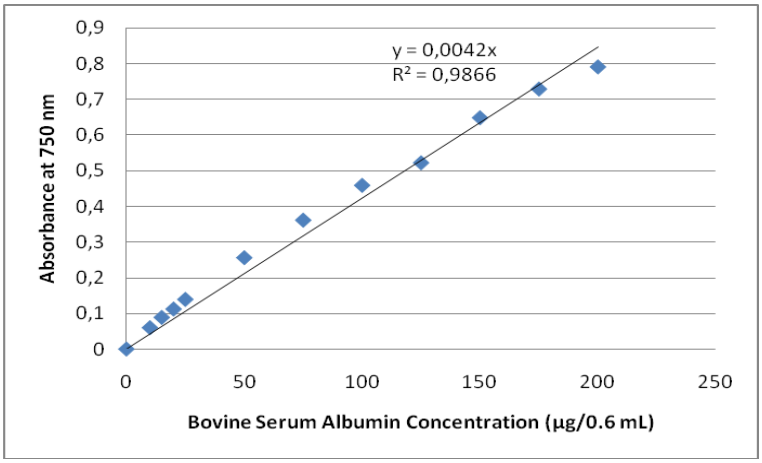

Figure B.3. Calibration curve for protein by using Lowry Method under phosphorus sufficient conditions

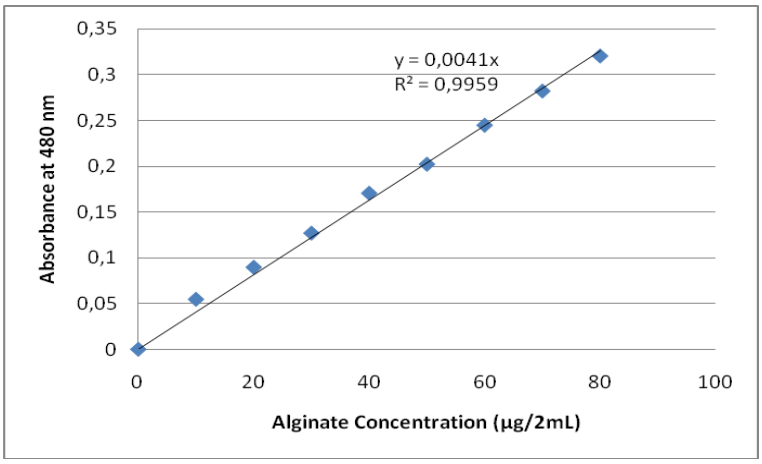

Figure B.4. Calibration curve for carbohydrate by using Dubois Method under phosphorus sufficient conditions