INVESTIGATION OF MAGNESIUM IONS EFFECT ON SLUDGE PROPERTIES IN PHOSPHORUS DEFICIENT BIOREACTORS

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ABSTRACT

INVESTIGATION OF MAGNESIUM IONS EFFECT ON SLUDGE PROPERTIES IN PHOSPHORUS DEFICIENT BIOREACTORS

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The activated sludge process efficiency depends on separation of microbial cells from treated wastewater. Separation can fail due to a number of problems. One of these problems is sludge bulking which is non-settling situation of biomass. Former studies showed that phosphorus deficiency caused filamentous sludge bulking with increasing magnesium ion concentrations. The main objectives of this study are to find out the effect of magnesium ions on sludge properties in phosphorus deficient medium and to determine if there is any bulking. Three different concentrations of magnesium (0.5, 5, 15 meq/L) were added to three bioreactors which contained phosphorus deficient medium. In first set C: N: P ratio was 100:5:0.05. In second set, C:N:P ratio was elevated to 100:5:1. At steady state, physical characteristics including sludge volume index (SVI), viscosity, turbidity and dewaterability were determined. Besides concentration of extracellular polymeric substances (EPS) as well as conductivity was measured. By using API kits, bacterial identification was achieved.

In first set phosphorus deficiency and increasing magnesium ion concentration caused filamentous bulking. Carbohydrate content of extracellular polymeric substance significantly increased by magnesium addition. Dewaterability of the system got worse and viscosity decreased. Sludge Volume Index (SVI) indicated severe bulking at all magnesium concentrations. By using biochemical tests microorganisms dominant in the system were determined

In second set, all of the parameters indicated healthy flocculation. By magnesium addition, EPSp and EPSc increased. Dewaterability and settleability, improved by the presence of phosphorus with close values measured at different magnesium concentrations. Nocardia related genera of Corynebacterium and Enteric microorganisms were identified.

Keywords: Activated sludge, magnesium, filamentous sludge bulking, extracellular polymeric substances, phosphorus

ÖZ

MAGNESYUM İYONLARININ FOSFOR YETERSİZLİĞİ OLAN BİYOREAKTÖRLERDE ÇAMUR ÖZELLİKLERİNE OLAN ETKİSİNİN İNCELENMESİ

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Aktif çamur ıslahının verimliliği mikrobiyal hücrelerin ardıllanmak amacıyla ayrılmasına bağlıdır. Ayrılma birtakım problemler nedeniyle bozulmuş olabilir. Bu problemlerden birisi biyokütlenin çökmemesi durumu olan çamur kabarmasıdır. Daha önceki çalışmalar fosfor yetersizliğinin, artan magnesyum iyonu konsantrasyonlarıyla birlikte filamentli çamur kabarmasına neden olduğunu göstermiştir. Bu çalışmanın ana hedefleri fosfor eksikliği olan besi ortamında magnesyum iyonlarının çamur özelliklerine olan etkisini ortaya çıkarmak ve çamur kabarması olup olmadığını belirlemektir. Fosfor eksik besi ortamı içeren üç biyoreaktöre üç farklı konsantrasyonda magnesyum (0.5, 5, 15 meq/L) ilave edilmiştir. Ilk aşamada KOİ/N/P oranı 100:5:0.05 olmuştur. İkinci aşamada, KOİ/N/P oranı 100:5:1'e yükseltilmiştir. Kararlı halde, çamur hacim indeksini (SVI), viskositeyi, bulanıklığı ve susuzlaştırılabilirliği içeren fiziksel

özellikler belirlenmiştir. Bunlarla beraber iletkenlikte olduğu gibi hücre dışı polimerik madde (HDP) konsantrasyonu da ölçülmüştür. API kitleri kullanarak bakteriyel tanımlanma başarılmıştır.

İlk sette, fosfor yetersizliği ve artan magnesyum iyonu konsantrasyonu, filamentli çamur kükmesine neden olmuştur. Hücredışı polimerik maddelerin (HDP) karbonhidrat içeriği, magnezyum eklenmesiyle önemli ölçüde artmıştır. Sistemin susuzlaştırılabilirliği kötüleşmiş, viskozite düşmüştür. Çamur hacim indeksi (ÇHİ) bütün magnezyum konsantrasyonlarında ciddi çamur kükmesini göstermiştir. Biyokimyasal testleri kullanarak, sistemdeki baskın mikroorganizmalar belirlenmiştir.

İkinci aşamada bütün parametreler , sistemin sağlıklı yumaklaşmasını gösterir şekilde iyileşmiştir. Magnezyum iyonu eklenmesiyle HDP'nin hem protein hem de karbohidrat içeriği artmıştır. Sistemin çökebilirliği ve susuzlaştırılabilirliği, değişik magnezyum konsantrasyonlarında yakın değerler elde edilecek şekilde iyileşmiştir. Enterik mikroorganizmalar ve Nocardia ile alakalı Corynebacterium cinsi mikrorganizmalar belirlenmiştir.

Anahtar kelimeler: Aktif çamur, magnezyum, filamentli çamur kükmesi, hücre dışı polimerler, fosfor

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LIST OF ABBREVIATIONS

BSA	: Bovine Serume Albumin
CER	: Cation exchange resin
CGY	: Casitone-glycerol-yeast extract
C/N	: Carbon to nitrogen ratio
C/N/P	: Carbon to nitrogen to phosphorus ratio
COD	: Chemical oxygen demand
CST	: Capillary suction time
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
SVI	: Sludge volume index
TKN	: Total kjeldahl nitrogen
VSS	: Volatile suspended solids
ZSV	Zone settling velocity

CHAPTER 1

INTRODUCTION

Activated sludge systems are known as the most conventional and popular method among several biological treatment processes. It is composed of aeration and further settling of microbial mass. Organic substrate is utilized and converted to carbon dioxide, water and new biomass in the aeration tank. After this utilization step, proper settling of the new biomass is essential for the efficiency of the treatment. Separation of microorganisms from the effluent is crucial. This separation and settling are highly dependent on the healthy bioflocculation of microbial species. Bioflocculation plays a vital role in this process (Pavoni *et. al,* 1972). As a result of this significance there are number of researches that focused on the dominant mechanism of bioflocculation or the microbial aggregation (Higgins *et al.*, 2002). Several theories for the bioflocculation were proposed over the years.

Floc structure in activated sludge systems is mainly composed of microorganisms, cell debris, cations and extracellular polymeric substances (EPS) (Eriksson and Alm, 1991; Bruus *et al.,* 1992; Higgins and Novak, 1997 a, b). Among them extracellular polymeric substances and cations were proposed to have interactions in order to contribute to the bioflocculation mechanisms. Due to the negative charge carried by the majority of EPS, cations are crucial for the floc structure in order to provide bridging to the negative sites on biopolmer network (Bruus *et al.*, 1992; Urbain *et al.,* 1993; Higgins and Novak, 1997a).

There are several researches in literature that dealt with the effect of cations on bioflocculation mechanism. However some of them could not reveal ultimate effect

of cations on flocculation due to the operation in batch mode (Higgins and Novak, 1997, a, b). In addition some studies investigated the effect of cations on the system having only mono culture bacteria. Since activated sludge system is composed of mixed cultures that contribute discrepant properties to floc structure, the results with monocultures could not reflect actual case (Kara, 2007).

Many researchers have focused on the effect of divalent cations in bioflocculation and therefore physical and chemical characteristics of the system. Higgins and Novak in 1997, proposed a floc model with greater contribution of the divalent cations such that lectin like proteins that were attached to bacterial surface were cross-linked with polysaccharides by the help of calcium and magnesium. The study conducted by Bruus *et al*. in 1992, indicated that the extracellular polymers may be alginate or another polysaccharide properties of which resemble to alginate and form a gel-like structure in the presence of calcium ions. Sanin and Vesilind in 1996, supported this proposal by the establishment of synthetic sludge flocs due to the addition of calcium and alginate. Higgins and Sobeck in 2002 found that sludge settling and dewaterability characteristics enhanced by addition of either magnesium or calcium.

Binding ability of the components of the extracellular biopolymers to divalent cations was studied as another issue in some other research. Calcium and magnesium were found to increase bound protein concentration of EPS (Urbain *et al.,* 1993; Dignac *et al*., 1998, Higgins and Novak, 1997, a).

It is known from the literature that flocculation of the microorganisms is dependent on many factors. Among them one of the most important is the concentration of the nutrients that is provided in the feed. Under nutrient deficient conditions macrostructure failure of the system which is called as sludge bulking occurs (Jenkins *et al.,* 1993). In addition to this, sludge bulking can differ in type with addition of different cations under nutrient deficient conditions. For instance under phosphorus deficiency with abundance of magnesium in the feed, filamentous bulking was reported (Turtin, 2005) whereas viscous bulking was observed under

phosphorus deficiency and calcium ion abundance in the system (Vatansever, 2005). However these studies were not detailed enough to identify all components related to bioflocculation.

This study is conducted to investigate the effect of magnesium addition on bioflocculation and sludge bulking at phosphorus deficient and phosphorus sufficient conditions. The semi-continuous reactors were used in order to reflect the effect of divalent cation addition more accurately than batch reactors. Physical, chemical and microbiological analyses were conducted during the study. It is desired to know under sludge bulking conditions if the improvement of flocculation can be achieved or not with divalent cation addition to the system. Besides, under phosphorus sufficient conditions the effect of magnesium was studied in the second set. By this way comparison of two systems could be done.

CHAPTER 2

LITERATURE REVIEW

2.1 Activated Sludge System and Its Components

One of the most common and conventional methods of the wastewater treatment is known as activated sludge process. This process contains several metabolic reactions such as synthesis, nitrification and respiration of the microorganisms; separation and settling of activated sludge solids; removing excessive amount of the sludge to be further processed in thickeners and recycling the required amount of the microorganisms back to the system.

The schematic representation of such a system can be seen in Figure 2.1 below. After the pre-treatment reactor, activated sludge process begins at aeration zone and continues at the settling tank.

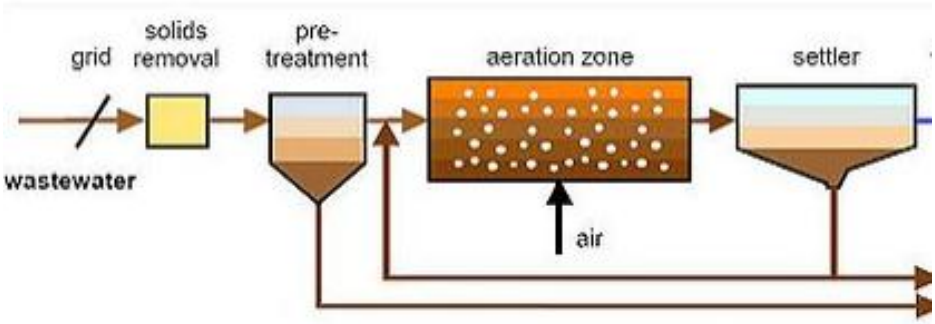


Figure 2.1 Schematic Representation of Waste Treatment System with Activated Sludge Component

In the aeration tank, the air is introduced to the system in order to achieve aerobic conditions and mixing. This step involves the utilization of colloidal and suspended organic material by the microorganisms. The products are mainly carbon dioxide, water and new biomass. In addition to this, the microorganisms convert ammonia nitrogen to nitrate nitrogen by the nitrification process. The efficiency of first step or substrate utilization phase depends on providing required environmental conditions to active biomass.

After the aeration tank, wastewater comes to secondary clarifier. In this step, main aim is to obtain clear supernatant by providing the effective settling of produced biomass which completed its metabolic role in the first step, other suspended and colloidal components. The effectiveness depends on the successful biosolids/liquid separation by bioflocculation and solids/liquid separation. Therefore bioflocculation plays a vital role in this process (Pavoni *et. al,* 1972).

After successive separation of the biomass from the supernatant, some of the settled sludge/biomass is returned to the beginning of the aeration tank in order to sustain same F/M. Moreover, some of the settled and excess biomass is wasted from the system in order to be handled in the thickeners.

Although all of the process can be seen as physical and chemical process, microorganisms play a major role in substrate utilization and settling steps of the activated sludge. Among the heterogeneous mixture of activated sludge that is composed of particles, colloids, organic polymers and cations, there is also microorganisms (Jorand *et al.*, 1995). As the biological components, system is comprised of bacteria, fungi, protozoa and rotifers. The dominance of the microorganism depends primarily on the environmental conditions, wastewater characteristics, process design and mode of plant operation (UCLA College of Letters and Science, 2010).

Although there are number of the microorganisms present, most recognizable one in the system is bacteria. The majority of the bacteria in the system are facultative, able

to live in the presence or absence of oxygen. In addition, the system is highly heterotrophic. *Arthrobacter, Citromonas, Flavobacterium, Pseudomonas, Alcaligenes, Achromobacter* is some of the genera that can be found in activated sludge (Jenkins *et al.,* 1993). Besides this, autotrophic bacteria which utilize ammonia nitrogen to nitrate nitrogen, found in smaller amounts compared to heterotrophic bacteria. This may be due to the slower growth rate of these types of bacteria. Most common autotrophic bacteria are identified as *Nitrobacteria and Nitrosamonas* (UCLA College of Letters and Science, 2010).

Besides several species of the bacteria there are also eukaryotic organisms in activated sludge process. One of these organisms is fungi most of which are strictly aerobe and can resist to low pH and nitrogen deficiency. Excessive proliferation of filamentous fungi was reported to cause settleability problems in settling tanks of activated sludge systems. Some species of the protozoa also have been identified in activated sludge. They are the indicators of oxygen availability in the system due to their aerobic features. However some types such as *amoebae* and *flagellates* are able to resist to anerobic conditions (Gerardi, 2006). Rotifers which are multicellular organisms are strictly sensitive to oxygen depletion and toxicity as well as the protozoa. They are found in a stable activated sludge environment and they seem as a recirculating wheel when they move (UCLA College of Letters and Science, 2010).

Aggregation of all these organisms is essential in the second step of the system. Activated sludge structure is composed of three micro-structures: bacteria, micro-colonies and flocs (Jorand, *et al.,* 1995; Snidaro *et. al,* 1997). First of all microorganisms or specifically bacteria stick to each other by a polymeric matrix and form the second level, micro-colonies. Then extracellular polymeric substances and cations bridge separate micro-colonies and form the final step, activated sludge flocs. The flocculated microbial aggregates called as flocs change in size from 10 to 1000 ε m (Andreadakis, 1993).

According to Jenkins *et al*., (1993), floc structure is composed of macrostructure and microstructure. Microstructure is formed by microbial aggregation, bioflocculation

and adhesion. Macrostructure is composed of filamentous microorganisms that form the backbone of the floc. Main floc forming heterotrophic bacteria are listed as *Achromobacter, Alcaligenes, Arthrobacter, Citromonas, Flavobacterium, Pseudomonas, and Zoogloea.*

2.2 Bioflocculation and Its Mechanisms

2.2.1. Bioflocculation

Bioflocculation which can be defined as the microbial aggregation is crucial for the effluent quality in biological wastewater treatment systems. The microbial aggregation takes place by the floc formation of microorganisms with the help of microbially produced extracellular polymeric substances. When the microbial aggregation is not achieved successfully, the solid/liquid separation in the secondary clarifier becomes ineffective and leads to the problems in dewaterability and settling. Due to decrease in dewaterability of the system the further removal of the water by thickening and other mechanical means become impossible. In addition, the main purpose of the treatment process becomes useless due to the settling problems. Due to this high significance in the overall process several researches have been performed for understanding the driving mechanisms of bioflocculation (Sobeck & Higgins, 2002).

Progresses that can be achieved in understanding the whole picture behind the microbial aggregation mechanisms have great importance in the biological wastewater treatment which is not limited to activated sludge process. These systems include:

- aerobic waste treatment containing the bacteria and other microorganisms;
- anaerobic waste treatment containing the bacteria and other microorganisms;
- stabilization ponds and nutrient-stripping processes containing algae and other microorganisms and
- the dewatering process of excess biomass (such as bacteria and algae) synthesized in the above types of biological treatment (Pavoni *et al*, 1972).

2.2.2. Bioflocculation Mechanisms

There have been several studies conducted in order to understand the major mechanism in the bioflocculation process for years. According to these studies, some of the bioflocculation theories have been proposed.

2.2.2.1. The Zoogloea Ramigera Theory

The first theory in this field is known as Zooglea Ramigera Theory which was studied by C.T Butterfield in 1935, H. Heukelekian and coworkers in 1939. They claim that bioflocculation ability of the activated sludge systems is dependent on the particular group of bacteria *Zoogloea Ramigera* due to their extracellular gelatinous matrix which keeps the microbial aggregates together. However the validity of the theory was lost by the further studies indicating that other bacterial species which were isolated from the activated sludge environment was able to make flocs (Mc Kinney, 1952; McKinney & Horwood, 1952; McKinney & Weichlein, 1953).In addition, majority of the scientists is in agreement that Zoogleal growths do not involve in microbial aggregation all the time (Friedman & Dugan, 1968).

2.2.2.2. Flagella Agglutination Theory and Protozoa Theory

In 1938, A. Pijper proposed that flagella interactions may be the reason for the agglutination of bacteria. However for the species not having flagellar interactions, bioflocculation occurred generally. In the same manner, protozoa were thought as a supporter for the microbial aggregation (Pillai, 1941; Barker, 1946). As in the case of flagella it was proved that many pure cultures were able to form flocs. Therefore, these two proposals seem to be limited and not inclusive rather than being completely wrong.

2.2.2.3. PHB (poly-beta-hyroxybutryric acid) Theory

K.Crabtree and his co-workers in 1965 and in 1969 considered PHB in the cells as the direct constituent responsible for the microbial aggregation. A study proposed the presence of PHB granules in some of the cells during bioflocculation although the direct correlation between aggregation and PHB could not be found (Friedman *et al.,* 1968). Many researchers mainly focused on the food reserving function of PHB which can be used during endogenous growth rather than bioflocculation potential (Macrae & Wilkinson, 1958; Rouf & Stokes, 1962).Therefore there was not a direct correlation between flocculation and PHB content of the cell and can not be considered as major bioflocculation mechanism.

2.2.2.4. Extracellular Polymeric Substances (EPS) and Bioflocculation

Several bioflocculation mechanisms are based on the formation of EPS**.** From the past research there appears to be a close relationship between bioflocculation and EPS. This relationship was firstly studied by R. E. Mc Kinney in 1953. He stated that the cell wall enveloped by polysaccharide could play a role in reduction of the surface potential and could increase the aggregation.

2.2.2.5. Filament Backbone Theory

The theory which was first proposed by D. S. Parker and his co-workers in 1972 necessitates the presence of filaments in bioflocculation. Once the microorganisms attach to each other and form flocs by the presence of polymer bridges, these attachments then bind to filaments. The floc strength becomes highly dependent on these filaments in a way that filaments constitute the backbone of the aggregation. The schematic presentation of filament backbone is seen in Figure 2.2 below

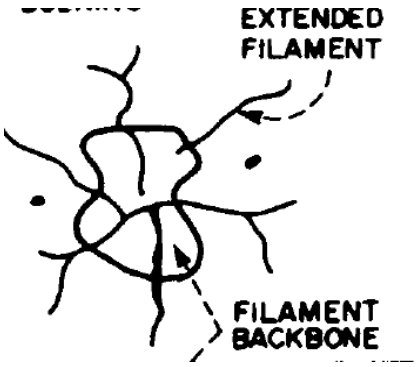


Figure 2.2 Depiction of the Filament Backbone (Sezgin *et al*., 1978)

2.2.2.6. Double Layer Theory (DLVO Theory)

Double Layer Theory, developed by Derjaguin, Landau, Verwey and Overbeek, can be regarded as the classical colloidal theory that assumes presence of electrical double layer around charged particles. In an electrolytic solution, oppositely charged ions are attracted to the surface of the charged particle therefore get closer; oppositely charged ions can either be distributed in different parts of the solution (Gregory, 1992). Around the charged particles there has been the formation of diffuse cloud of ions due to electrostatic attraction and ionic diffusion. This cloud of ions is described as the electrical double layer (Kruyt, 1952). Double layer is mainly composed of two layers, Stern layer and Diffuse layer. Double layer is demonstrated in Figure 2.3. The counter-ions are strongly attached to the surface of the charged particle and forms Stern layer. The outer layer, named as Diffuse layer, contains counter-ions which are repelled by the Stern layerôs counter ions. The attraction by the particle continues but repulsion is stronger and hence these counter ions seem less tightly associated with the particle (Adamson, 1990). The density of the ions in the diffused layer reduces going through the bulk liquid until it becomes equivalent to that of bulk liquid. Because of the presence of double layer and hence repulsion, the adjacent particles can not coalescence with the charged particle.

When the ionic strength increases, the double layer size decreases and hence the attraction of other particles increases. From this theory it can be concluded that by

the addition of the cations to activated sludge systems, the double layer compression takes place and bioflocculation is favored. This idea was supported by some of the researchers. C. P. Cousin and J. J. Gnaczarcyzk in 1998, studied sodium effect; found out an increase in floc size and deduced it to working of this theory. In addition, A. Zita and M. Hermansson in 1994 found the association of floc stability with ionic strength and DLVO theory and studied the effect of calcium and potassium ions.

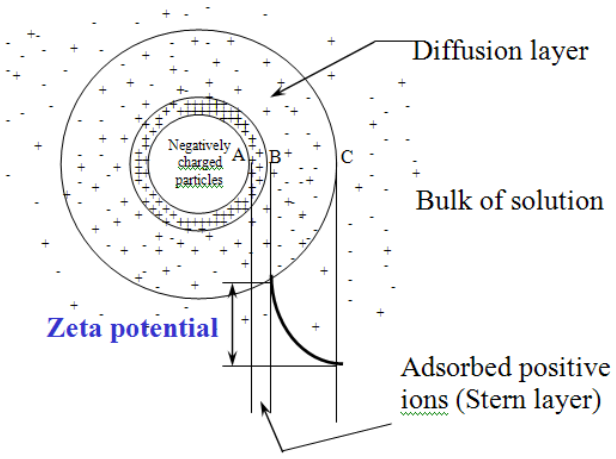


Figure 2.3 Demonstration of the Double Layer

2.2.2.7. Polymer Bridging Model

According to polymer bridging theory, long-chain polyelectrolyte with high molecular weight, support the interaction of bacterial cells, other individual cells and particles by acting just as a bridge and hence cause aggregation of bacteria. In order to achieve flocculation net electrostatic surface charge should be equal to zero (Tenney & Stumm, 1965; Ries & Meyers, 1968). However one of the studies conducted by Pavoni *et al.*, 1972, found out that surface potential reduction was not solely necessary for microbial flocculation. The polymers might be physically or electrostatically bond and in the end bridging of the cells of the dispersion into three-dimensional matrix occurred. The same study supported the polymer bridging theory in a way that the microbial flocculation increased by the increase in exocellular polymer to microbial mass ratio.

2.2.2.8. Divalent Cation Bridging Theory (DCB)

Y. Tezuka and R. E. Mc Kinney firstly developed this theory. As can be seen in Figure 2.4, divalent cations attach anionic parts of the biopolymers and thus form bridges between the bacterial colonies and flocs, making stable floc matrixes (Tezuka, 1969; McKinney & Horwood, 1952).

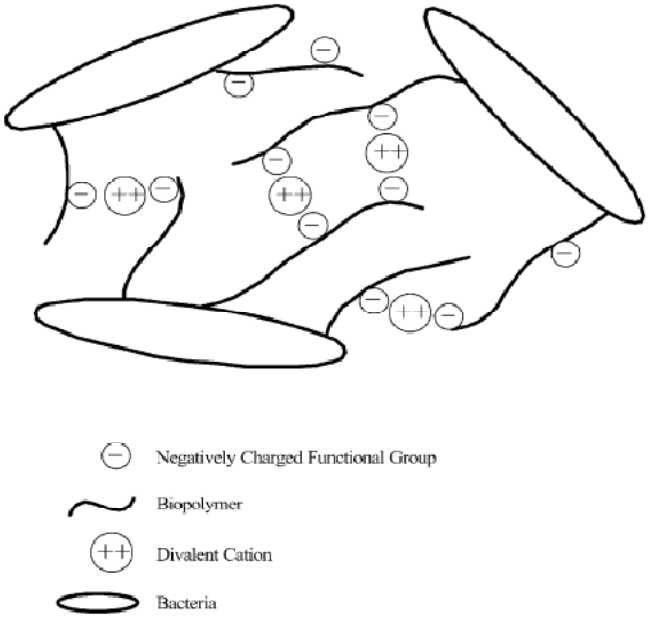


Figure 2.4. Depiction of the DCB Theory

Higgins and Novak, (1997 a) supported the idea behind this theory, after adding one of monovalent cations, sodium, floc properties deteriorated due to ion exchange with divalent cations.

2.2.2.9. Alginate Theory

This theory was first developed by Bruus *et al.*, in 1992. It is known from some of the studies that *Azotobacter sp.* and *Pseudomonas aeruginosa* bacteria is capable of synthesizing one of the polysaccharides, alginate, in activated sludge systems (Nunez, *et al.*, 2000; Davies & Geesey, 1995). When the calcium ions are found in the system, alginate form alginate gels. In this theory it is assumed that the

exocellular polysaccharides are composed of alginates and by the addition of calcium, binding of particles, calcium and negatively charged alginate surfaces takes place. Due to this specific type of cation and polymer binding, addition of other cations just as magnesium and sodium resulted in deterioration in sludge characteristics by the exchange of ions with calcium (Bruus *et al.*, 1992). Another study conducted by Sanin and Vesilind in 1996, supported this theory using the synthetic sludge containing alginate. Magnesium did not give the same result as in the case of calcium. The working principle of the alginate theory is similar to divalent cation bridging theory. However DCB is available for any kind of divalent cation rather than specifically calcium and its binding to alginate specifically.

In 2002 D Sobeck and M. Higgins studied the best fitting model between three mechanisms by measuring sludge characteristics by adding calcium, magnesium and sodium concentrations to continuous reactors separately. DCB was found to be the best fitting model. Floc characteristics of calcium and magnesium showed similar values, enhancing floc formation. DLVO theory failed due to the deterioration of floc by addition of the monovalent cation. There was not superiority of calcium reactors over to magnesium reactors and hence the validity of the alginate theory was lost partially. The specificity of calcium ions could not be explained by these results.

In addition to all of these theories, it is believed that hydrophilic and hydrophobic properties of the EPS play a role in bioflocculation mechanism. Higgins and Novak (1997 a) proposed that hydrophobic parts just as the amino acids of polymers were essential constituents in activated sludge and biopolymers can bind through hydrophobic parts. In addition when the hydrophibicity of the floc increased the sludge became easily settleable since it was easily separated from the hydrophilic, polar parts (Urbain *et al.*, 1993).

2.3 Extracellular Polymeric Substances (EPS)

2.3.1 Definition of EPS

Majority of bacteria have polymeric structures, lying outside of their cell walls. These polymeric structures are comprised of ï homo and ï heteropolysaccharides, humic compounds, some types of polypeptides, polyalcohol, lipids and nucleic acids. Extracellular polymeric substances (EPS) are the common name given to these polymeric structures (Pavoni *et al.,* 1972; Urbain *et al.,* 1993). They have a significant role in bioflocculation mechanism.

In addition, EPS has a highly hydrous, gel-like structure and it has an often charged network into which microorganisms are embedded. In this EPS network, some dissolved substances in water and particulate substances can be seen due to sticky nature of the polymer (Sanin *et al.,* 2006).

It can be regarded as these polymers may be in the form of discrete capsule that is strongly attached to cell wall / surrounding the cell wall or in the form of a jelly-slime structure that is not strictly adhered to cell wall or not adhered to it They are released out of the cell wall by active transport or cell lysis (Sutherland, 1972; 1977 and 1990). They have high molecular weight and mass of total EPS constitutes approximately 80% of the mass of activated sludge (Frolund *et al.,* 1996).

G H. Yu and his co workers in 2008 proposed EPS had different partitions according to separation principles. When EPS is distributed in bulk solution after settling of sludge, this portion is named as supernatant EPS. The remaining part that is embedded in sludge matrix is composed of EPS in slime form and bound EPS fractions. Slime EPS has loose binding to flocs and can not persist to washout. Bound EPS, on the other hand, is a discrete covering layer with a distinct margin at the outside of the cell wall. In addition it is a double layer structure which is named as loosely bond EPS (LB-EPS) and tightly bound EPS (TB-EPS) based on extraction

procedure (Poxon & Darby, 1997; Ramesh *et al.*, 2006; Li & Yang, 2007; Yu *et al.*, 2007).

2.3.2 Composition of EPS

As stated before, the extracellular polymeric substances are composed of polysaccharides, proteins, lipids, nucleic acids and humic substances (Eriksson & Alm, 1991; Urbain *et al.*, 1993; Frolund *et al.*, 1996). Although these basic constituents remain same, the dominant composition can change according to different conditions. There are a number of studies investigating the dominant part of EPS but there is not an agreement between these results due to the conditions. For instance, some of the research regarded polysaccharides as the most important component in EPS taking part in flocculation (Horan & Eccles, 1986; Bruus *et al.*, 1992, Jorand *et al.*, 1995). On the other hand, others reported that exocellular protein concentration was higher than the polysaccharide concentration and played the most important role in flocculation (Tenney & Verhoff, 1973; Brown & Lester, 1980; Barber & Veenstra, 1986; Urbain *et al.*, 1993).

By the characterization of the EPS, it was found that some portion of the extracellular polysaccharides was made up of uronic acids (Brown & Lester, 1980; Forster, 1971; Frolund *et al.*, 1996; Horan & Eccles, 1986). Uronic acids are known to be polyanionic due to carboxyl group on its surface which determines the basis for having an interaction with cations present in the system. This property makes the cations possible to form complexes with extracellular polymeric polysaccharides (Christensen, 1989; Sutherland, 1990). In addition, same examination was applied for the exocellular proteins. Proteins were composed of amino acids such as glutamic and aspartic acid which contain carboxyl group that contributes to negative charge of bioflocs (Dignac, *et al.*, 1998; Higgins and Novak, 1997 a, b, c).

At neutral pH range, extracellular polymers possess net negative charge that will soon interact with divalent cations to form bridges (Nguyena, *et al.*, 2008). In

addition strong association between calcium ions and exocellular protein had been found in some of the studies (Urbain *et al*., 1993; Higgins and Novak 1997 a). However due to presence of mixed culture in activated sludge systems it seemed plausible that there would be differences in uniformity of extracellular metabolites produced by the culture. In addition, different type of substrate utilization may change the dominant composition but major components in the EPS structure remained same (Pavoni *et al.*, 1972).

2.3.3 Factors Affecting EPS Production and Bioflocculation

Many factors affect the EPS composition, EPS production and bioflocculation. Environmental conditions, genotype and physiological factors among those. The nutrient composition, C/N, C/P ratio, pH, temperature and agitation speed can be identified as the environmental factors. These are also essential for the process efficiency (Salehizadeh & Shojaosadati, 2001).

The physiological state of the microorganisms is one of the factors that affect the microbial aggregation and EPS production. In his study Pavoni *et al.*, (1972) discovered that exocellular polymer production and therefore biological flocculation reached its optimum value when the endogenous growth phase was obtained. The validity of this data did not change with various biological systems such as aerobic-anaerobic systems mainly comprising heterotrophic-autotrophic microorganisms respectively. However in the example of *Z. Ramigera*, production of the flocculant terminated after 90h in stationary phase (Norberg & Enfors, 1982). Surprisingly, flocculation related with *S.griseus* was not dependent on growth phase (Shimofuruyauji *et al.*, 1996).

The proportions of the organic concentrations provided in the feed seemed to change the EPS production and composition. When nitrogen is limited and the carbon sources are in excess amounts, it has been shown that the cells tend to accumulate large amounts of various polymers. With a remarkable increase in C/N ratio,

majority of bacteria increase the production of carbonaceous compounds and reduce the cellular protein and nucleic acid synthesis. Under these conditions, substantial amounts of EPS are produced (Sanin *et al*., 2006)

At lower C/N ratio, all carbon sources were utilized by microorganisms in biomass synthesis and nitrogen was used for protein synthesis. Microorganisms probably did not tend to produce extracellular polysaccharides. However higher amounts of extracted proteins were observed which can function inside the cell or found in the extracellular medium (Sanin *et al*., 2006).

Another operational parameter, mean cell residence time (MCRT), had an influence on the EPS production. According to previous studies, larger amounts of EPS were produced due to endogenous metabolism of microorganisms at higher MCRTs than lower MCRTs (Pavoni *et al*., 1972; Chao & Keinath, 1979; Steintuch, 1987).

At higher MCRT values the protein component predominated. At low MCRT (high F/M), there is high quantity of food. However microorganisms do not have the sufficient time to consume most of the carbon source. They tend to convert excess carbon source to EPS. At higher MCRT, food is in lower quantities; microorganisms utilize it in biomass synthesis. They just secrete the EPS to outside which could be used for microbial protection. At the stationary growth phase of microorganisms, cell lyses takes place and therefore abundance of proteins at higher MCRTs might be explained by this reason (Sanin *et al*., 2006). As MCRT increases, total EPS amount increases. By an increase in MCRT; protein component of extracted EPS increases. On the other hand, there is not a significant change in carbohydrate content (Sanin *et al*., 2006).

Since the system is dependent upon the microorganisms behavior temperature ranges are also significant. There is an optimum temperature range for obtaining higher concentration of EPS. Mixed culture *R-3* (Kurane & Matsuyama, 1994), *Bacillus sp. PY-90* (Yokoi *et al.*, 1995), *Flavobacterium sp.* (Endo et al., 1976), *Bacillus sp. DP-152* (Suh et al., 1997) were reported to produce optimum EPS concentration at a

temperature of 30°C whereas *Zoogloea MP6* was able to produce EPS at 20°C (Kakii et al., 1996).

As well as the temperature, pH affected EPS production. The optimum pH range for the EPS production varied for different species. For instance, *C.xerosis* could produce EPS at lower pH (Esser & Kues, 1983) whereas *A. Sojae* produced biopolymers in the alkaline pH.

2.4 Cations

Main cations that are found in activated sludge systems are sodium, potassium, ammonium, calcium, magnesium, iron and aluminum. Ions have three fundamental functions in the cell:

i) Being coenzymes or metal cofactors
ii) Transferring of electrons in oxidation-reduction reactions
iii) Serving as regulators of osmotic pressure (Gerardi *et al.,* 1994).

Besides this, they are regarded as one of the major components of the activated sludge flocs as well as the microorganisms and extracellular polymeric substances (Bruus *et al.,* 1992; Higgins and Novak, 1997 a).

It was known from previous studies that exocellular biopolymers and cations took part in flocculation process. According to some of the bioflocculation mechanisms, negative sites on exocellular biopolymers were binded by the help of cations and therefore supported flocculation (Tezuka, 1969; Novak & Haugan, 1978; Bruus *et al.,* 1992). Not having a proportional cation concentration in the feed would lead to formation of weak and dispersed flocs (Park, 2002).

2.4.1 Divalent Cations

Many researches have been conducted for understanding the role of divalent cations on flocculation. Calcium and magnesium might be needed for adhesion of certain monocultures of certain bacteria (Tezuka, 1969; Lodeiro *et al,* 1995).

A floc model in which divalent cations were involved has been proposed (Higgins and Novak, 1997 b). Lectin like proteins that were attached to bacterial surface cross-linked with polysaccharides by the help of divalent cations (calcium and magnesium). Network was formed by binding the exocellular polysaccharides and proteins. The illustration of the floc model can be seen in Figure 2.5

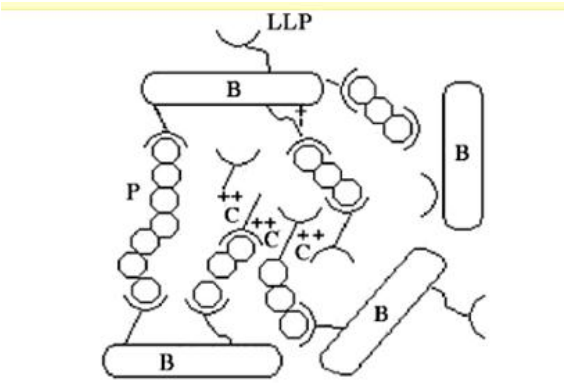


Figure 2.5 Roles of Biopolymers and Divalent Cations on Bioflocculation(B:Bacteria,LLP:Lectin-like protein,P:polysaccharide,C^{++}:Divalent cation (Higgins and Novak,1997 b)

The removal or extraction of calcium ions by using EGTA (Bruus *et al.,* 1992), EDTA (Kakii *et al*., 1985) and cation exchange resin (CER) (Keiding & Nielsen, 1997) led to increase of turbidity and worsening of filterability and settleability. At the same time, the study conducted by Bruus *et al.*. in 1992 ,indicated that the extracellular polymers may be alginate or another polysaccharide whose properties resemble those of alginate and form a gel-like structure in the presence of calcium ions. Further, another study conducted by Sanin and Vesilind in 1996, enhanced this

proposal by forming stable synthetic sludge flocs due to the addition of calcium and alginate into a suspension of stable particles.

Magnesiumôs role on bioflocculation was studied as well. Higgins and Novak, (1997 a) observed in their study that the system required calcium and magnesium for acceptable physical characteristics whereas the same system required the addition of magnesium in the second trial. Further study revealed the same system was dominated by different type of bacteria at different time intervals and therefore the requirements for the cations altered. It was concluded in some of the studies the change of the physical characteristics of the system to the absence of calcium and magnesium depended on the type of the bacteria dominant in the system (Tezuka, 1969; Endo *et al.,* 1976; Lodeiro *et al.,* 1995). Higgins and Sobeck (2002) found that sludge settling and dewaterability characteristics were enhanced by addition of either magnesium or calcium.

The effect of the divalent cations differed on the sludge characteristics although they have similar properties in some of the studies. For instance, by the addition of calcium ions, the bound water content was reduced in an earlier study. On the other hand magnesium ion had no such an effect on the bound water content (Forster and Lewin, 1972). The same study had also found out that the extracellular polymers would rather prefer calcium ions than magnesium ions to have interactions.

Binding ability of the components of the extracellular biopolymers to divalent cations is another important issue that has been discussed. It was found that by the addition of calcium and magnesium ions, bound protein concentration of the EPS increased (Urbain *et. al.*, 1993; Dignac *et al*., 1998, Higgins and Novak, 1997 a).

On the contrary one of the studies conducted by Nguyena *et al.*, (2008) supported the previous findings that divalent cation concentration caused a decrease in supernatant EPS carbohydrate concentration in semi-continuous and batch reactors. In same manner, the bound EPS carbohydrate concentration increased by increasing calcium concentration.

2.4.2 Monovalent Cations

In activated sludge systems, higher concentratios of sodium resulted in the deterioration in settling and dewaterability of sludge (Novak and Randall, 1986; Bruus *et al.,* 1992). Up to 5meq/l of sodium SVI decreased .Above 5 meq/L sludge volume index (SVI) which is an indicator of sludge settleability increased and up to 20 meq/L, the system had reached such a state that SVI could not be measured due to deflocculation (Higgins and Novak, 1997 a).

Due to displacement of divalent cations within the floc with high sodium concentrations according to ion exchange process, poor settling and dewatering took place. Floc structure weakened due to removal of divalent cations, cation bridging function destroyed (Tezuka, 1969; Novak and Haugan, 1978; Bruus *et al.,* 1992). When the soluble cation concentrations of magnesium, calcium and sodium were measured after the addition of sodium, it was observed that sodium brought about an increase in soluble calcium and magnesium concentration meaning that release of calcium and magnesium from biopolymer network due to sodium (Higgins and Novak, 1997 a).

When another monovalent cation, potassium concentration amount was increased, SVI or settling properties improved due to formation of very large flocs which was capable of settling rapidly. On the other hand dewatering properties got worse (Higgins and Novak, 1997 a).

As it was explained before, cation bridging mechanism is one of the mechanisms of bioflocculation. Ionic charge, ion size and radius of hydration shell of the cations are the factors that influence on the binding ability of cations. It is known that cations with higher valency, large size and thin hydration shell may easily get closer to charged sites of surfaces and form bonds with negative charged sites of EPS (Piirtola *et al.,* 1999). Among four cations, magnesium, calcium, sodium and potassium, potassium has the lowest hydration shell radius (Mg^{2+} > Ca^{2+}>Na^{+}>K^{+}). Due to this

fact, potassium becomes more easily dehydrated, moves to charged sites of EPS and forms stronger bonds with these sites compared to sodium ions. One of the studies conducted by Rengasamy and Naudi (1998), found out relative flocculation power of cations within the following order: $Ca^{2+} > Mg^{2+} > K^{+} > Na^{+}$. It can be derived from this data that sodium is the weakest flocculator due to larger hydrated shell radius, single charge and small size.

In addition to this, the ability of the cations to be incorporated in floc matrix depended on the functions of the cations in the cell. It was known that potassium ions were responsible for maintaining osmotic pressure in the cell (Gerardi *et al.*, 1994). Therefore it acted in the intracellular medium rather than magnesium and calcium ions which were in extracellular medium and helped in binding of flocs (Sanin *et al.*, 2006). As it happened in the sodium case, another monovalent cation ammonium caused deterioration in floc characteristics (Novak, 2001).

2.4.3 Monovalent ion / divalent ion (M/D) Ratio

Higgins and Novak, (1997 a) found out that, physical properties of the sludge got worse when the proportion of monovalent to divalent cations were greater than 2. Therefore they regarded M/D as an indicator to determine for floc characteristics as well as the amount of individual concentrations of the cations.

2.4.4 Trivalent Cations

There were not enough investigations that focused on the concentration of trivalent cations on activated sludge systems although these cations were having higher concentrations in these systems. At an acid treatment of the activated sludge systems near a pH value of 3, iron and aluminum could not be extracted from the sludge matrix whereas calcium and magnesium could be extracted (Kakii *et al.*, 1985). In other studies it was shown that ferric iron had high affinity for binding protein in sludge (Murthy *et al.*, 2000; Muller, 2001).

The number of the studies investigating the effect of aluminum which was commonly used as coagulants for removing natural organic matters was limited. One of the studies found out that alum was able to remove humic substances from the swamp water at pH of 5 to 7. Since the humic substances are one of the major constituents of EPS there was the possibility of having an interaction between EPS and alum (Lu *et al.*, 1999).

Nguyena *et al* (2008) researched the contribution of a trivalent cation with calcium ion to flocculation. The samples containing both calcium and alum had higher flocculation than the samples containing only calcium. Use of these cations increased the bound polysaccharide content as expected.

In the study conducted by Yu *et al* (2009) it was observed that in tightly bound EPS extracted samples, the contribution of trivalent cations to sludge matrix was higher than calcium and magnesium ions.In another study the availability of iron and aluminum caused improvement of activated sludge settling in a way by reducing number of the filamentous bacteria and preventing bulking (Agridiotis *et al.*, 2007).

2.5 Activated Sludge Properties Related To Bioflocculation

2.5.1 Dewaterability

The conversion of organic compound to less harmful inorganic compounds in activated sludge process is not enough for the process efficiency. The biosolids should be separated from the system by settling. In addition the settled sludge or the sludge that is going to be wasted should release its water content easily and hence should not contain higher amounts of water in its content. Since the thickened sludge is only 3-5 % solids when there are difficulties in dewaterability, the cost of sludge handling increases dramatically. Therefore it is true to conclude that sludge dewatering is the bottleneck of the sludge handling operation (Karr and Keinath, 1978).

The dewaterability of sludge is generally determined by identifying rate of filtration and bound water content of the sludge. In sludge, water is composed of ñboundò water and ñfreeò water (Lee *et al*., 1994, 1995; Wu *et al.,* 1998). The latter one is easy to remove from sludge by mechanical means whereas the former one is held in floc matrix, bound to sludge particles firmly and hence cannot be removed by simple mechanical means (Lee *et al.,* 1994; Colin *et al.,* 1995). Bound water can be defined as sum of water hold in capillaries and voids inside the sludge flocs and between them (interstitial water), internal water in bacterial cells and water chemically/physically bound in the sludge and surface water that is adsorbed to wet sludge surface (Jin *et al.,*2004).

The dewaterability of sludge can be assessed by applying several analyses. Capillary suction time (CST) measurements, specific resistance to filtration (SRF) and bound water measurements are some of those. Specific resistance to filtration was first developed by Coakley *et al*. (1956). By applying Darcyôs equation, through a porous medium pressure drop for a flow was analyzed. Although there have been some modifications of the technique and image of the SRF over the years (Christensen and Dick, 1985) the measurements were not successful in estimating the actual vacuum or pressure filter performance (Vesilind, 1988). Moreover it was time-consuming (Novak and Knocke, 1987).

Due to its practical use, CST measurements are generally preferred in activated sludge systems. It measures time that is required for the sludge to permeate through the filter paper for a constant distance. For the studied specific sludge sample, CST and rheological properties of sludge depended on suspended solids concentration (Mikkelsen and Keiding, 2002). Higher values of the CST are the indication of poor dewaterability and filterability (Smollen, 1990; Lin *et al.,* 1996; Higgins and Novak, 1997 a, b; Murthy and Novak, 1999; Lee and Liu, 2000).

Floc characteristics and floc properties affect the dewaterability of the sludge (Karrand *et al.,* 1978; Novak *et al.,* 1988; Sorensen *et al.,* 1995). Flocs contain

extracellular polymeric substances, organic and inorganic molecules as well as microorganisms (Frolund *et al.*, 1996). One of these constituents, EPS, is able to bind a portion of the water in the sludge although major part of the water is trapped by cell walls in order to be used intracellular (Keiding *et al.*, 2001; Eriksson *et al.*, 1992). Since EPS and its network are negatively charged, the osmotic pressure would cause high amount of water uptake into the floc matrix (Keiding *et al*., 2001). As a result there is a strong relation between EPS concentration and dewaterability (Houghton *et al*., 2001; Mikkelsen *et al.*, 2002).

According to the study conducted by Mikkelsen and Keiding (1995), by the increase in concentration of total extractable EPS, CST of the sludge decreased. This result might be obtained due to the fact that easily extractable EPS binds flocs and water in the bulk sludge weakly. As a result smaller values in CST, which is found to be mostly related with free water content in the bulk sludge (Jin *et al.*, 2004), are obtained in analysis. On the contrary, Kang *et al.*, (1989) indicated that by the addition of extracted EPS to system, dewatering characteristics decreased. In the study carried out by Sanin et. al. (2000), the extraction of calcium ions caused the release of extracellular polymers and therefore induced deflocculation. In addition, the filterability decreased. Some of the studies concluded that a certain concentration of EPS was required for enhancing filterability of sludge (Durmaz and Sanin, 2003; Houghton *et al*., 2001). However, after exceeding this certain amount of concentration, the dewaterability decreased due to the ability of EPS matrix for trapping of the water (Houghton *et al*., 2001).

The EPS composition influences the dewaterability as well as the EPS concentration. Water binding depended on the hydrophilic interactions and hydrogen bonding. By the contribution of other molecules such as the ï OH and ï NH bonds with hydrogen bond, water bonds were formed. In this sense, proteins and polysaccharides of the EPS contribute to water binding (J. Schmitt *et al.*, 1999; Jin *et al.*, 2004). There is some contradiction about positive or negative correlation of these constituents. Bowen and Keinath (1984) found that carbohydrate, protein and surface lipids had positive impact on dewaterability. Protein component of the EPS was found to be

more hydrophobic and hence became easily separated from the hydrated media and filterability eased (Sanin and Sesay, 2004). This result was in agreement with some of the studies (Sanin *et al.*, 2006, Jin *et al.*, 2004; Higgins and Novak, 1997 a, b, c). On the contrary, elevation of CST and difficulty in dewaterability of the sludge was observed by the release of protein to solution (Murthy and Novak, 1998, 1999; Novak *et al.*, 2001).

Higher concentrations of more hydrophilic component, EPS carbohydrate, resulted in deteriorations of the dewaterability (Sanin *et al*., 2006; Murthy and Novak, 1999).
In addition to EPS characteristics and concentration, the particle size distribution has a significant impact on dewaterability. In the case of chemical coagulants, the effectiveness of the coagulation depended on the capability of the coagulants to increase the particle size (EPA, 1974). It was found out from the investigation of particle size distributions in activated sludge treatment systems, most influential particle size belonged to supracolloidal particles in the range of 1 to 100µm. There was negative correlation between the increase of the concentration of the particles in this size range and dewaterability. This size range caused the clogging of the filter medium and sludge cake (Karr and Keinath, 1978). On the other hand, Novak *et al.*, (1988), found out that particle size smaller than 40 µm resulted in poor dewatering.

There are number of studies regarding the effect of cations on dewaterability. Due to presence of divalent cation bridging mechanism the flocculation eases with the addition of cations such as calcium and magnesium. As a result the dewaterability of the sludge improved (Higgins and Novak, 1997 a). The presence of trivalent cations such as aluminum and iron enhanced dewaterability as well (Keiding and Nielsen, 1997; Knocke *et al*., 1996; Colin and Gazbal, 1995; Higgins and Novak, 1997). Apart from the multivalent cations, monovalent cations seemed to affect sludge dewaterability negatively. Especially higher concentrations of the sodium resulted in bad filterability (Novak and Randall, 1986; Bruus *et al.*, 1992). This result was attributed to the displacement of monovalent cations with divalent cations that the bridging between floc components destroyed. The presence of the monovalent

cation, potassium resulted in poor dewaterability as traced by SRF values whereas the settleability was not affected (Higgins and Novak, 1997 a).

2.5.2 Settleability

The effective settling of the activated sludge biosolids is one of the key factors for effective treatment in activated sludge systems. In the case of ineffective settling, the quality of the effluent gets worse. Therefore settling characteristics is crucial for the success of the overall system.

Settling of the system is determined by Sludge Volume Index (SVI) and Zone Settling Velocity (ZSV). SVI is defined as the volume in milliliters occupied by 1g of suspension after 30 minutes of settling (Dick and Vesilind, 1969). SVI value bigger than 150 is taken as indication of deterioration in settling (Jenkins *et al.*, 1993).

Many factors have been identified to affect the settling. These are listed as organic loading of the system (Sürücü, 1982; Chao and Keinath, 1979; Barahona and Eckenfelder, 1984), mean cell residence time (Bisogni and Lawrence, 1971; Sürücü, 1982; Chao and Keinath, 1979), physical, chemical nature of the floc surface and physiological and biochemical nature of the flocs (Forster *et al.*,1976; Smith and Novak, 1982; Lee, and Ganczarczyk, 1986;Horan and Shanmugan, 1986). As a result there is a strong relationship between the settleability and flocculation.

Physiological and biochemical nature of flocs and therefore settleability seemed to be strongly affected by pH, temperature and dissolved oxygen concentration (Sanin and Sürücü, 1989). When the sludge temperature increased from 15 to 35°C, settleability decreased due to structural changes in polysaccharidic and proteinaceous part of the EPS. In addition, increasing the pH of the system lead to an increase in settleability since the addition of the anions caused an increase in reactive sites of the EPS and enhanced flocculation. Low concentrations of DO caused highly turbid supernatant and not properly measured settleability parameters (Sanin and Sürücü, 1989).

There are different findings of different studies concerning the EPS concentration and settleability. When the EPS concentration increased, zeta potential increased due to negativity of EPS. Then the electrostatic repulsion forces between negatively charged floc components became stronger as in the case of DLVO theory. This resulted in poorer compaction and settling (Zita and Hermansson, 1994). Increase of the EPS concentration had negatively affected flocculation (Wilen *et al.,* 2008). Higher concentration of the biopolymers avoided the flocculation of bacterial mass and caused poor settling (Harris and Mitchell, 1975; Kakii *et al*., 1989; Urbain *et al*., 1993; Liao *et al*., 2001).

The dominant component of the EPS also affected the settleability in some of the studies. Higgins and Novak, (1997 a), had positively correlated the bound protein concentration and settleability. Liao *et al.* (2001) and Urbain *et al.* (1993) found similar results indicating the improvement of settleability with higher protein content.

Increased polysaccharide concentration seemed to negatively influence settleability of the sludge (Urbain *et al.* 1993, Randall *et al*., 1971; Forster, 1971). In the same manner a high C/N ratio of 43 caused an increase of the carbohydrate portion of EPS under nutrient deficient conditions. This led to an increase in SVI, indicating poor settleability and sludge bulking condition (Durmaz and Sanin, 2001).

Divalent and monovalent cations played role in settleability. Bruus *et al.* (1992) and Higgins and Novak (1997 b) indicated that excess concentrations of the monovalent cations caused deterioration in floc structure and settleability. However this conclusion was not same for all the monovalent cations. Excess sodium caused poor dewaterability, settling and bioflocculation (Higgins and Novak, 1997 a) whereas potassium led to worsening of dewaterability while there was not a problem in settling (Novak *et al*., 1996; Higgins and Novak, 1997 a).

Addition of the divalent cations such as calcium and magnesium improved sludge settling proving the validity of the divalent cation bridging mechanism. Moreover

monovalent/divalent ratio that is bigger than 2 caused problems in settling (Higgins and Novak, 1997 a).

Moreover floc density and floc particle size affected the settleability. Stokeôs law explained the relationship between the floc size and density and settleability. By the increase of the floc density and floc size the settling properties improved. Plants reported good settling properties have floc densities in the range of 1.025 ï 1.035 g/mL range. The floc density and floc size was shown to be one of the main factors influencing the settling characteristics (Higgins and Novak, 1997 a and Murthy and Novak, 1997).

2.5.3 Rheology

Rheology can be described as the science concerning about the relationship between stress and deformation (strain). One of the rheological parameters, viscosity, is used for understanding the ñfluidityò of a fluid (Young et al., 1994). It can be mainly described as the ñresistance of the fluid to flowò or the proportionality constant between the shear stress and shear rate of a fluid element which can be formulated as below:

$$\tau \propto \frac{du}{dy} \tag{2.1}$$

Where Űis the shear stress, du/dy is the shear rate. When there is a linear relationship between the shear stress and the shear rate the fluid is said to be Newtonian fluid which can be formulated as below:

$$\tau = \mu \frac{du}{dy} \tag{2.2}$$

Here, the definition for the viscosity, μ, is valid that viscosity is proportionality constant. Newtonian viscosity is constant under a certain temperature and pressure (Hou and Li, 2003). However activated sludge is known to obey non-Newtonian behavior (Hou and Li, 2003; Sozanski *et al.,* 1997). The viscosity of sludge depends on a shear rate gradient under certain pressure and temperature (Dentel, 1997; Spinosa and Lotito, 1997).

The ratio of shear stress to shear strain gives the apparent viscosity in non-Newtonian fluids (Young *et al.,* 1994). Apparent viscosity in sludge is the reflection of the internal and external forces acting within the sludge flocs and also it indicates the deformation of the floc under stress (Dentel *et al.*, 1997; 2000).

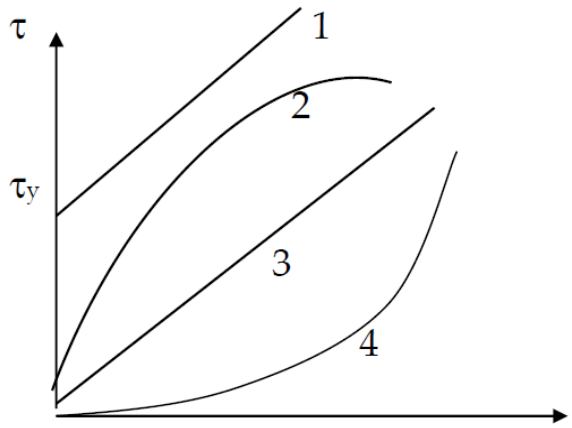


Figure 2.6 The Rheograms of Different Fluids (Vesilind, 1979)

There are different types of Non-Newtonian fluid behavior as can be seen in Figure 2.6 above. 1 denotes Bingham plastic; 2 represents Pseudo-plastic; 3 is the Newtonian fluid and 4 is the dilatant fluid.

The Bingham plastic shows neither fluidity nor solidity. Up to a certain point with a finite shear stress the material does not move. However once the yield stress is

exceeded the fluid starts to flow (Young *et al.,* 1994). Bingham plastic behavior is formulated as follows:

$$\tau = \tau y + \eta \frac{du}{dy}$$

(2.3)

Where Ű= yield stress and Ẽ is the plastic viscosity.

According to the studies, activated sludge rheology was mostly reported to be the pseudo plastic (Lotito and Spinosa, 1997; Moeller and Torres, 1997; Proff and Louhmann, 1997; Krauth and Staab, 1992).This behavior is formulated as follows:

$$\tau = K\left(\frac{du}{dy}\right)^n$$

(2.4)

Where K= fluid consistency index and n= flow behavior index which is a number less than 1. In ñshear thinningò or pseudo plastic behavior, apparent viscosity decreases when shear rate is increased (Young *et al.,* 1994).

Pseudo plastic behavior of sludge can be attributed to bioparticulate structure of the activated sludge. These particles flocculate in a large network. When increasing shear rate is applied to sludge, flocculated network gets disrupted and viscosity of sludge decreases (Rosenberger *et al.,* 2002). When the MLSS concentration is in increasing amounts and there is a cross-linkage between particulates, by the increase in shear rate, network disrupts and water is released between the particles and hence viscosity of the sludge decreases (Proff and Louhmann, 1997; Dentel, 1997).

Some of the results for activated sludge showed pseudo plastic behavior with an initial yield stress which is a combination of Bingham plastic and pseudoplastic flow (Battistoni, 1997; Slatter 1997; Günder, 1999; Mikkelsen, 2001). In addition to all of

these fluid types, there is the presence of dilatant fluid or ñshear thickening fluidsò in which there is a positive relation between the viscosity and shear rate. Mathematical expression of shear stress vs. shear rate relationship for dilatants fluids is the same of that of pseudo plastic fluid. The only difference n is bigger than 1 (Young *et al*., 1994).

In the study conducted Günder (1999) it was found out that increased viscosity is the result of increased EPS concentration. When the polysaccharides and proteins were removed by some procedures just as the enzymatic treatments, there is an expectation of a decrease in viscosity (Sanin and Vesilind, 1994). Moreover by the addition of the metal ions to activated sludge systems the bound water content and therefore viscosity decreases (Forster, 1983). However, by the extraction of calcium ions and deflocculation, the apparent viscosity increased surprisingly in one of the studies (Sanin *et. al.*,2000). On the contrary, the recent studies regarded the reduction in viscosity due the release of kinetically immobilized bound water content within the flocs. Therefore when the flocs deflocculate, this released bound water become one of the factors that support the fluid flow through the flocs, with the assumption that the particles are rigid, non-interacting and non-mobilized (Sanin *et al.*, 2000; Hiemenz, 1986; Logan *et. al*., 1988). However activated sludge flocs are not completely immobilized and rigid (Logan *et. al.*, 1988). Therefore the abundance of loose and extended activated sludge flocs around the EPS was concluded as the reason for a slight increase in viscosity (Sanin *et al*., 2000).

2.6 Bulking

Sludge bulking can be named as macrostructure failure in which settling and compaction of sludge occur slowly. There are two types of sludge bulking: filamentous bulking and zoogleal (viscous) bulking.

2.6.1 Filamentous Bulking

A certain amount of filamentous bacteria is necessary for healthy flocculation in activated sludge systems. However overabundance of this type of bacteria results in sludge bulking problems. Lack of the filamentous bacteria with low SVI, high turbid supernatant is indicating of pin-point floc formation (Jenkins *et al.,* 1993).

In normal conditions filamentous microorganisms establish the basis of a backbone that enhances binding of polymers and microbial flocs (Sezgin *et al.*, 1978). When dominance of these filaments prevails, most common and serious type of the bulking in activated sludge systems known as filamentous bulking occurs. Filaments in the case of filamentous bulking behave in two ways in floc in order to interfere in settling:

- Inter-floc-bridging: filaments extend from the floc surface in a way that holds the other particles apart from the floc;
- Open-floc structure: the filaments grow inside the floc and attach to other filaments. Floc gets larger and contains internal voids that prevent floc from settling (Jenkins *et al.*,1993)

Sludge bulking condition can be understood through the analysis of SVI and can be observed at SVI Ó 150 (Jenkins *et al.,* 1993). There are several factors causing filamentous bulking. One of the factors that cause filamentous bulking is reported as nutrient deficiency (phosphorus and nitrogen). A recent study by Peng *et al*. (2003) showed that nitrogen deficiency provided by the feed in activated sludge systems is not only responsible for the viscous bulking but also filamentous bulking.

According to researches, 20-30 different filamentous microorganisms were identified to be found in activated sludge systems. *Sphaerotilus natans, Microthrix parvicella, type 1701, Nocardia spp. ,Haliscomenobacter hydrossis, Nostocoida limicola I, II & III, type 021N, type 0961, Thiothrix I and II,type 0581, Beggiatoa spp., type 0092,type 0914 type 0411,type 0041, type 1863,type 0675, fungi, type 1851, actinomycetes, type 0803* were identified as filaments that cause bulking and foaming

There are several environmental causes of the filaments growth in activated sludge systems. The main causes that can enhance the production of specific type of the filaments were identified for some of the cases. The cause of the filamentous microorganism growth was studied by using three different methods. In the first method, filaments were isolated in pure culture and their competitive growth regimes were observed. The study succeed in finding the cause of *S.natans,type 1701,Haliscomenobacter hydrossis, type 021N, Thiotrix I and II and Microthrix parvicella* species. Secondly the thousands of activated sludge samples were examined and the filaments were identified under the microscope in order to obtain a database. The negative and positive associations between filaments were identified. Thirdly, the trial and error methods in activated sludge systems with bulking problems helped to show some causes of the overgrowth of some of the filaments (Jenkins *et al.,* 1993).

When the system goes under low dissolved oxygen concentration, *Sphaerotilus natans, type 1701 and Haliscomenobacter hydrossis* are found dominantly in the sludge. In order to prevent this, minimum concentration of 2mg/L of oxygen should be provided to system.

Low F/M is responsible for the over growth of *type 0041,type 0675,type 1851* and *type 0803*. Under the competitive conditions of substrate, filamentous microorganisms are more successful to utilize substrate than floc formers.

Type 021N,Thiothrix I and II,Nostocoida limicola I,II,III,type 0914,type 0411,type 0961,type 0581and type 0092 growth was attributed to the septicity conditions in the system.

Some carbohydrates containing glucose, maltose and lactose in their composition improve the growth of filamentous bacteria. Easily biodegradable substrates just as fatty acids, aminoacids and alcohols can enhance production of some types whereas slowly biodegraded substrates are preferred by other types. It has been found that

under the presence of Grease and oil, *Nocardia spp., Microthrix parvicella* and *type 1863* are able to predominate.

Nutrient deficiency also causes bulking conditions. Under nitrogen *deficiency type 021N, Thiothrix I and II* filamentous growth is observed whereas under phosphorus deficiency *Nostocoida limicola III, Haliscomenobacter hydrossis, Sphaerotilus natans* growth takes place.

As a solution for controlling the filamentous bulking, hydrogen peroxide, ozone or chlorine is added to return activated sludge stream. By this way filamentous microorganism are selectively killed. The filaments inside the floc which form the basis for healthy flocculation are not affected by oxidants whereas the filaments extending out of the flocs become vulnerable to oxidants.

In order to enhance bioflocculation and therefore remove bulking, additional flocculants such as synthetic organic polymers lime and iron salts can be added to activate sludge. However these flocculants have no effect on filamentous microorganismôs growth (UCLA Website, 2003).

2.6.2 Zoogleal (Viscous Bulking)

Zoogleal bulking or viscous bulking is indicated by poorly settling, compacting and viscous activated sludge in which the microbial cells are in a dispersed position in extracellular mass. It is mainly caused by the failure of microstructure so that the excessive production of extracellular material takes place. (Jenkins *et al*, 1993). Due to excessive production of the extracellular polymers, the viscous bulking sludge contains higher amounts of the hydration water. As a result, the excessive polymers and its hydration water prevent the compaction of sludge and make it difficult to handle (Horan *et al.*, 1986).

In slime or Zoogleal bulking, Z*oogloea* overgrowth is seen to an extent that reduces sludge dewaterability and settleability. Correspondent organism responsible for the

bulking is thought to be *Zoogloea ramigera*. Zoogloea overgrowth is observed at high F/M ratios and in low oxygen concentrations so that the alcohols and organic acids are in excess amounts in the system (Michael, 2003). At high levels of viscous bulking, carbohydrate levels can be up to 70% as glucose on a sludge dry weight basis whereas the wastewater treatment plants contain 14-18% of total carbohydrate under normal conditions (Jenkins *et al.*, 1993)

Similarly, in the study conducted by Peng *et al.* (2003), the C/N ratio effect to sludge bulking was investigated while the phosphorus concentration provided was kept in adequate amount. It was found out that at BOD/N ratio of 100/3; at first, the excessive growth of filamentous microorganisms and then the viscous zooglea formation and hence viscous bulking took place. When the nitrogen concentration was decreased to provide BOD/N 100/0.94, serious viscous bulking problem was observed.

Besides the ratio of C/N/P, the presence of the cations may change the type of bulking. In the study of Vatansever and Turtin, (2006), the effect of two divalent cations, calcium and magnesium, on sludge bulking at phosphorus deficient conditions was investigated. It was seen from photomicrographs that Magnesium provided in the daily synthetic feed induced the production of filamentous bulking whereas Calcium enhanced the dominance of zoogleal bulking. When the phosphorus was provided in sufficient amounts and there was an excess carbon source at a ratio of C/N=43, sever viscous bulking was observed (Durmaz and Sanin, 2001).

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Set-Up and Reactor Operation

The experiments were composed of two sets. In the first set, microbial culture in bioreactors were exposed to phosphorus deficient conditions. In the second set, they were grown under sufficient phosphorus concentration.

Six semi-continuous activated sludge reactors, each having a capacity of 2L, were operated during two sets of the study. The reactors were seeded with wastewater taken from the primary settling tank effluent of Tatlar Ankara Central Wastewater Treatment Plant. The schematic presentation of the operating reactors can be seen in Figure 3.1

Continuous supply of oxygen was provided to reactors by the air pumps in order to have the dissolved oxygen concentration at a minimum value of 3mg/L. Moreover air pumps provided mixing conditions in the reactors. The sludge age (θ_c) of the reactors was 8 days. In order to sustain the sludge age, everyday 250 mL of sludge was wasted from each reactor. The mixed cultures in bioreactors were fed with synthetic feed solutions and distilled water.

In this study, one of the aims was to investigate the effect of increasing magnesium ion concentrations to sludge characteristics. Therefore during two sets, three different magnesium ion concentrations, 0.5, 5 and 15meq/L, were applied to six semi

continuous reactors, each pair having same magnesium ion concentration. Reactors having 0.5meq/L magnesium ion concentration was used as control reactors.

In order to feed the bioreactors, the following procedure was done everyday. First of all, mixing of each reactor was provided. Then 250mL of sludge from each reactor was wasted and the mixed cultures in bioreactors were left to settle for 2 hours. After settling, the supernatants were siphoned off. New feeding solutions were added to system and remaining part was filled with distilled water to 2L level.

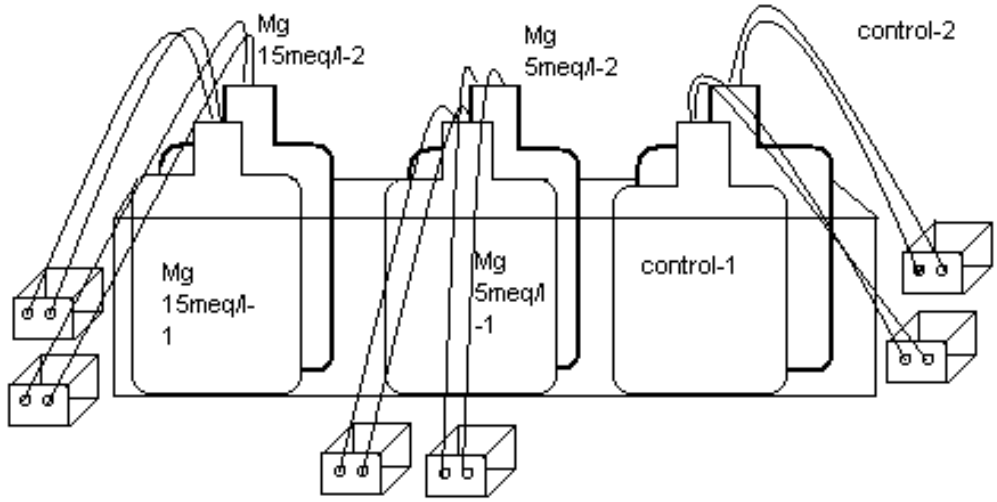


Figure 3.1. Schematic Representation of the Reactor Set-Up

3.1.1. Phosphorus Deficient Conditions

It is known from literature, there are many factors that cause sludge bulking. One of them is caused by the nutrient deficiency in the system. In order to observe if sludge bulking is occurring in the first set-up, phosphorus given to the system was kept in lower amounts. BBL Biosate peptone was used as the only phosphorus source in the system. The synthetic feed content which was provided to system everyday can be seen in Table 3.1 below.

In addition to these constituents, mentioned in the Table-3.1, $MgSO4.7H_2O$ was added to pair of the reactors in three different concentrations (123mg/l, 1230 mg/l and 3690 mg/L) in order to sustain magnesium ion concentration in 0.5, 5 and 15 meq/L.

COD of synthetic feed solution was measured as 1115 mg/L and TKN of the feed was found to be 60.2 mg/L. In addition to this, peptoneôs phosphorus content was 0.624mg/l indicating phosphorus deficiency in the feed solution. C/N ratio was 18.5 in terms of COD/TKN. In this set, it was desired to obtain COD: N: P ratio near to 100:5:0.05 in order to provide balanced C/N but deficient phosphorus.

The temperature was kept at 25ŰC and pH in the reactors was maintained at 7±0, 5 in order to satisfy optimum growth conditions. The neutral pH range was obtained by containing tris buffer in synthetic feed.

Table 3.1 the Composition of the Synthetic Feed Medium Given to the Reactors

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
$NaHCO_3$	105
Peptone	60
KCl	37.25
$CaCl_2.2\ H_2O$	36.75
Tris Buffer	36.3

Under these environmental conditions and feed provided, the reactors reached steady state after 80 days (10 ъ c). The steady state situation was decided by measuring the

MLSS and MLVSS concentrations of the wasted sludge once in two days. When the measurement values converged to each other in these subsequent measurements, chemical, physical and biological analysis were conducted.

3.1.2. Phosphorus Sufficient Conditions

In the second set of the experiments, sufficient phosphorus concentration was provided to the reactors. Required phosphorus concentration was provided by increasing the concentration of BBL Biosate peptone. It was aimed to operate the reactors at COD: N: P ratio 100:5:1 which is known as a balanced feed. In order to provide synthetic feed to the reactors near to this value, a few trials were done to obtain the corresponding COD, TKN concentrations. From analysis COD value was found to be 1429 mg/L and TKN was 78.08 mg/L while the phosphorus concentration was 11.44 mg/L.The concentrations of the constituents of the synthetic feed can been seen in the Table 3.2

Table 3.2 the Composition of the Synthetic Feed Medium Given to the Reactors

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
$CaCl_2.2\ H_2O$	36.75
Tris Buffer	18.15

As in the first set of the experiments, magnesium concentration in the form of $MgSO_4.7H_2O$ was added to each pair of the reactors in three different concentrations (123 mg/L, 1230 mg/L and 3690 mg/L).

Water bath, in which the reactors were laid, was adjusted to 25ŭC. In this set, the pH range was 7.8±0, 3. The second set reached to steady state conditions at 60 days (7.5 ɒ c). After determination of steady state conditions, chemical, physical and biological analyses were begun.

3.2 Analysis Conducted At Steady State

When the reactors reached steady state at two sets, chemical, physical and microbiological analyses were conducted.

3.2.1. Chemical Analyses Conducted At Steady State

Chemical analyses within the scope of this thesis study were constituted by extracellular polymer extraction, carbohydrate, and protein analysis of extracted EPS and conductivity measurements. As soon as the extracellular polymer extraction process ended, the carbohydrate and protein analysis were carried out.

3.2.1.1. EPS Extraction and Protein Carbohydrate Analysis

3.2.1.1.1. EPS Extraction

Chemical, physical and physico chemical methods were developed in order to extract extracellular polymeric substance (EPS) from sludge. In order to conclude that the extraction method is effective, the technique should be strong and effective enough to extract the polymers at the highest range and should not disrupt the structure of cell and cause cell lysis. In the case of the occurrence of a cell lysis, intracellular polymers transport to extracellular phase of the cell and therefore inaccurate

concentration and composition of the extracellular polymeric substance can be obtained (Sanin and Sesay, 2006).

During two sets, one of the chemical extraction techniques, CER (Cation Exchange Resin) method which was first discovered by Frolund et al. (1996) and then developed by Durmaz and Sanin (2001), was applied to sludge samples. Strongly acidic cation exchange resin (CER) (Dowex 50x8, 20-50 mesh) in Na- form was used. Before using this chemical, the cation exchange resin was exposed to washing procedure in order to get rid of contaminants. Firstly, MLVSS values of each reactorôs sludge samples were determined. Then by accepting that 100g of CER is required for one gram of VSS, necessary CER concentrations for each reactor samples were calculated. Therefore determined CERs were weighed; they were washed and stirred with 2L of phosphate buffer saline solution for an hour at 120 rpm. Phosphate buffer saline (PBS) solution was composed of 4g/l of NaCl, 0.1g/L of KCl, 0.06g/L of KH_2PO_4 and 0.859g/L of $Na_2HPO_4.7H_2O$. After termination of the stirring procedure, washed CER was filtered and dried at room temperature for one day.

As the first step of the polymer extraction procedure, 200mL of daily 250mL wasted sludge was centrifuged for 15 minutes at 3500 rpm. Remaining sludge was used for MLSS and MLVSS analysis. Centrifugate was discarded and remaining sludge was resuspended by using PBS solution and finally put into jar test beakers in which the dried CER was already placed. The beakers were filled with PBS until 200 mL level. There were also two control beakers which were put into same procedure. The first one was CER control beaker which contained CER and PBS without addition of sludge to check the possible releases from CER. Second one was sludge control, containing sludge and PBS without having CER to evaluate how much of EPS was released by only mixing when CER was absent. The jar test beakers for each reactor sludge and two control beakers were put into jar test apparatus and stirred at a rate of 120 rpm for 300 minutes.

After the end of the stirring process, all of the beakers were waited about 30 minutes without any intervention until the two separate phases were obtained inside the beaker. The supernatant parts of the solution were pipetted and again went under a centrifugation process at 3500 rpm for 15 minutes. The centrifugated sample was further used in the analysis of carbohydrate and protein.

3.2.1.1.2. Carbohydrate Analysis

Phenol-sulfuric acid method (Dubois *et.al.,* 1956) was used in the carbohydrate analysis of extracted polymer and soluble polymer. In this method phenol (80% by weight) and sulfuric acid (reagent grade 95.5%) were used as reagents in order to determine concentrations of sugar which were in micro level

2 mL of each extracted polymeric samples was pipetted into test tubes. In order to assure the validity of results and minimize errors due to contamination by cellulose lint, the samples in test tubes were run in triplicate. A 0.05 mL of phenol (80% by weight) was added into test tubes containing 2mL of extracted samples. Following this, 5mL sulfuric acid was added directly to liquid surface taking into care the avoidance of collision of acid to test tube sides. After adding all of the necessary reagents, the test tubes were waited for 10 minutes at room temperature. At the end of ten minutes, all of the test tubes were vortexed and incubated for 15 minutes at 30°C in order to obtain stable yellow-orange color. As a last step in this procedure, each sampleôs absorbance was read in Pharmacia LKB Novaspec II Spectrophotometer at 480 nm. According to the calibration curve constructed before the carbohydrate analysis, by using the alginate as standard, carbohydrate concentration of each sample were calculated. (Dubois et al., 1956). The constructed calibration curves for two sets are demonstrated in Appendix B

3.2.1.1.3. Protein Analysis

In determination of protein concentration, a method developed by Lowry *et al*. (1951) was applied due to the sensitivity of this method to smaller concentrations of protein or highly diluted protein. During the experiments four different reagents were used which were described below:

- Reagent A: containing 2 percent of Na_2CO_3 which was dissolved in 0.1N NaOH.
- Reagent B: 0.5 percent $CuSO_4.5H_2O$ in 1 percent sodium potassium tartarate.
- Reagent C: obtained by mixing 1mL of Reagent B and 49mL of Reagent A
- Reagent D: obtained by mixing of 10mL of Folin- Ciocalteuôs phenol reagent with 10mL of distilled water.

A 0.6mL from each reactor samples was taken into tubes. As in the case of the carbohydrate analysis, the samples were run in triplicate. Then 3mL of Reagent C was added to sample containing test tubes and they were let to stand with no action for 10 minutes at room temperature. At the end of the waiting period, 0.3mL of diluted folin, Reagent D, was added to each tube and vortexed sufficiently. The tubes were waited for 30 minutes at room temperature with the appearance of a blue color. As the last step, the samples were read at 750 nm in Pharmacia LKB Novaspec II Spectrophotometer. The exact concentrations of protein of extracted samples were calculated by using the calibration curve. Calibration curve of each experiment of two sets were constructed by using Bovine Serum Albumin as Standard. Calibration curves are seen in Appendix B

3.2.1.2. Conductivity

In order to flow the dissolved ion concentration in the reactors the conductivity measurements were done. The measurements were carried out by using probe of Cyber Scan PC 510 pH/conductivity meter. Before wasting 250 mL of daily sludge,

the reactors were mixed well as it was mentioned before. After wasting this volume of sludge, the shaken reactors were allowed to settle for 2 minutes. Then the probe was submerged to reactors. When the values seen on the monitor was converged to a constant number at nearly 25°C, this constant value was recorded.

3.2.2. Physical Analyses Conducted At Steady State

3.2.2.1 Viscosity

The viscosity of the sludge at two sets was measured with rotational viscometer Brookfield LVDVII+ with ultra low viscosity adapter. The measurement at viscometer was taken at the end of 1 minute rotation. At different shear rates (1.83, 3.67, 7.34, 14.7, 36.7, 73.4 s^{-1}), the shear stress of each sample was recorded. At the same time, MLSS concentrations of each reactor samples were determined. Moreover 25, 50 and 75 % dilutions of each sampleôs MLSS were done. Dilution was obtained by using Phosphate buffer saline (PBS) solution, composition of which is described above. Same procedure was applied to diluted samples in order to read the shear stress. By getting these data set, shear stress vs. shear rate graphs were drawn in order to understand the fluidity of sludge.

In addition to this, from the values recorded at viscometer, apparent viscosities of each sample at different concentrations were determined. After that the apparent viscosity versus measured MLSS concentration graph of samples were plotted.Viscosity of sludge was read at 1500 mg /L of MLSS concentration.

3.2.2.2. Sludge Volume Index (SVI)

One of the most important parameters indicating the settleability of sludge is Sludge Volume Index (SVI). As a procedure, the reactors were shaken and 1L of them was poured into 1L of graduated cylinder. The sludge was settled for 30 minutes. The volume of the settled sludge solids was recorded at the end of the half an hour. At the days that the SVI analysis conducted, the MLSS concentrations were also

determined. By getting MLSS and the volume, SVI was calculated according to formula below:

$$\text{SVI} = \frac{\text{settled volume } (\frac{\text{mL}}{\text{L}}) \times 1000}{\text{MLSS concentration } (\frac{\text{mg}}{\text{L}})} \quad (3.1)$$

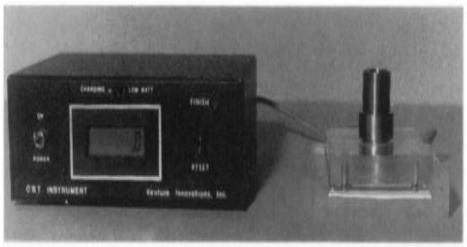
3.2.2.3. Turbidity

Turbidity measurements were done by using Hach Turbidimeter 2100N. The SVI and turbidity analysis were conducted at the same days. The reactors were shaken and poured into 1L of graduated cylinder as it was indicated in SVI part. When the sludge in the cylinder was settled for 1 hour, 19-20mL of the supernant at the top was taken by the help of the pipettes from top. Supernant was poured into turbidimeter tubes.

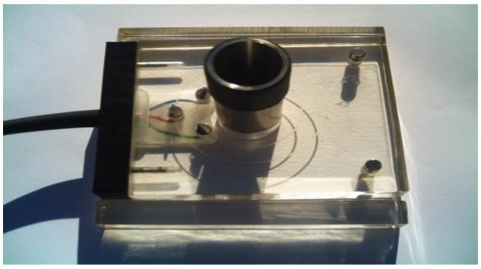
3.2.2.4. Capillary Suction Time (CST)

Due to its rapid and simple use, capillary suction time (CST) test is used for comprehending the dewaterability potential of wastewater sludge (Vesilind, 1988). Moreover it is applied as a method for understanding the effect of chemical conditioners such as cationic polymers on sludge dewaterability (Baskerville, 1977; Swanwick, 1972).

In the experiments, Type 304 M Triton Electronics Capillary Suction Timer was used in CST analysis and Standard Method 2710G was applied (APHA, 2005). This device which can be seen in Figure 3.2 below is mainly composed of two plastic blocks, a stainless steel collar, three electrical contacts that are positioned in the upper plastic block, an electrical timer and filter paper. The thickness and type of the filter paper is also significant. In CST analysis Whatman 17 Chromotographic paper was used as filter paper.



(a)



(b)

Figure 3.2 a) The Basic Appearance of a CST Device (Vesilind, 1988) b) Part of the Triton Electronics Type 304M CST with its Stainless Steel Collar, Plastic Blocks with Contact Points of Them

The sludge sample from each reactor was mixed well and this mixed sample was poured into the stainless steel collar. Liquid phase in sludge began its movement through the paper, making a circular wet blot shape in the paper. When the interface moved and therefore passed the center of the stainless collar about 1.59 cm, it contacted the sensors or the two contact points. This was the time that the electrical timer counting began. When the liquid circular movement continued and passed the two contact points about 0.7 cm, it contacted the third point on the block. This was the end of the electrical timer work. The written value in the electrical timer was recorded and determined as CST in seconds.

3.2.3. Microbiological Analyses Conducted At Steady State

When steady state was achieved in two sets, microscopic analysis was conducted in order to determine type of microorganisms in sludge and to assess the structure of flocs. Photomicrographs of sludge flocs were taken under the microscope.

Microscopic analyses were conducted and photomicrographs were taken by using Leica DFC 280 device.

3.2.3.1. Analysis Conducted for Bacterial Identification

In order to understand which type of the bacteria or genus is predominant in sludge samples API 20E and API Coryne test strips manufactured by bioMerieux, Inc. were used. Each identification system required culturing and isolation of the bacteria.

3.2.3.2. Quantification of Bacteria of the Wastewater Sludge

It is known that most of the microorganisms are heterotrophic in activated sludge environment. Therefore quantifying heterotrophic bacteria in the sample of reactors is essential. In order to achieve this, the spread plate technique was applied in this study. Before applying the technique, Casitone-Glycerol-Yeast (CGY) agar was chosen as the growth media and therefore prepared to be poured into empty petri dishes. CGY agar was prepared by mixing 5g/L of casitone, 5g/L glycerol, 13g/L agar and 1g/L agar and then autoclaving of the mixture. After this procedure the prepared agar was poured into petri dishes near bunsen burners in order to keep aseptic conditions.

As soon as the agar plates prepared, the technique began to be applied. First of all, the samples were required to be diluted with the aim of counting 30-300 colonies on petri plates. Serial dilutions were performed by using 0.1% peptone. When the required serial dilutions were performed, 0.5 mL of diluted samples with the highest dilution factor was transferred to CGY agar plates by using a sterile pipette. Streaking was achieved by using the bottom part of the test tubes. Test tubeôs bottom part was passed quickly through the Bunsen Burner flame. Then the inoculation process began by streaking the bottom part of tube back and forth across the plate in order to distribute the bacteria as evenly as possible. After assuring that the bacteria

were spread through all the plates, the plates were inverted, ready for the incubation. The plates were incubated at 35°C until the colonies were countable on the plates.

The incubated plates were examined in order to distinguish and choose different colonies. The different colonies were determined and they were again re-streaked on the CGY agar plates to obtain isolated colonies. Streak plate technique was applied. Colony at CGY agar plate, obtained by the procedure of spread plate technique was taken by the help of a loop. Before this process, the loop was sterilized.

As soon as the loop cooled in seconds, full loop of determined colony was picked. Then from the initial site of inoculation the loop of bacteria was spread by passing from one side to another. The loop was sterilized again and from the end point of the first streaks, other streaks were made through the other side of the CGY agar plates. Same procedure was repeated through the unstreaken parts of CGY agar plates as illustrated in Figure 3.3 below.

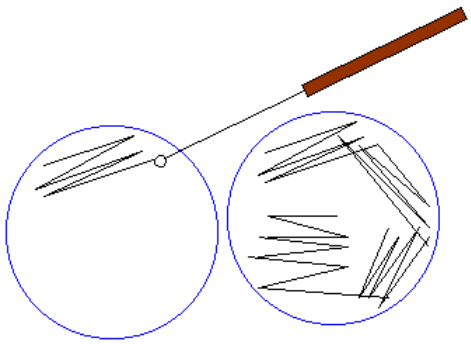


Figure 3.3 Streaking Process

After the inoculation, the streaken plates were incubated for 24 hours at 35°C. The result was the colony formed from a single bacterial cell fallen to agar plates. The isolated bacteria were picked for the use of API 20E and API Coryne.

3.2.3.3. Additional Tests Required for Using the Appropriate API Test

Before using API tests directly, some preliminary tests were applied in order to assure the use of appropriate type of the API kits. There was the necessity of determining gram positivity/negativity of isolated bacteria. Moreover catalase and oxidase tests were applied to determine the genus of isolated bacterium.

3.2.3.3.1. Catalase Test

The catalase test detects the presence of catalase enzyme in bacteria, by the decomposition of hydrogen peroxide to release oxygen and water. Due to aerobic breakdown of sugars, the hydrogen peroxide is formed as an end product. By the presence of catalyse enyzme the peroxide can decompose. By dropping one drop of 3% H_2O_2 to isolated cultures and waiting about a minute, the catalase set finished. When the bubbles formed it could be said that the reaction was positive. When the bubbles were not formed the reaction could be said as negative. This test was applied to isolated bacteria before the inoculation procedure at API kits.

3.2.3.3.2. Gram Staining

One of the basic methods which makes the bacterial cells visible under the microscope is gram staining. Loopful of isolated bacteria was placed on the top of an aseptic clean slide on which 1-2 drops of distilled water was injected. By staying the slide on a constant position over the bunsen burner the water on the slide was dried. Moreover the fixing of the material on the slide was provided. Then staining of the slide with crystal violet and maintaining the stain for 1 minute on slide was done. After pouring off the stain and washing slide with water, the slide was subjected to Gramôs iodine solution for 1minute.At the end of the pouring off the iodine the slide was decolorized by washing it with acetone /alcohol for 30 seconds. After washing the acetone remaining, this time, the slide was flooded with safranin counter strain for 30 minutes and finally washed with water. The procedure was repeated for all of the isolated bacteria. After the end of this procedure the stained samples were

examined under the microscope. According to the color retained in the peptidoglycan layer in the periplasm of the cell, positivity/negativity was determined. The cells were said to be gram-positive when the purple color was retained in the thick layer of peptidoglycan. Gram negative cells were pink in color due to not retaining the dark stain in thin layer.

3.2.3.3.3. Oxidase Test

By use of the oxidase test it can be understood that bacteria use oxygen as an energy source or not by the presence or absence of cytochrome oxidase enyzme. Firstly, the oxidase reagent was poured into a filter paper then isolated culture was distributed on the reagent. The test lasted about 30 seconds to 1 minute. When the color change to purple was observed during mentioned time interval, the bacteria was said to be oxidase positive.

3.2.3.4. The API 20E System

In order to apply API 20E Kits manufactured by bioMerieux, Inc., the isolated culture should be Gram negative rod shaped (bacillus) bacterium. API 20E Kit apparatus as can be seen in Figure 3.4 is composed of 20 individual cupules in which different reagents are placed in order to specify the metabolic capabilities, genus and species of enteric bacteria in the family of Enterobacteraceae.

Figure 3.4 API 20E Kit Apparatus

The cupule set was placed under humid plastic strip. The isolated cultures were taken to 0.85% NaCl solution by the help of the sterilized loop and by using vortex, homogeneous mixture was obtained. Citrate utilization (CIT), gelatinase activity (GEL) and acetoin production(VP) cupules were completely filled with the prepared homogeneuos mixture.

Arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC) and urease activity (URE) and H2S activity cupules were half-filled with the suspension medium and then the remaining parts were overlaid with mineral oil with the aim of carrying out the anaerobic reactions. Other cupules were filled with suspension medium. After putting these necessary mediums, the cap of the plastic strip was closed and incubation lasted about 18-24 hours at 37°C. At the end of the incubation period, the reactions were evaluated as positive or negative according to the manual and some further reagents were added to some of the cupules. After another 24 hours of incubation due to the addition of further reagents, final evaluation of the reactions was done. The results were converted to Analytical Profile Index and from software database, the genus or the species of the isolated cultures were determined.

3.2.3.5. API Coryne System

API Coryne is a standardized system that determines the genus or the species of the Corynebacterium in 24 h period. It is composed of 20 microtubes of dehydrated substrates that demonstrates the enzymatic activity or fermentation of the carbohydrates. API Coryne Apparatus can be seen in Figure 3.5. In order to apply API Coryne tests, gram staining and catalase tests of the isolated cultures were performed. Although the CGY agar plates were used in obtaining pure cultures in API 20E system, the mixed cultures obtained by spread plate technique was re-streaken again on Blood Agar (5% sheep blood) in API Coryne System. In this case, the pure culture on blood agar was taken by the loop into the 3mL of API bacterial suspension medium. The addition of culture to medium lasted until the turbidity of

the homogeneous medium was greater or equal to 6 McFarland. First nine cupules until the urease activity cupule as demonstrated in Figure below were completely filled with this suspension. Then Urease activity (URE)ôs only tube part was filled with suspension. Gelatine hydrolysis cupule and tube were entirely filled with suspension medium.

Figure 3.5 API CORYNE Test Kit

Remaining suspension was transferred to GP medium (0.5mL) and mixed well. Last 9 reactionsô (0-GLYG) tubes were filled this new suspension. The cupules of O-GLYG and URE were filled with mineral oil for anaerobic reactions. After 24 hours of incubation at 36±2°C, the required reagents were added in drops in order to observe the color changes by the occurrence of reactions in the cupules. The evaluation of the positivity/ negativity of the reactions were done. Finally, the results were converted to Analytical Profile Index and from software database, the genus or the species of the isolated cultures were determined.

3.2.4. Other Measurements

3.2.4.1. MLSS and MLVSS

Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentration experiments were conducted once in two days in order to determine the steady state conditions. Besides this, at steady state conditions MLSS and MLVSS were continued to be measured since the results were used in analysis of viscosity, polymer extraction, SVI and CST. MLSS and MLVSS were measured by using Method 2450D and 2540E (APHA, 2005). MLSS and MLVSS concentrations of two sets with respect to time can be observed in Appendix A.

3.2.4.2. COD

At steady state conditions, chemical oxygen demand of the reactors was also determined in order to define organic content of system. Before using the closed reflux colorimetric method (EPA Method, 410.4), the samples were centrifuged in order to use the supernatant part of the solution. By taking the supernatant part and making necessary dilutions if necessary, the samples were put into the HACH digestion solutions for COD. Then the kits were allowed to be heated for 2 hours in Hach COD reactor. In the end, the values were obtained by using Hach DR 2000 spectrometer.

3.2.4.3. pH

In order to keep the system at optimal conditions for the mixed culture, the pH measurements were conducted twice a day. Cyber Scan PC 510 pH meter /conductivity meter was used.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results of the Reactors at Phosphorus Deficient Conditions

Most of the industrial wastewater does not contain sufficient amount of phosphorus (Eckenfelder, 1966; Wu, 1972). The problems in the operational conditions of the activated sludge due to this fact are surpassed by adding phosphorus to the system. However the effect of changing cation concentrations under this condition is not studied. The possibility of improvements of sludge characteristics by increasing cation concentration to phosphorus limited reactors is of interest. Therefore the first part of this study evaluates the effect of magnesium concentration under phosphorus deficient condition. The results of the first set conducted with varying magnesium cation concentration will be helpful for understanding the progress of sludge characteristics.

As aforementioned, the phosphorus deficient conditions were provided to the reactors by keeping the COD/P ratio near to 1800 (1115mg/L/ 0.624mg/L) whereas the COD/TKN ratio was kept at a nearly optimum value of 18.5 (1115mg/L/60.2mg/L). At phosphorus deficient conditions the effect of three different magnesium ion concentrations (0.5, 5, 15 meq/L) that were supplied to each replica reactors were examined. Physical, chemical and microbiological analyses were conducted. During the analyses it was recognized that replica reactors with minimum magnesium ion concentration which are named as the control reactors, did not get similar results. In addition one of these reactors was extremely different in

appearance, much darker as if it was anaerobic than the other reactors. Consequently the results of this reactor were not taken into consideration during the measurements. The results from other reactors were the averages obtained from the two replica reactors.

4.1.1 Results of the Chemical Analyses

Chemical analysis of wastewater is a useful tool for understanding the characteristics of wastewater and condition of the treatment process (Water Environment Federation, 2008). In addition it provides information about concentration of the specific substances and organic content of the wastewater (Tchobanoglous & Burton, 1991). As chemical analyses, effluent soluble chemical oxygen demand (COD), EPS concentration, carbohydrate content of EPS (EPSc), protein content of EPS (EPSp) and conductivity measurements were conducted in both sets.

4.1.1.1 Chemical Oxygen Demand (COD) Measurements

Effluent COD measurements were carried out in order to evaluate the reactor operation efficiencies. In this set all of the effluent COD values as can be observed in Figure 4.1 were higher than the influent COD of 1115mg/L. This may result from the non settling conditions of sludge. In every day procedure, after 2 hours of settling of sludge, supernatant part was siphoned off and daily feed of a total volume of 250 mL was provided to the reactors as aforementioned. Since sludge did not settle properly in all of the reactors of this set, due to the sludge bulking under deficient phosphorus concentration, supernatant partition to be siphoned could not be distinguished. Although ineffective siphoning occurred, new daily feed was continued to be provided to the system. As a result of that, organic content of the system which was expected to be consumed daily, accumulated. This resulted in an effluent COD extremely higher than the influent COD.

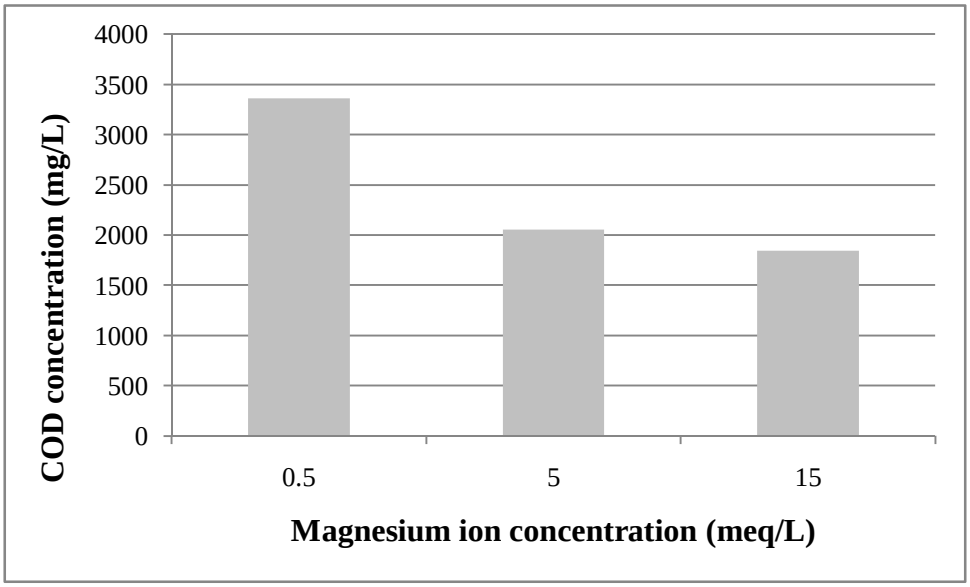


Figure 4.1 Effluent COD Values versus Magnesium Ion Concentration

4.1.1.2 Results of the Extracellular Polymeric Substance Extraction, Carbohydrate and Protein Analysis

Among several extracellular polymeric substance extraction methods, Cation Exchange Resin (CER) method was used as one of the chemical extraction techniques due to its higher efficiency and being mild not to cause cell lysis (Frolund *et al.*, 1996; Liao *et al.*, 2001; Durmaz & Sanin, 2001). Following the extraction procedure, carbohydrate and protein analyses were conducted in order to measure proteinaceous and carbonaceous part of the EPS.

The results are depicted in Figure 4.2 below. As can be seen protein content of the EPS increased by increasing magnesium concentration in the feed. At the concentration of 5 and 15 meq/L of magnesium, carbohydrate content was much higher than protein content. On the other hand there was not a significant difference between the two constituents in the control reactor (0.5meq/L). Addition of magnesium ion caused the increase of bound carbonaceous part of EPS in the composition.

The dominancy of carbohydrate concentration of EPS is in agreement with the previous study conducted by P.T.Hoa *et al.*, in 2003 that with the C/N/P ratio of 100/5/0.5 and 100/7/0.5, carbohydrate content of the EPS was much higher than the protein content indicating the high degree of impact of phosphorus on EPS_c. As a result of such an increase in carbonaceous matter, sludge settling and dewaterability characteristics got worse (Shin, et al., 2001; Morgan et al., 1990; Hoa *et al.,* 2003). In addition to these, comparatively lower values of the protein can be attributed to the fact that the phosphorus deficiency in the system affects the energy consuming processes just as the protein synthesis whereas the carbohydrate synthesis is not affected negatively by the starvation of this nutrient (Wu & Okrutny, 1982). In addition to effect of phosphorus starvation, magnesium ion seemed to contribute to the carbohydrate content increase in total EPS concentration in this set.

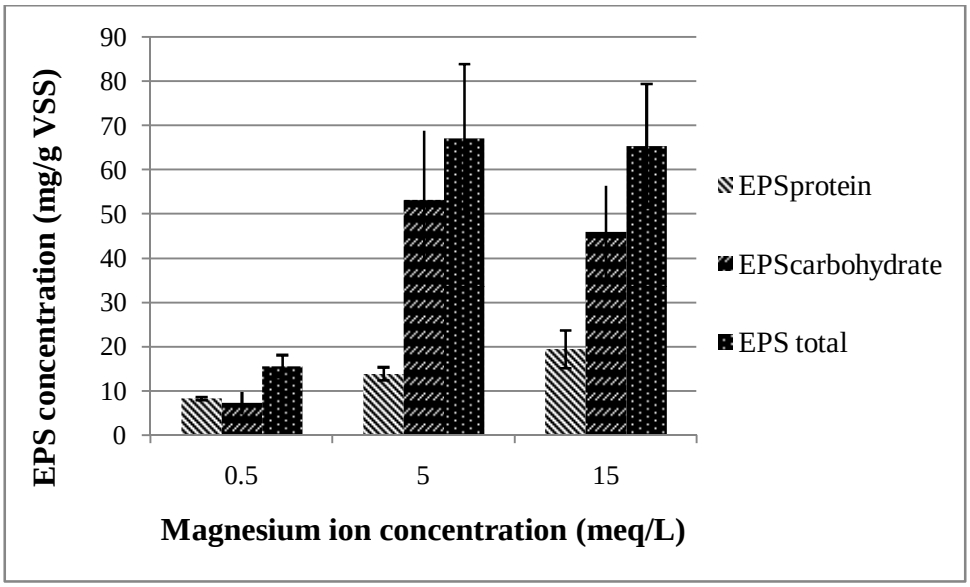


Figure 4.2 EPS_P, EPS_c and total EPS Concentration Versus Magnesium Ion Concentration

In the study conducted by Yu *et al.,* 2009, EPS fractions which were aforementioned were separated in order to understand which fraction of EPS acts as the best flocculant. In addition, the polysaccharide and protein content of these fractions were measured. Protein content of slime, supernatant and LB-EPS fractions was much

lower than the carbohydrate content whereas TB-EPS had highest protein content compared to carbohydrate content and showed best flocculating ability.

It may be speculated that lower concentration of protein observed in this set can be the indication of the absence of tightly bound EPS although the total EPS concentration is higher. Despite the fact that there is high concentration of total EPS in the system for the higher magnesium concentrations, healthy flocculation cannot be achieved. This result may be attributed to absence of EPS which has lower flocculation capability. This finding needs further investigation.

4.1.1.3 Conductivity

As stated before, electrical conductivity is a measure of level of ion concentration of a solution which is generally expressed with Siemens/m (S/m) or micro-Siemens (mS/cm). Conductivity versus magnesium ion concentration graph for the first set is illustrated in Figure 4.3 below. The cause of increase of dissolved ion concentration in solution with respect to changing concentrations of magnesium is dependent only on magnesium ion since only the concentration of the magnesium is altered in the synthetic feed in three conditions.

As can be observed there was not one to one increase in conductivity with the increase of magnesium ion concentration. For instance, by the increase of magnesium ion concentration from control to 5 meq/L, conductivity of the system increased only about 1.45 times. This might result from the fact that certain functions of the cation in the cell contributed to incorporation of cation into the cells. In addition some amount of cation provided in the feed might have been incorporated into floc structure.

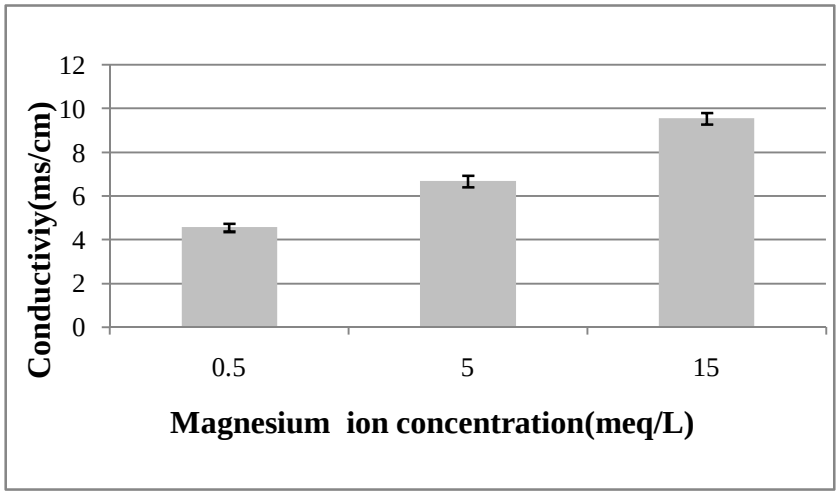


Figure 4.3 Conductivity of the Sludge with respect to Magnesium Ion Concentration Provided in the Feed.

4.1.2 Results of the Physical Analyses

Physical analyses are significant as well as the chemical analyses in order to determine the sludge characteristics. Physical analyses included Sludge volume Index (SVI), Capillary Suction Time (CST), viscosity and turbidity measurements in both of the sets.

4.1.2.1 Settleability

Settleability of the system was determined by measuring sludge volume index (SVI) which is a simple and quick test to show sludge settleability. SVI results absolutely indicated sludge bulking with values higher than 150 (Jenkins *et al.*, 1993). Extremely higher values of SVI reported in this set are the direct result of the lack of phosphorus concentration in floc structure. Under nutrient deficiency, filamentous microorganisms having higher substrate uptake rate than the floc formers (Chudoba *et al.*, 1973) grow in excess amounts and they stretch out of the flocs, forming expanded floc structure. As a result compaction and settling of sludge got worse by overgrowth of filamentous microorganisms (Novak *et al.*, 1993).

The relationship between SVI and changing magnesium ion concentration is graphed in Figure 4.4. SVI decreased from 470 to 330 by the transition from control reactor to 5 meq/L of magnesium ion concentration. At 15 meq/L, SVI value increased back to the value of 460.

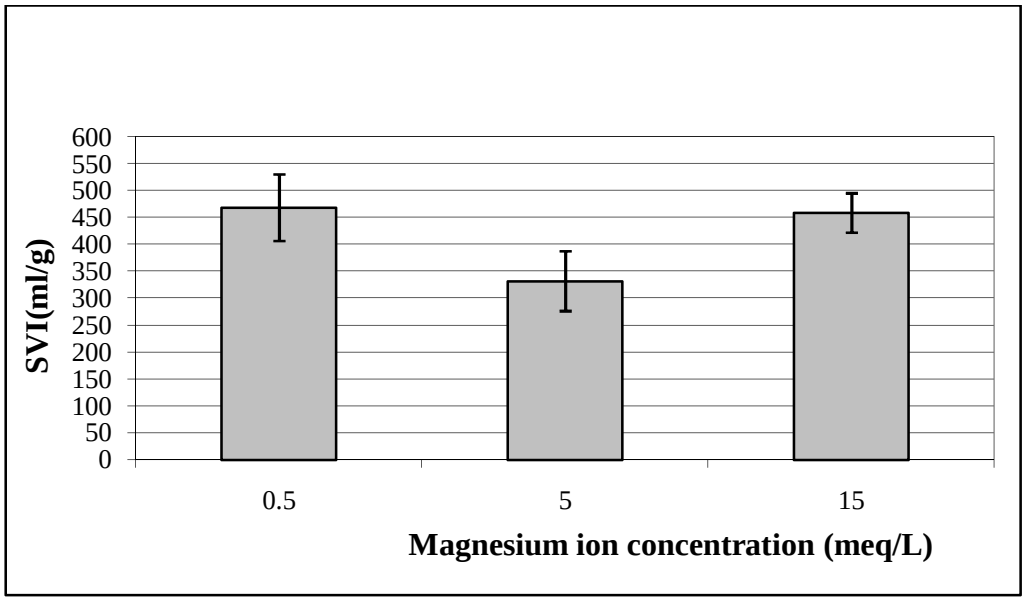


Figure 4.4 SVI Values at Different Magnesium Concentrations

Sludge settleability is known to be affected from variety of parameters. Among them extracellular polymeric substance concentration and composition were reported to be strongly related with settleability and hence SVI. However there are contradictory results in literature. Positive relation between EPS concentration and SVI was reported (Urbain *et al.*, 1993; Eriksson and Alm, 1991; Liao *et al.*, 2001) whereas negative correlation was found in some of the studies (Goodwin & Forster, 1985; Yun *et al.*, 2000). As stated in the latter case, EPS contributed positively to settleability of the sludge whereas decrease of it led to increase of SVI in this set.

EPS composition also acts on the settleability of the sludge. In transition from control reactor to 5 meq/L of magnesium concentration, carbohydrate content of the sludge increased in greater amounts whereas protein content increase was comparatively smaller. SVI value decreased to 330 during this period. From 5 to 15

meq/L carbohydrate content decreased from 53 to 45 mg/g VSS while SVI increased to 460. This result does not support the studies of Urbain *et al*., (1993) and Sanin *et al.*, (2006) that stated the negative correlation between EPS_c and settleability.

4.1.2.2 Dewaterability Results

Dewaterability of the activated sludge samples were determined by measuring Capillary Suction Time (CST) of each set. This setôs data with respect to alterations in magnesium ion concentration is given in Table 4.1 below. In this set, there was a great difference in CST values between different concentrations of magnesium ion. Lowest CST or, highest dewaterability was observed at the lowest concentration of the magnesium. When the concentration of magnesium in the feed was increased about 10 times, dewaterability tremendously got worse. By an increase of the concentration from 5 meq/L to 15 meq/L, CST continued to increase. Higher CST values mean it is much more difficult for sludge to release its water. For full scale operations this means that this type of sludge is harder to dewater.

Table-4.1 CST Values with respect to Magnesium Ion Concentration

CST (seconds)	**Magnesium Ion Concentration(meq/L)**
31.885	0.5
234.477	5
291.96	15

Although CST is preferred for its practical use to determine dewaterability in activated sludge plants, it is known that CST has a unique characteristic for a specific sludge solids concentration (Vesilind, 1988) and therefore it cannot be considered alone as a measure of the dewaterability. CST and viscosity has been found to be strongly depended on the solids concentration of the sludge (Mikkelsen and Keiding, 2002). As a result, normalized CST, which can be obtained by dividing the CST value to MLSS of the sludge, can be used for understanding the dewaterability potential of the sludge. In each set, normalized CST with changing magnesium

concentrations was calculated and the results for the first set can be seen in Figure 4.5 below. Similar increasing trend in normalized CST as in the case of CST was determined with respect to changing concentration of magnesium. As can be observed, normalized CST and CST values were significantly higher at the concentrations of 5 and 15 meq/L of magnesium.

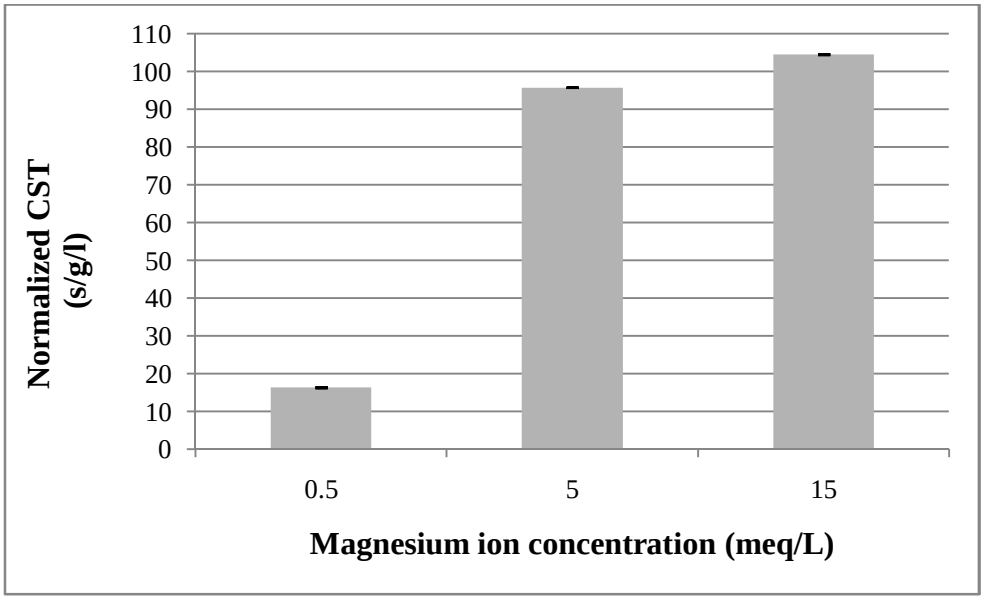


Figure 4.5 Normalized CST Measurements with Respect to Magnesium Ion Concentration

It was known that addition of divalent cations enhance dewaterability by establishing dense and compact floc structures due to the bridging across negatively charged biopolymers (Murthy, 1998). However in this set, addition of magnesium did not result in an improvement in dewaterability. Therefore it is true to say that there is another factor that influences on dewaterability in this set.

Since there is a strong relationship between the EPS_p/EPS_c and dewaterability as well as the total concentration of the EPS, these values with respect to magnesium ion concentration were considered. Lowest EPS concentration of 15.6 mg/g VSS was obtained for the concentration of 0.5 meq/L with EPSp/EPSc of 1.14. The highest dewaterability or the lowest CST value at this concentration was in compliance with

the previous studies (Higgins and Novak, 1997 a, b, c) that higher EPS_p/EPS_c contributed positively to sludge dewaterability. When the concentration of magnesium ion increased to 5 meq/L and then 15meq/L, production of higher concentrations of EPS_c was stimulated in the system which was reported to worsen the dewaterability due to its hydrophilic properties (Murthy and Novak, 1999).

In addition to these findings, some of the research previously focused on the portion of the EPS and its relationship with dewaterability. Loosely bound EPS and dissolved EPS was found out to be one of the most important part in affecting filterability of sludge negatively (Rosenberger and Kraume, 2002). Therefore it may be speculated that the possibility of formation of loosely bound EPS and soluble EPS rather than tightly bound EPS in this set may be the reason for obtaining these higher CST values.

4.1.2.3 Rheology

As one of the physical analyses viscosity measurements were conducted in each set. Shear stress versus shear rate graphs were plotted in order to determine which flow model best fits studied sludge. The sample rheograms from this set can be seen in Figure 4.6. All of the flow models were tried and power law equation indicating pseudo plastic behavior was determined as the best fitting model for this sludge. In pseudo plastic fluid the relationship between shear stress and shear rate were as formulated below:

$$\tau = K\left(\frac{du}{dy}\right)^n \tag{4.1}$$

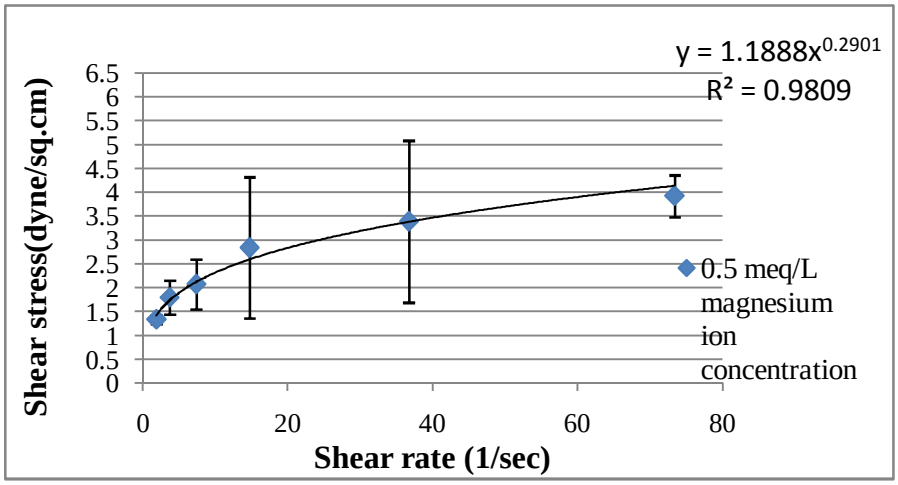
Where n represents flow behavior index which is smaller than 1 for pseudo plastic fluids.

From the fitting equations the correspondent values of n were 0.2901, 0.7841and 0.8275 for control, 5 and 15 meq/L of magnesium ion concentrations respectively. It

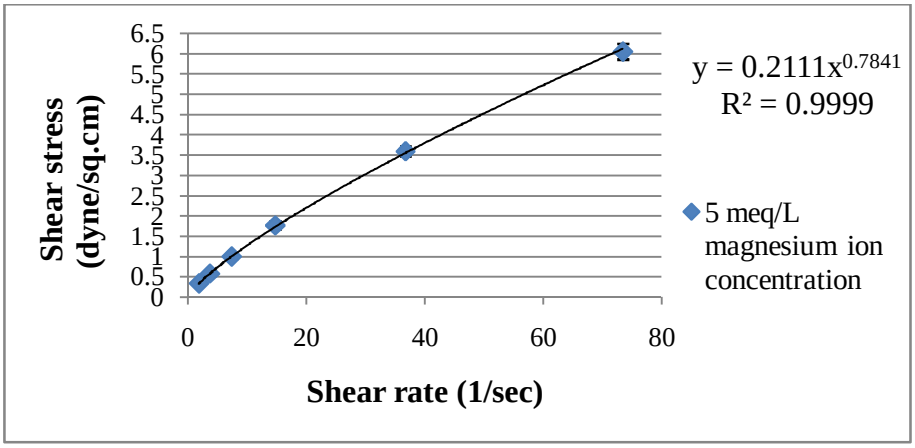
is known that when n value converges to 0, sludge gets closer to non-Newtonian behavior (Durmaz & Sanin, 2003). By the increase from 0.5 to 5 meq/L and then 15meq/L, sludge samples got closer to Newtonian behavior due to the increase of n value.

When the relationship between the apparent viscosity and MLSS concentration at a fixed shear rate of 73.4/sec was studied, the reactors were best fitted to exponential relationship just as in the case of the previous study conducted by Durmaz and Sanin in 2003. The results are depicted in Figure 4.7 below. Reactor with 15 meq/L of magnesium concentration showed smaller increase in viscosity with change in MLSS concentration whereas other two concentrations showed sharper increase in viscosity respect to MLSS.

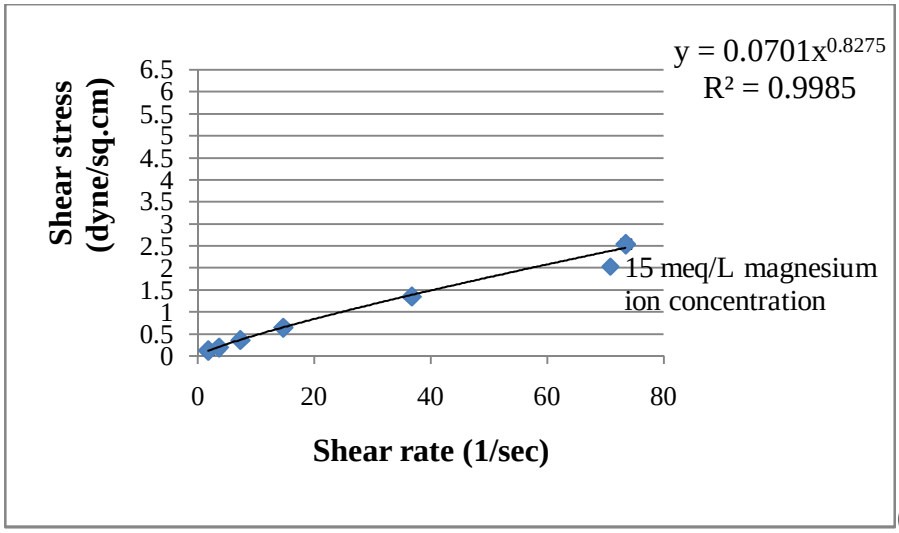
In addition, the apparent viscosity of the sludge at a determined MLSS concentration of 1500mg/L with respect to magnesium ion concentration was plotted as can be seen in Figure 4.8. Apparent viscosity seemed to decrease significantly by the change in concentration from 5 to 15 meq/L whereas the decrease was rather smaller from 0.5 to 5 meq/L. Previous studies stated that dissolved ion concentration or conductivity decreased viscosity by decreasing bound water content of the flocs (Tixier *et al.*, 2003; Sanin, 2002). The decrease of the viscosity for magnesium ion concentrations from 0.5 to 15 meq/L was in agreement with the previous studies.



(a)



(b)



(c)

Figure 4.6 Typical Rheograms for **a.** Control Reactor at 1851 mg/L **b.**5meq/L Reactor at 2260 mg/L **c.**15meq/L Reactor at 2800 mg/L MLSS Concentration

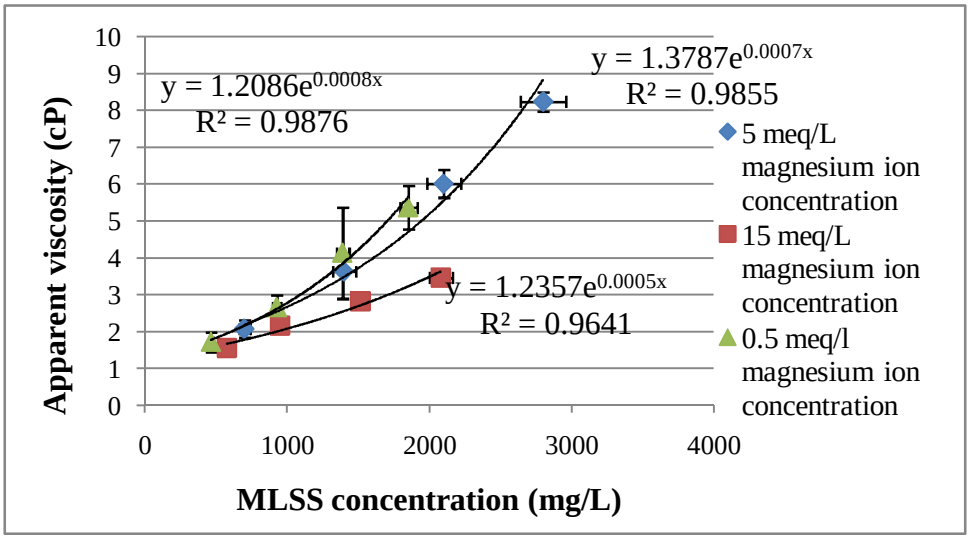


Figure 4.7 Apparent Viscosity Values with Respect to Magnesium Ion Concentration

Earlier studies found significant contribution of EPS to the viscosity of sludge in a way that by the increase of extractable and removed polymer from floc, viscosity of sludge decreased. Moreover higher EPSc was found to increase the viscosity of the sludge significantly (Sanin, 2002). Contrary to this finding, apparent viscosity decreased although carbohydrate portion of the EPS increased. The effect of higher values of dissolved ion concentration at this set seemed to affect the system more than the EPS.

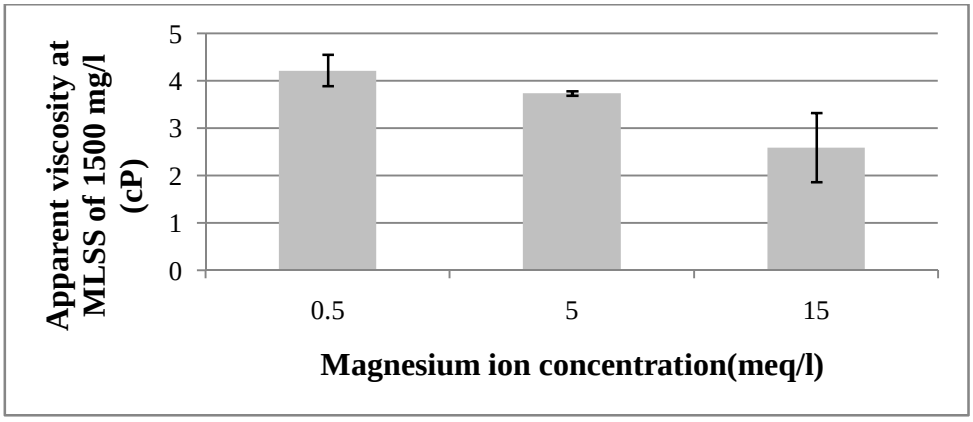


Figure 4.8 Apparent Viscosities versus Magnesium Ion Concentration at a Fixed MLSS Concentration of 1500mg/L

4.1.2.4 Turbidity

Turbidity measurements were conducted by taking supernatant part of the samples after settlement of the reactors in 1L graduated cylinder. Turbidity values ranged between 260-900 NTU that indicated very turbid samples can be seen in Figure 4.9 below. This may result from the presence of dispersed microorganisms and colloids that could not settle and hence got retained in the supernatant part under nutrient deficient conditions.

In one of the studies it has been found out that there was correlation between filterability and turbidity such that by the filterability increase, turbidity decreased (Bruus *et al.,* 1992). This study supports this finding that by the worsening of filterability from 0.5 to 5 meq/L. As opposed to this study at 15meq/L of magnesium ion concentration although CST continued to increase turbidity decreased.

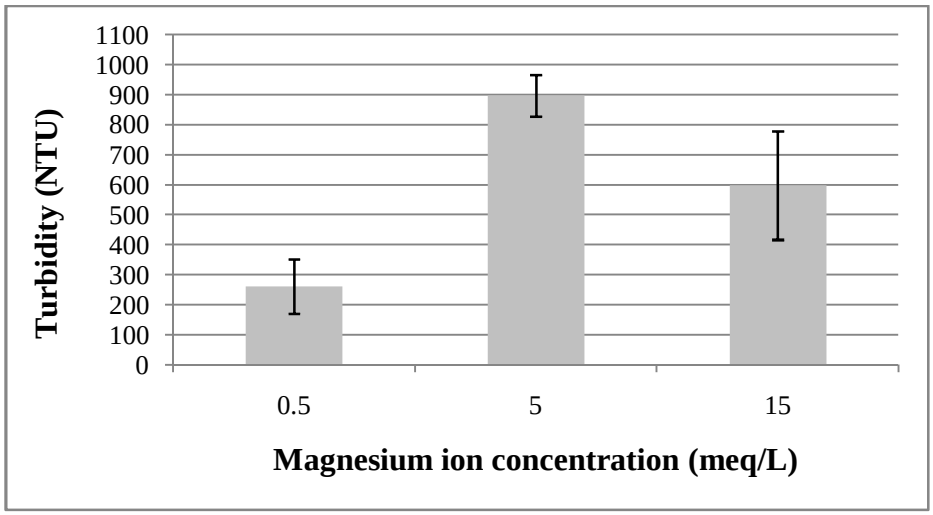


Figure 4.9 Effluent Turbidity Values versus Magnesium Ion Concentration

4.1.3 Results of the Microbiological Analyses

In each set, after conducting physical and chemical analyses, microbiological analyses were carried out. Firstly microscopic analysis was done in order to understand the effect of magnesium ion and phosphorus starvation on floc formation. For each concentration of magnesium ion photomicrographs were taken.

When the photomicrographs were examined for all of the reactors, it was observed that flocculation mechanism was not adequate due to phosphorus deficiency. There were spaces between small floc structures indicating the absence of structures contributing to flocculation. The results for each concentration are depicted in Figure 4.10, 4.11 and 4.12 below.

In the control reactor, the dominance of the zooglea bacteria was observed. Filamentous bacteria could not be observed in high amounts. This may result from the fact that the concentration of calcium ion was in equimolar quantities with magnesium ion in control reactor. Calcium ion which is known to cause viscous/zoogleal bulking in phosphorus deficient conditions (Vatansever, 2005), seemed to reduce the effect of magnesium ion and therefore caused induced formation of higher amounts of zooglea rather than the filamentous bulking.

At the concentration of 5meq/L the dominance of microorganisms changed in a way that there was overabundance of filamentous microorganisms. When floc structures were examined in a closer view filamentous microorganisms tended to extend from the floc structures rather than acting between floc surfaces. As a result filamentous sludge bulking occurred in the reactors. This result is in parallel with the results of the physical and chemical analyses.

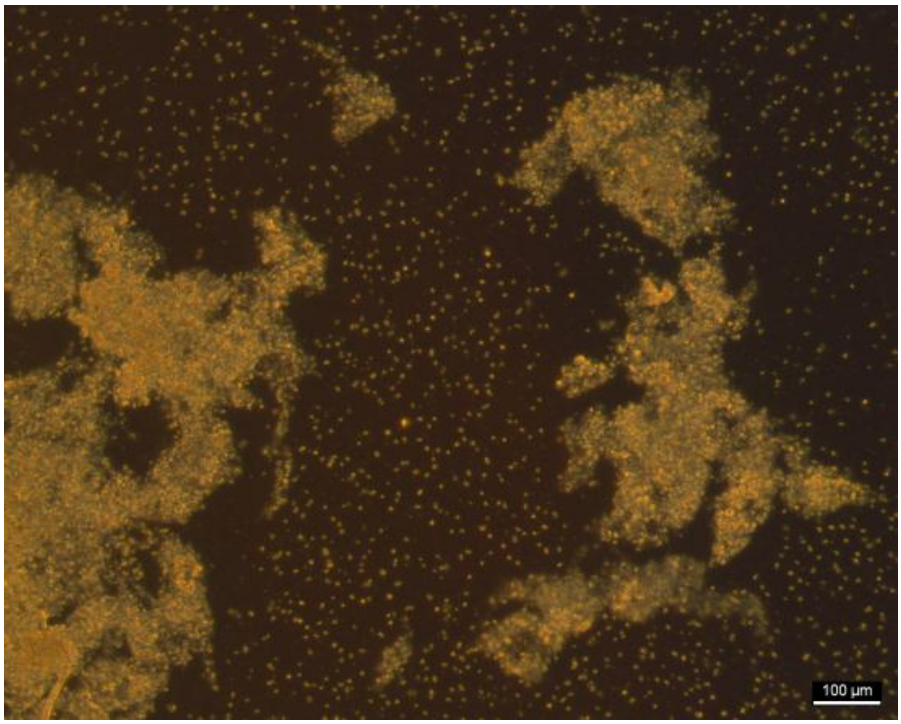


Figure 4.10 Photomicrographs of control reactor containing 0.5meq/L Mg under 4 × magnification

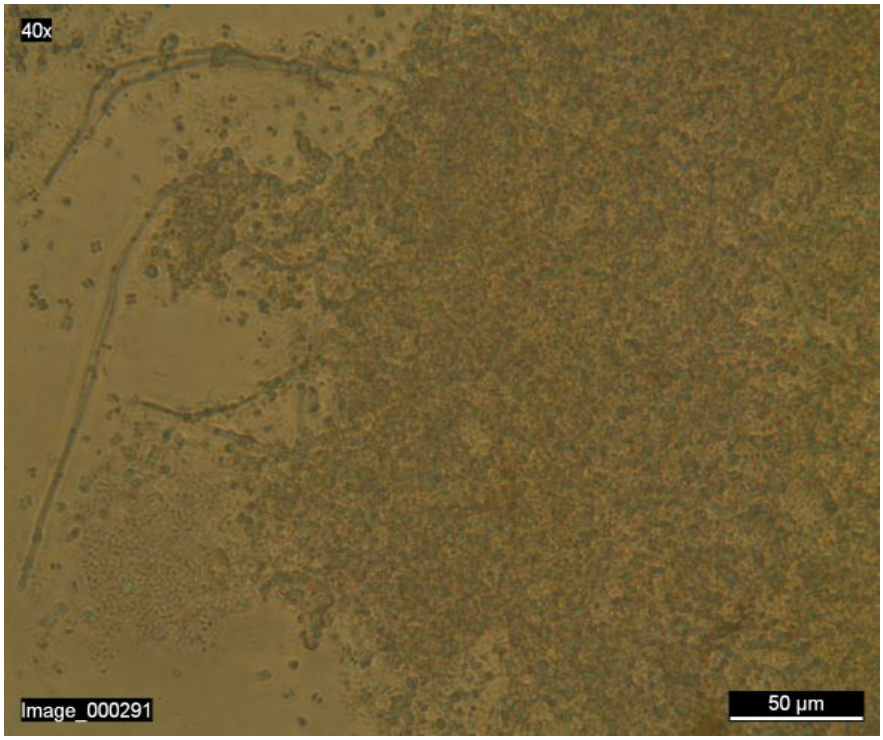


Figure 4.11 Photomicrographs of reactor containing 5meq/L Mg, under 40 Magnification

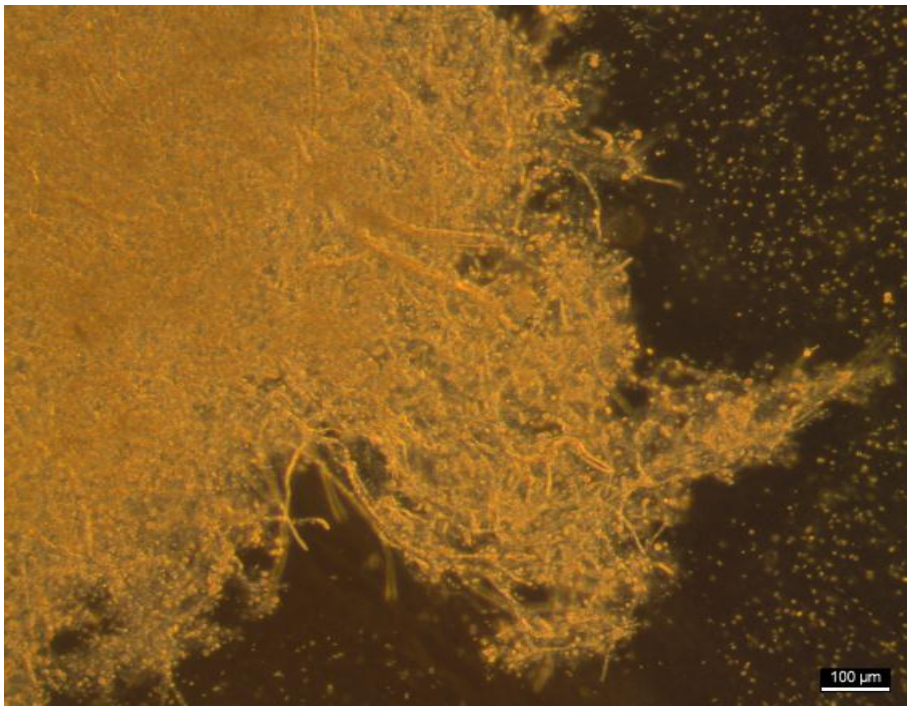


Figure 4.12 Photomicrographs of reactor containing 15meq/L Mg, under 4 □ Magnification

Excessive growth of filamentous microorganisms was observed at the concentration of 15 meq/L as well as 5 meq/L. Filamentous bulking resulting mostly from the filamentous bacteria obtained under phosphorus deficiency at higher concentrations of magnesium ion, was supported by the previous study conducted by Turtin, in 2005, that magnesium ion under phosphorus deficiency caused filamentous sludge bulking at concentrations of 5, 10 and 20 meq/L.

In addition to photomicrographs, the biochemical tests were performed during analyses period. Casitone glycerol yeast agar (CGY) was chosen for cultivation and isolation of heterotrophic bacteria. In addition to this, total hetetorophic bacterial count was calculated after making necessary dilutions and applying spread plate technique. Under phosphorus deficient conditions total hetetorophic bacterial count was found to range between $6*10^6$ and $2*10^8$ CFU/mL as can be seen in the Table 4.2 below.

Table 4.2 Total Heterotrophic Bacteria Count Results

Reactors	Counting (cfu/mL)
Control (0.5 meq/L)	$6*10^6$
5 meq/L	$1*10^7$
15 meq/L	$2*10^8$

It is known from the literature studies that it is not possible for one single medium to support all nutritional types of heterotrophic bacteria present in activated sludge (Prakasam & Dondero, 1967 a b; Lighthart and Oglesby, 1969; Unz and Davis 1975). However highest median counts were obtained for the enumeration of the bacteria of activated sludge when CGY agar was used as growth media. (Pike *et al.,* 1972). In addition CGY agar was considered as the most generally useful medium for cultivation and isolation purposes of the activated sludge bacteria (Banks & Walker, 1977). It is used in several activated sludge bacteria isolation procedures including filamentous bacteria and polyphosphate accumulating bacteria isolation (Richard, *et al.,* 1985; Mubyana & Letsamao, 2002).

Before culturing and isolating the activated sludge bacteria, it was essential to choose which biochemical test was appropriate for determination of activated sludge bacteria. In the end it was decided to use biochemical profiling by API kits that became extremely popular in recent years for the implementation in activated sludge samples (Bezuidenhout *et al.,* 2002).

It is known from the previous study that API 20 NE, API 20 E and API Coryne were used for the identification of bacteria in activated sludge (Eusébio *et al.*, 2004). In another study API 20 E and API 20 NE were used for identification of the polyphosphate accumulating bacteria (Bux & Kasan, 1999). In this study, API 20 NE, API 20 E and API Coryne test strips were chosen for the identification of microorganisms in activated sludge samples.

When activated sludge bacteria of municipal source was studied, most frequently found genera was belonging to the family of *Enterobacteriaceae* which are known to be gram-negative which do not form spores (Younos, 1987). Applicability of the API 20E tests were studied for the lab scale activated sludge in one of the studies and it has been found out that API 20E is applicable for determination of dominating microorganisms in the system in particular gram-negative bacteria (Juang & Morgan, 2001). As a result API 20E was chosen in this study in order to determine this major family found in activated sludge samples.

API Coryne was selected to be used since it was known that API Coryne determines microorganisms belonging to the genus of *Corynebacterium* (API web site). Genera such as *Corynebacterium, Arthrobacter* are identified as the related genera of the *Nocardiaforms* or *actinomycetes* which are known as the filamentous microorganisms in the activated sludge system (Gerardi, 2006).

Before applying these tests, some preliminary tests such as gram staining, oxidase and catalase tests were performed for each isolated bacteria. In order to use API 20E effectively, bacteria that will be identified should be gram negative whereas bacteria should be gram negative and oxidase positive for the identification in API 20NE. Apart from these two strips, bacteria that will be characterized by API Coryne should be gram positive and catalase test is compulsory for the use of the kit. During these preliminary tests, it was realized that oxidase test was always negative for all of the isolated bacteria. As a result of that API 20NE test could not be applied in two sets.

When all of the procedures were followed and API 20E strips were inoculated for 48h, following results were obtained. The identified species and genus that belongs to gram-negative Enterobacteriaceae family can be seen in the Table 4.3 below. Detailed results can be observed in Appendix-C.

Table 4.3 Results of API 20E Biochemical Kits

Organism identification	API 20E profile no	Accuracy
Pantoea spp 2 Pantoea spp 4	1 2 4 4 5 7 3	70.4% 25.7%
Citrobacter youngae	3 6 4 4 5 1 2	99.8%
Enterobacter sakazakii	3 3 4 4 7 7 3	93.4%
Serratia liquefaciens Serratia marcescens	5 3 0 7 7 6 3	69.0% 30.8 %
Serratia liquefaciens	7 3 0 7 7 6 3	96.0%
Citrobacter braakii	3 6 4 4 5 5 3	92.9%

As can be seen in results *C. Youngae, C. Braakii, S. Liquefaciens and E. Sakazaakii* were identified with highest accuracy using inoculation in API 20E sets. In addition *Pantoea, Citrobacter, Serratia* genus could be identified from the tests.

Although it was decided to use API Coryne, the preliminary tests of the isolated samples did not result in gram positivity and therefore API Coryne kits could not be applied for the determination of Corynebacterium in the first set with phosphorus deficiency.

4.2 Results of the Reactors at Phosphorus Sufficient Conditions

In the second set of the experiments, sufficient phosphorus concentration was provided to the reactors by keeping the COD/P ratio near to 120 (1429mg/L/ 11.44mg/L) whereas the COD/TKN ratio was kept at a nearly optimum value of 18.3 (1429 mg/L/78.08mg/L). Changing magnesium ion concentrations and analyses in order to understand the sludge characteristics remained as the same as in the case of the phosphorus deficient conditions. All replica reactors gave results similar to each other which were proved by smaller standard deviations. Therefore in the calculation of the physical and chemical parameters average of the replica reactors were taken.

4.2.1 Results of the Chemical Analyses

4.2.1.1 Chemical Oxygen Demand (COD) Measurements

Effluent COD measurements were considered as well as the first set. In this set, all of the effluent COD values as can be observed in Figure 4.13, were well below the influent COD which was 1429 mg/L. Effluent COD values were very close to each other for each magnesium ion concentrations. The removal efficiency of the system was 96.1, 95.8 and 95.5% for 0.5, 5 and 15 meq/L of magnesium ion concentration respectively. These values indicate high efficiency of the system.

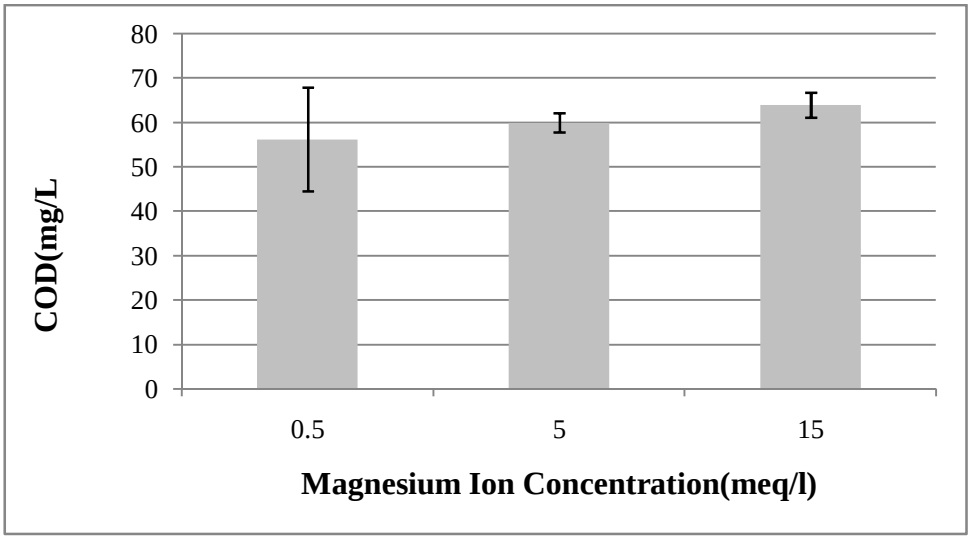


Figure 4.13 Effluent COD Values versus Magnesium Ion Concentration

4.2.1.2 Results of the Extracellular Polymeric Substance Extraction, Carbohydrate and Protein Analysis

CER extraction method for the extraction of EPS and Dubois and Lowry methods for the analysis of carbohydrate and protein content of EPS were applied in the second set as well as the first set. Carbohydrate and protein standard curves for this set is in Appendix-B

The results can be observed in Figure 4.14 below. Protein content of the EPS was higher than the carbohydrate content for all of varying magnesium ion concentrations as opposed to the first set. By increasing concentration of the cation, protein content tended to increase. Concentration increase of the proteinaceous part of the EPS was much sharper with a magnesium ion concentration increase from 5 meq/L to 15 meq/L.

Carbohydrate component of EPS did not show a continuous increasing or decreasing trend during the analysis. Carbohydrate content tended to decrease by increasing concentration from 0.5 meq/L to 5 meq/L. On the other hand its concentration increased by the increase of magnesium from 5 to 15 meq/L. Similar to the trend in carbohydrate content of the EPS, total EPS concentration did not have an increasing trend in all of the concentrations. It decreased by the change of magnesium ion concentration from 0.5 meq/L to 5 meq/L whereas its concentration increased significantly from 5 to 15 meq/L.

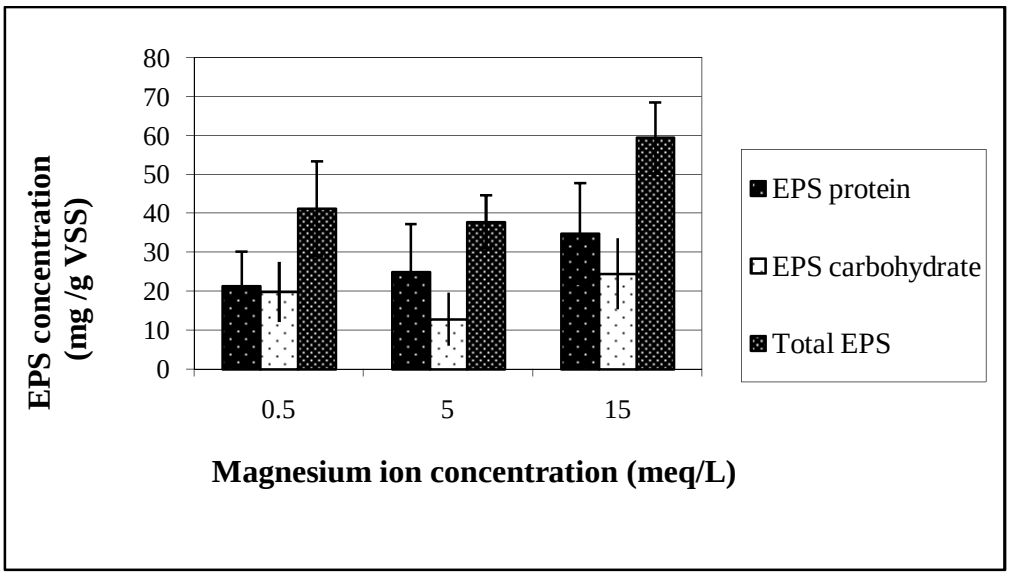


Figure 4.14 EPS_P, EPS_C and total EPS Concentration Versus Magnesium Ion Concentration

At nearly same C/N/P ratio, synthetic feed components for the control reactor, environmental and operational conditions such as the sludge age, higher concentration of the protein component was obtained with respect to carbohydrate concentration during analysis after applying CER extraction method for the extraction of EPS (Durmaz & Sanin, 2001; Sanin *et al.,*2006). The result for the lowest magnesium ion concentration is in agreement with these previous studies. Moreover in literature some of the studies conducted by Urbain *et al.,* 1993, and Dignac *et al.,* 1998, resulted that protein content of EPS was in positive association with calcium and magnesium ion concentrations. EPS results in this set are in agreement with these studies.

In addition to these, the study conducted by I.Turtin in 2005, is in compliance with this finding that more specifically, increasing magnesium ion concentration led to the increase in the content of extracellular protein concentration rather than carbohydrate concentration. The result of the study is particularly most comparable with this study since sufficient phosphorus concentration was provided to the reactors as well as this study with same sludge age and environmental conditions.

There has been found positive correlation between the protein concentration and cation concentration in a way that divalent cations have more tendency to bind protein than carbohydrates (Higgins and Novak, 1997 a). As a result, good level of dewaterability and settleability in the study was attributed to bound protein content. It may be speculated that increasing protein concentration by an increase in magnesium concentration in this set may be the indication of bound protein of EPS in floc matrix.

4.2.1.3 Conductivity

The conductivity of the solution in the reactors with respect to increasing magnesium ion concentration can be seen in Figure 4.14. As it can be expected, by an increase of the magnesium ion provided to the feed, electrical conductivity of the solution increased. However the ratio of the increase was not directly proportional with the

increase ratio of magnesium ion. This may be indication of the use of magnesium ion in the floc structure and cell activities.

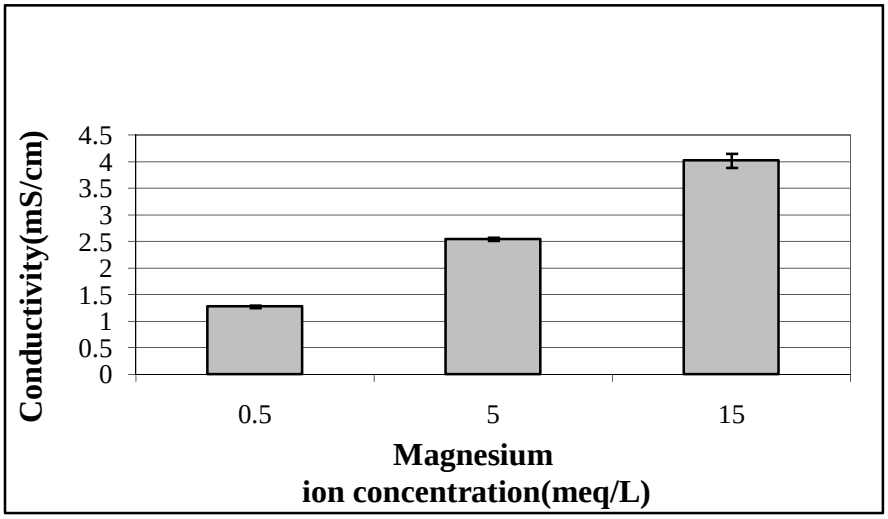


Figure 4.15 Conductivity of the Sludge with Respect to Magnesium Ion Concentration Provided In the Feed.

4.2.2 Results of the Physical Analyses

4.2.2.1 Settleability

In order to examine the settleability of the sludge, sludge volume index was measured. SVI as in the case of first set. The values smaller than 120 mL/g was reported to be well settled sludge whereas the ones higher than 150 mL/g indicated sludge bulking (Jenkins *et al*, 1993).

The change in SVI with respect to the magnesium ion concentration can be observed in Figure 4.16. Each value, ranging from 63 to 71 mL/g indicated very good settling sludge. SVI value decreased from 71 to 63 by increase of magnesium from 0.5 to 5 meq/L. However further addition of magnesium from 5 to 15 meq/L resulted in an increase in SVI. However these fluctuations in SVI are insignificant and can be considered nearly same. It is clear that at sufficient phosphorus concentration all of

the reactors settled well regardless of changing cation concentration. In addition, results are in parallel with the photomicrograph views that will be presented in proceding parts. In photomicrographs it is evident that there is the formation of larger and healthy floc structures which is in close relationship with well settling of sludge.

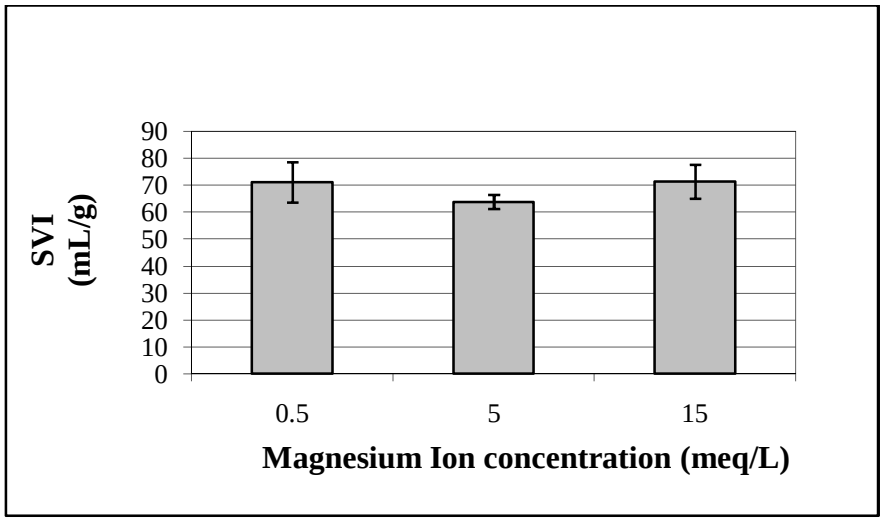


Figure 4.16. SVI Values at Different Magnesium Concentrations

4.2.2.2 Dewaterability Results

CST values were obtained by using Capillary Suction Timer and the results can be seen in the Table 4.4 below. The CST value, decreased about approximately 1.5 seconds by the increase of the magnesium ion concentration from 0.5 to 5 meq/L. On the other hand, at the increase of the ion concentration from 5 to 15 meq/L, increase with 1.2 seconds was observed in CST values. These small differences in CST measurements can be considered as same. In addition all CST values are very small indicating good filterability of sludge

Table 4.4 CST Values with Respect to Magnesium Ion Concentration

CST(seconds)	Magnesium Ion concentration (meq/L)
10.5825	0.5
9.0975	5
10.2275	15

Due to the dependency of the CST on solids concentration, normalized CST values in unit of s/(g/l), were also calculated. The results can be seen in Figure 4.17 below. The normalized CST values ranged between 3.89 to 5.17 s/(g/l) indicating good dewaterability for all magnesium ion concentrations. There was not a significant change in dewaterability resulting from addition of magnesium. On the other hand presence of sufficient phosphorus concentration affected system positively.

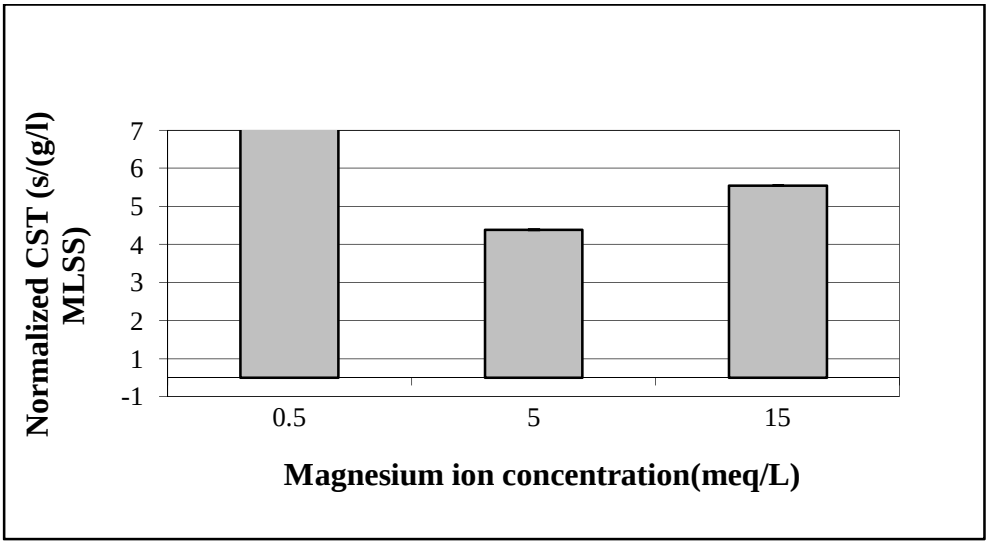


Figure 4.17 Normalized CST Measurements with Respect to Magnesium Ion Concentration

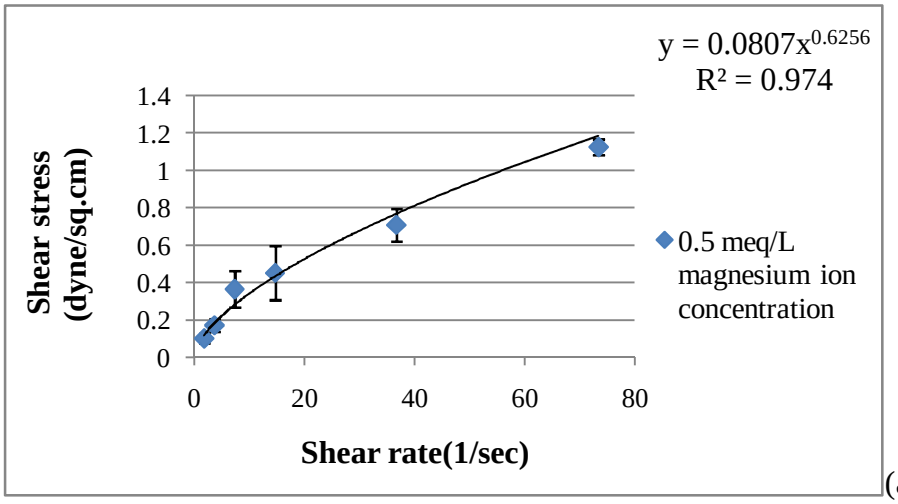
4.2.2.3 Rheology

As in the case of the first set, shear stress vs. shear rate graphs were plotted as can be seen in Figure 4.18. Pseudo plastic behavior was observed with best fitted power law

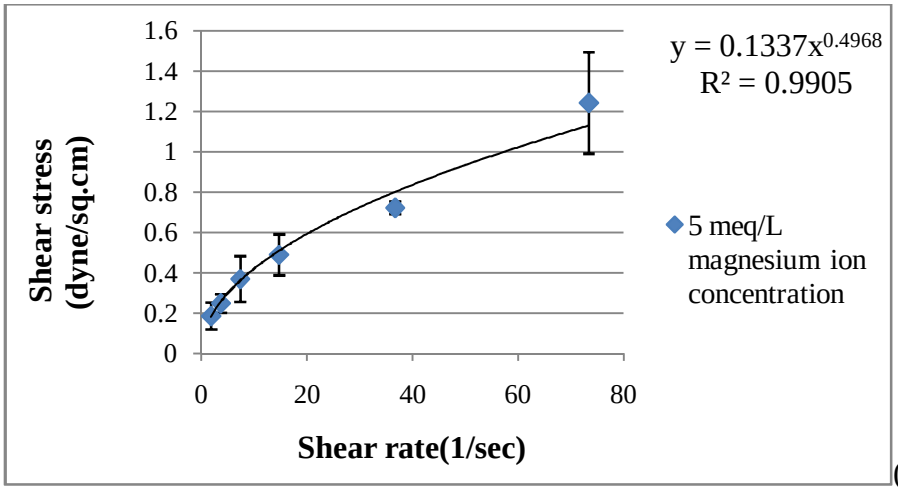
equation. n values were 0.6256, 0.4968 and 0.6087 for the reactors containing 0.5,5 and 15 meq/L of magnesium ion concentration respectively. The n value deviated from the Newtonian behavior more at the concentration of 5meq/L. At the concentration of 0.5meq/L, fluid got closer to 1 and thus Newtonian behavior.

At a constant shear rate of 73.4sec^{-1} apparent viscosities of the sludge with respect to changing MLSS concentration were obtained. As can be seen in Figure 4.19, apparent viscosity increased with the increase of MLSS concentration at all of the magnesium concentrations. The reactors showed similar characteristics and therefore got approximately similar fitting exponential equation coefficients.

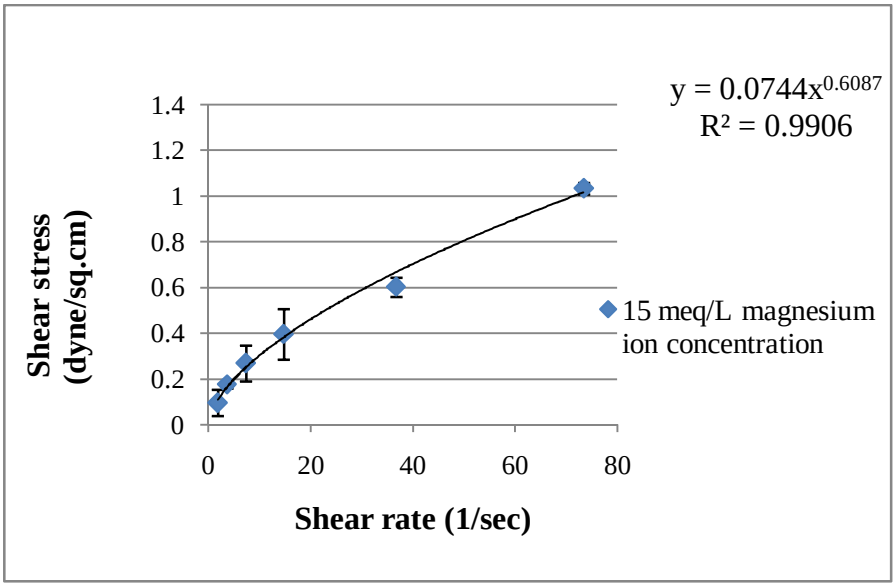
At a fixed concentration of 1500mg/L of MLSS for all of the reactors, the relationship between the apparent viscosity and concentration of the magnesium ion were studied. In Figure 4.20 apparent viscosity increased slightly by the increase of magnesium ion concentration from 0.5 to 5meq/L whereas decrease of the viscosity was reported with an increase of the concentration from 5 to 15meq/L. However all these changes are in decimal units and therefore effect of magnesium ion on viscosity is insignificant in this set. Sufficient phosphorus concentration provided lower viscosity values indicating pumpable sludge in operational conditions.



(a)



(b)



(c)

Figure 4.18 Typical Rheograms for **a.** Control reactor at 2331mg/L **b.**5meq/L reactor at 2296mg/L **c.**15meq/L reactor at 1946mg/L MLSS Concentration

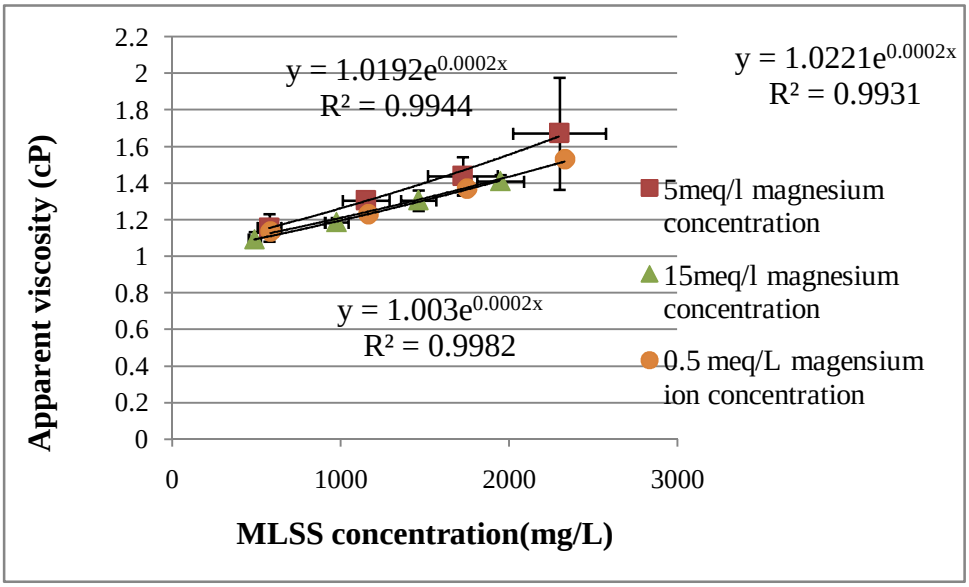


Figure 4.19 Apparent Viscosity Values with Respect to Magnesium Ion Concentration

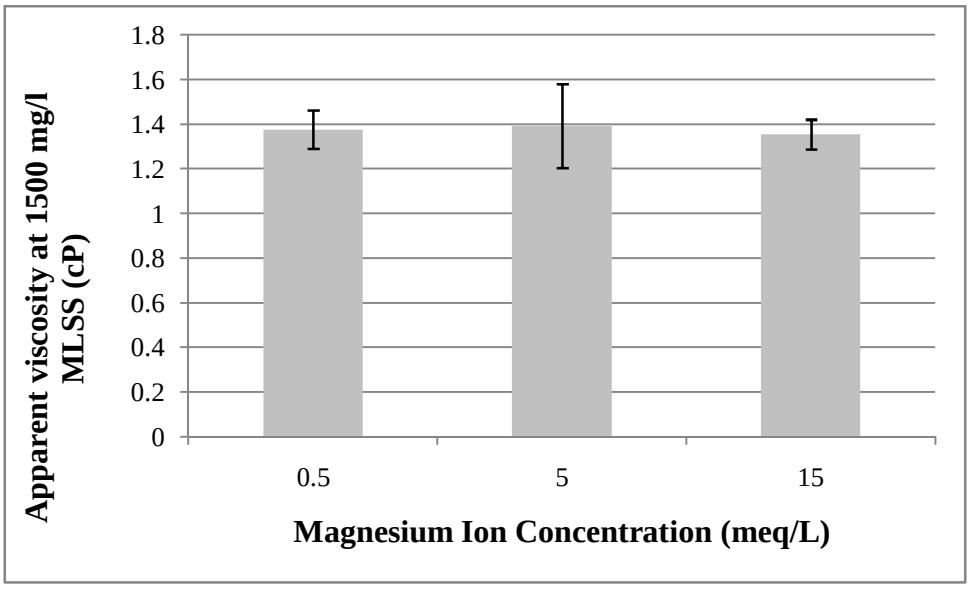


Figure 4.20 Apparent Viscosity vs Magnesium Ion Concentration in the Fixed MLSS Concentration of 1500 mg/L

4.2.2.4 Turbidity Measurements

Turbidity measurements were conducted by taking supernatant part of the samples after settlement of the reactors in 1L graduated cylinder. Turbidity values were very low, ranged between 32.4-40.8 NTU as can be seen in Figure 4.21. This is the indication of well settled sludge and not suspended particles in the system. There is a direct relationship between the enhancement of bioflocculation and turbidity decrease in the system. After the start of the endogenous growth and EPS production, bioflocculation is enhanced, smaller particles and microbial particles in suspension decrase and therefore turbidity decrease (Pavoni *et al,* 1972; Tenney *et al,* 1969).

In addition, the turbidity values did not alter significantly with respect to changing magnesium ion concentration in this set. In the study conducted by Bruus *et al.*, (1992), negative correlation between filterability and turbidity was observed. Results do not support the previous studyôs finding.

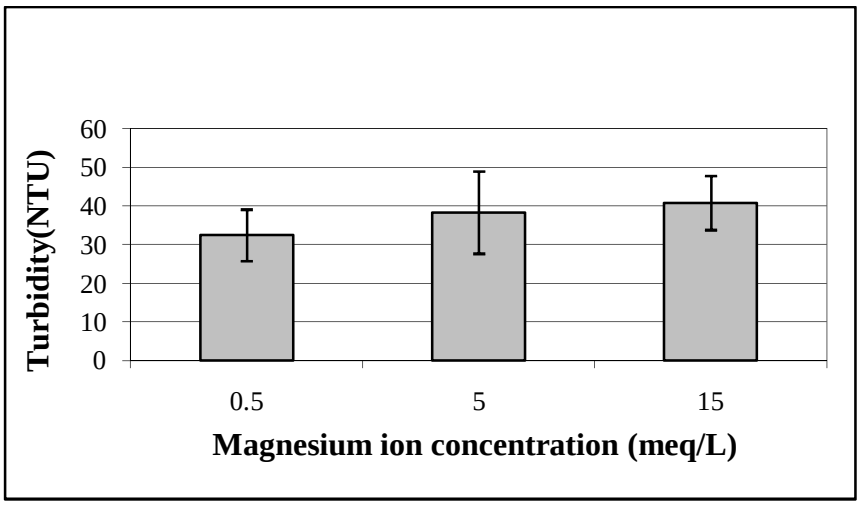


Figure 4.21 Effluent Turbidity Values versus Magnesium Ion Concentration

4.2.3 Results of the Microbiological Analyses

In the first stage of these analyses, samples from different concentrations of Magnesium were analyzed under microscope and their photomicrographs were taken. The results are illustrated in Figures 4.22, 4.23 and 4.24 below.

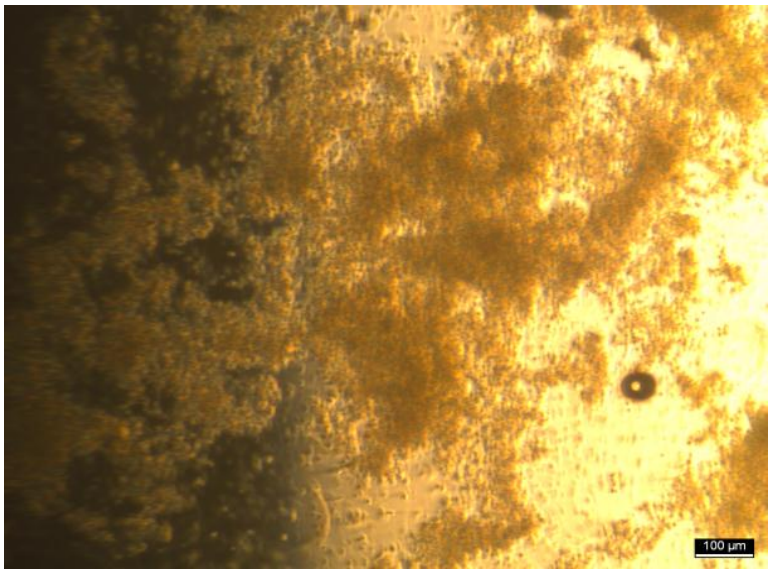


Figure 4.22 Photomicrographs of control reactor containing 0.5meq/l Magnesium under *4* × magnification

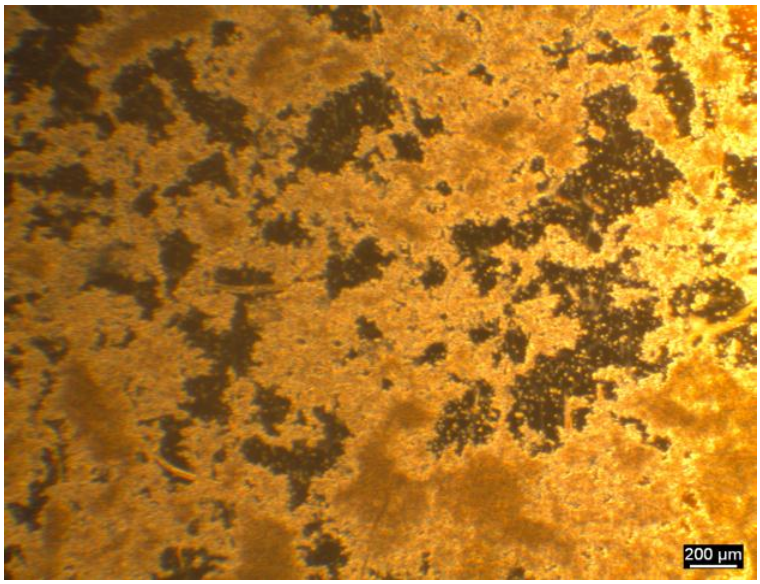


Figure 4.23 Photomicrographs of Reactor Containing 5meq/l Magnesium under *4* × Magnification

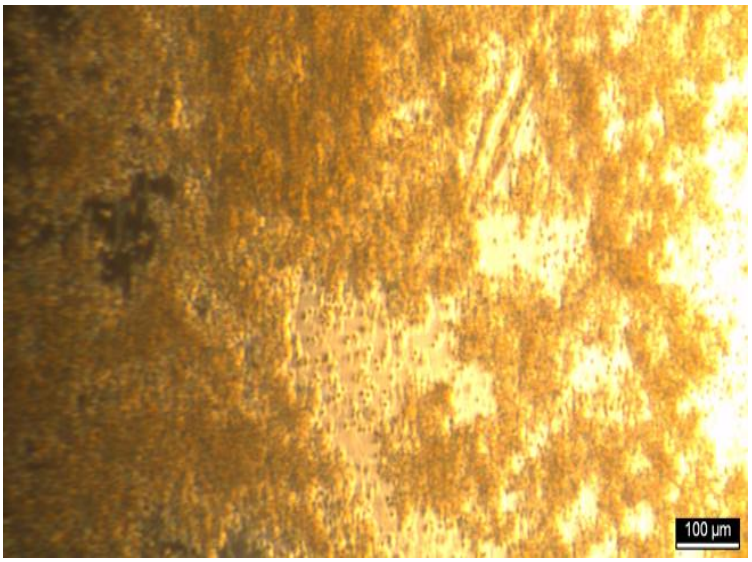


Figure 4.24 1) Photomicrographs of reactor containing 15meq/l Magnesium under 2) under *4* × Magnification

Abundance of the phosphorus at sufficient concentration led to an increase in the efficiency of the bioflocculation mechanism in all of the varying magnesium ion concentrations. As can be seen in Figures 4.22, 4.23 and 4.24, larger flocs were formed due to the balanced distribution of filamentous microorganisms and floc forming bacteria. According to microscopic analysis, it is observed that filaments involved in the interactions of flocs rather than stretching out of the floc. Therefore the colonial microorganisms and flocs tended to gather together and form larger flocs. There was not a significant distinction between the varying concentrations of magnesium and appearance of the floc at these concentrations. The results of these analyses were in parallel with physical and chemical results that all of the parameters indicated well settled, dewaterable and bioflocculated sludge at all of the studied concentrations.

In this set same procedure was applied for the cultivation and isolation of the bacteria as in the case of phosphorus deficient conditions. Under sufficient phosphorus conditions, total hetetorophic bacterial count was found to range between $6*10^6$ and $8*10^7$ CFU/mL as can be seen in Table 4.5 below.

Table 4.5 Total Heterotrophic Bacteria Count Results

	Counting (CFU/mL)
Control	2*108
5meq/l	8 *107
15meq/l	6*106

API 20E and API Coryne strips were used for the isolated species obtained from the reactors with sufficient phosphorus concentration. Results of the API 20E can be seen in Table 4.6 below. The positivity/ negaitivity of the reactions and detailed results can be observed in Appendix-C.

As can be observed *E.sakazakii* could be determined with the highest accuracy. There was the presence of *Pseudomonas* family with possibility of *P. fluorescens/putida, P. Aeruginosa* and *P. Luteola* species. However exact specie could not be determined by the use of API 20E alone. Availability of *Pseudomonas* confirms the photomicrographs taken for the second set. *Pseudomonas* were identified as the main floc formers responsible for the bioflocculation in activated sludge system (Gerardi, 2006).

In particular *Pseudomonas fluorescens* were found to be responsible for the polyphosphate accumulation from the system with higher uptake rates (Bux and Hassan, 1999; Gunther, 2009). Under sufficient phosphorus concentration and with the presence of magnesium, the polyphosphate granules produced by polyphosphate accumulating bacteria was reported to be formed (Schönborn, 2001).

Table 4.6 Results of API 20E

Identified genus	API 20E profile no	Accuracy
Enterobacter sakazakii	3 3 4 5 5 7 3	91.1%
Pseudomonas fluorescens/putida	2 2 0 0 0 0 0	44.5%
Pseudomonas aeruginosa		27.9%
Pseudomonas luteola		20.0%

In this set, there is the presence of Gram Positive bacteria As result, API Coryne biochemical kits could be implemented. The results of API Coryne reactions are tabulated in the Table 4.7. Negative and positive reactions can be observed in Appendix-C. Accuracy of the identified species were higher. *Corynebacterium* and *Arthrobacter* were known to be the related genera of *Nocardioforms* which are known to be filamentous microorganisms (Gerardi, 2006).

Table 4.7 Results of API Coryne

Identified genus	API Coryne profile no	Accuracy
Brevibacterium spp	3 1 0 0 6 0 4	94.9%
Arthrobacter spp	7 7 6 2 0 0 4	99.9%
Corynebacterium propinquum	5 1 0 0 0 0 4	91.2%

4.3 Comparison of the Phosphorus Deficient and Phosphorus Sufficient Conditions

4.3.1 COD Results

COD results for each set can be seen in Table 4.8. Under sufficient phosphorus concentration effluent COD concentration indicated high efficiency of the system. However COD concentration did not change significantly with respect to changing magnesium concentration.

Under phosphorus deficiency all of the reactors faced severe non settling conditions and as a result of that daily procedure for siphoning and adding new feed to medium could not be operated effectively. Organic content was added continuously to the system without having a chance to consume it. Hence effluent COD values were much higher than influent COD indicating badly operating system.

Table 4.8 Effluent COD concentration in phosphorus deficient and phosphorus present conditions

Magnesium Ion Concentration (meq/L)	Effluent COD Concentration (mg/L)	
	P ï Deficient Condition	P ï Present Condition
0.5	3365.33	56.25
5	2055.3	60
15	1844	64

4.3.2 Results of the Extracellular Polymeric Substance Extraction, Carbohydrate and Protein Analysis

From the results given in Table 4.9 it is obvious that there are significant differences in two sets. In the first set, most important result was the higher concentration of the EPS_c with respect to EPS_p at higher the magnesium concentrations. Lower concentrations of the protein in the first set were the result of phosphorus deficiency due to requirement of energy in its synthesis. The carbohydrate synthesis was not affected negatively by phosphorus deficiency. This result is in agreement with previous studies. In addition to this, higher magnesium ion concentration seemed to stimulate synthesis of carbohydrate type polymers under phosphorus deficiency.

Table 4.9 Composition and concentration of EPS under phosphorus deficient and phosphorus present conditions

	Magnesium Ion Concentration (meq/L)	Protein Concentration (mg/g VSS)	Carbohydrate Concentration (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	13.9	53.2	67.09	0.26
	15	19.48	45.98	65.45	0.42
P Present Condition	0.5	21.35	19.94	41.23	1.07
	5	24.97	12.9	37.87	1.94
	15	34.92	24.58	59.51	1.42

Under sufficient phosphorus concentration stimulation of the synthesis of protein with increasing magnesium ion concentration was one of the most important finding. This result is in agreement with the previous study conducted by Turtin (2005).

In addition to these findings, it is obvious that total EPS concentration was higher under deficient phosphorus concentration. This resulted from over production of carbohydrate in deficient case. Due to higher concentrations of the total EPS and carbohydrate with other factors, flocculation could not be achieved effectively (Harris and Mitchell, 1975). Result of increased EPS concentration under phosphorus deficiency resembles to the results obtained under nitrogen deficiency in literature (Durmaz and Sanin, 2003). It may be speculated that higher concentration of carbon with respect to other nutrients such as nitrogen and phosphorus provided in the feed, may result in over production of total EPS: However it seems that what is more important is not the total quantity but the ratio of EPSc/EPSp. Under phosphorus sufficient condtions this ratio improved significantly to a value of around and over 1.

4.3.3. Conductivity Results

Results are compared in Table 4.10. The expected increasing regime of the dissolved ion concentration with increasing concentration of magnesium was observed in both sets. Although conductivities tended to increase in two sets, conductivity of the

wastewater in each reactor with changing concentration of magnesium was much higher in the first set than in the second set. This is probably due to incorporation of the magnesium ion in flocculation process in the second set. Magnesium ion may be found in the floc matrix rather than being in the solution. Also inability of sludge tē settle in the first set made the siphoning process ineffective and each day a significant quantity of unremoved supernatant remained. This caused a built up of dissolved ions in the fisrt set which reflected to the measured conductivity values.

Table 4.10 Conductivity values under sufficient and deficient phosphorus concentration

Magnesium Ion Concentration (meq/L)	Conductivity (ms/cm)	
	P ï Deficient Condition	P ï Present Condition
0.5	4.57	1.275
5	6.68	2.54
15	9.56	4.02

4.3.4 Settleability

SVI values of phosphorus deficient and phosphorus present reactors can be seen in Table 4.11. Results indicate that under sufficient phosphorus concentration sludge settles well regardless of cation concentration. This is the indication of healthy floc structure formation. These results are in parallel with photomicrograph views that dense floc structures showed healthy flocculation of the system. On the other hand, first set was faced with severe bulking conditions due to the deficiency of phosphorus. Because of filamentous bulking, sludge samples got higher SVI values in all magnesium concentrations.

Table 4.11 SVI values with respect to magnesium concentration under phosphorus deficient and phosphorus present conditions

Magnesium Ion Concentration (meq/L)	SVI (mL/g)	
	P ï Deficient Condition	P ï Present Condition
0.5	468	71.1
5	331.8	63.8
15	458	71.3

4.3.5 Dewaterability Results

When two sets were compared with respect to normalized CST values, as in Table 4.12 it is obvious that by the abundance of phosphorus, system dewaterability characteristics improved and acceptable ranges were obtained for dewaterability in the second set. Filterability increase is the indication of healthy floc formation and floc structure. However the results for the second set indicated there is not an obvious improvement in dewaterability by the increase of cation concentration. Sufficient phosphorus concentration was enough for proper dewatering of the system. This result does not supported by the literature studies that reported improvements in dewaterability by addition of divalent cations (Higgins & Sobeck, 2002).

Table 4.12 Normalized CST values with respect to magnesium concentration under phosphorus deficient and phosphorus present conditions

Magnesium Ion Concentration (meq/L)	CST (s/ (g/L))	
	P ï Deficient Condition	P ï Present Condition
0.5	16.39	5.17
5	95.8	3.9
15	104.59	5.06

In the first set all the CST values indicated worse dewaterability conditions mainly affected by nutrient deficiency. Any improvement in sludge characteristics with respect to magnesium ion concentration could not be observed. On the contrary at higher concentrations of magnesium ion, the dewaterability got worse possibly due to higher concentrations total EPS and hydrophilic EPSc.

4.3.6 Viscosity Results

In both of the sets non- Newtonian pseudo plastic behavior of sludge was observed. This result is in accordance with previous studies indicating that most wastewater sludge represents pseudo plastic behavior (Lotito and Spinosa, 1997; Moeller and Torres, 1997; Proff and Louhmann, 1997; Krauth and Staab,1992).

At a fixed MLSS concentration of 1500 mg/L, the results of rheological properties of two sets are compared as in Table 4.13. Viscosity of sludge samples significantly decreased by the presence of sufficient phosphorus concentration in the system. However any contribution of magnesium could not be observed. The values were identical with changing magnesium concentration. In the first set apparent viscosities were higher possibly due to improper flocculation of the system.

Table 4.13 Apparent viscosities at different magnesium concentrations under phosphorus deficient and phosphorus present conditions

Magnesium Ion Concentration (meq/L)	Apparent Viscosity (cP)	
	P ï Deficient Condition	P ï Present Condition
0.5	4.22	1.36
5	3.74	1.39
15	2.6	1.35

4.3.7 Microbiological Analyses

There were significant differences in photomicrographs of two sets as well as the differences in physical and chemical properties of the sludge. Under phosphorus deficiency magnesium ion concentration at 5 meq/l and 15 meq/l tended to cause overproduction of filamentous microorganisms and filaments that stretch out of the weak and small floc structures. As a result problems in compaction and settling took place. Microscope analyses were in compliance with physical parameter, SVI, which is known as the best practical indicator of sludge bulking.

When sufficient phosphorus concentration was provided to the system, healthy bioflocculation occurred. At photomicrographs, filaments were not stretching out of the flocs to prevent gathering of floc components. They were observed inside floc structures. As opposed to the first set, flocs were larger in size. In this set SVI values were all below 120 indicating good settleability.

When two sets were compared with each other, there were exact differences for the identified species and genus. First of all in the first set, selected API Coryne test strip could not be applied since none of the identified species was gram positive. Secondly, when API 20E test results which are used for the identification of most predominant bacteria family in the activated sludge, *Enterobacteriaceae*, were

compared with each other; number of identified species and genus decreased in phosphorus sufficient conditions. In addition species and genus changed significantly due to different nutrient conditions. *Citrobacter, Serratia, Pantoea and Enterobacter sakazakii* were identified in first set, whereas *Pseudomonas and E. sakazakii* could be identified in the second set. *E. Sakazakii* was the only common specie in two sets.

An important result of the second set, was the presence of *Pseudoamona*s family, which are known as the floc formers in activated sludge systems (Gerardi, 2006). Therefore it was found in the phosphorus sufficient system where we also observed healthy flocculation occurred. In the second set *Nocardioform or actinomycetes* family related genera of *Corynebacterium* was identified in the samples. *C.propinquum, Arthrobacter spp and* Brevibacterium spp were determined.

CHAPTER 5

CONCLUSION

This study examined effect of magnesium ion concentration on sludge physical, chemical and microbiological characteristics under phosphorus deficienct and sufficient conditions. As a result following conclusions were obtained:

Under deficient phosphorus concentration,

- All of the measured parameters indicated non flocculating and badly operating system for all magnesium concentrations.

- Carbohydrate concentration of EPS was much higher than the protein concentration at increasing magnesium ion concentration. Due to energy requirement for the protein synthesis, cells could not produce protein in higher amounts. In addition, increase of magnesium concentration resulted in stimulation of production and binding of carbohydrates in EPS network.

- Dewaterability of the system got worse by increasing concentration from 0.5 to 5 and 15 meq/L. This possibly resulted from the tremendous increase of carbohydrate concentration in EPS due to the binding with magnesium.

- Viscosity of the system decreased by increase of magnesium ion concentration. This may result from the increase of dissolved ion concentration provided in the feed.

- Settleability of the system clearly indicated sludge bulking conditions. Filaments stretched out of the flocs and caused difficulties in settling of the system.
- Photomicrographs showed filamentous bulking under 5 and 15 meq/l of magnesium ion concentration. A number of microorganisms belonging to
- Enteric family was identified in this set. However with the chosen biochemical kit, filamentous microorganisms that might have caused bulking could not be observed.

Under sufficient phosphorus concentration,

- All parameters indicating physical and chemical characteristics of sludge improved significantly with respect to the phosphorus deficient concentration.

- Magnesium ion stimulated the production and binding of the protein content of EPS. In all of the concentrations, protein content was higher than carbohydrate yielding EPS_p/EPS_c ratio higher than 1. However for the concentration increase from 5 to 15 meq/L, carbohydrate production and binding was also stimulated.

- Physical characteristics of the system did not change significantly with varying cation concentration as opposed to previous literature studies.

- Dewaterability of the system did not change much with changing magnesium ion concentration. This indicated that floc structure is already good regardless of magnesium concentration.

- Settleability values indicated well settling sludge at all of the magnesium ion concentrations. All the SVI values measured in this set were close to each other and indicated non bulking sludge.

- Apparent viscosity values at all of the studied concentration were small indicating low resistance of sludge to flow. All the values measured were almost identical at different magnesium ion concentration. In addition non Newtonian pseudoplastic behavior was observed similar to the case of phosphorus deficient conditions.

- From photomicrographs, it is clearly evident that healthy flocculation took place in all of the reactors. Identified microorganisms changed due to different nutrient concentrations. Nocardiaform related microorganisms were identified by API Coryne in sufficient phosphorus concentration. In addition some floc forming bacteria was identified in this set.

CHAPTER 6

RECOMMENDATIONS

In future according to results obtained in this set following investigations can be done:

- In order to explain effect of magnesium ion on floc structure more extensively additional analyses such as surface chemical analyses and not conducted physical and chemical analyses can be done.

- Microbiological characterization can be conducted by using biotechnological techniques just as 16sRNA sequencing method for identification of filamentous microorganisms. In addition additional conventional tests can be applied for microbiological characterization at the species level.

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APPENDIX A

Solids Concentrations of Reactors under Deficient Phosphorus and Sufficient Phosphorus Conditions

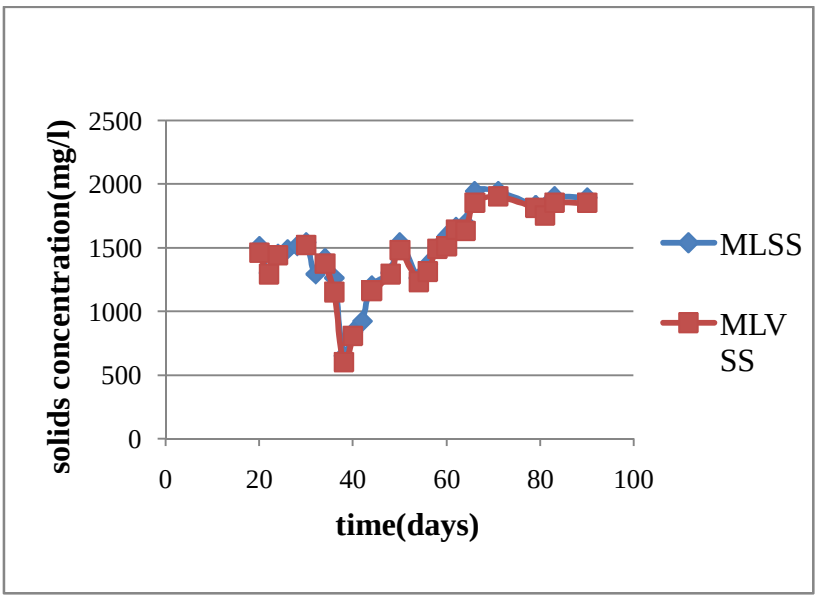


Figure A.1 Solids Concentration versus time graph for 0.5meq/l of Magnesium under deficient phosphorus concentration

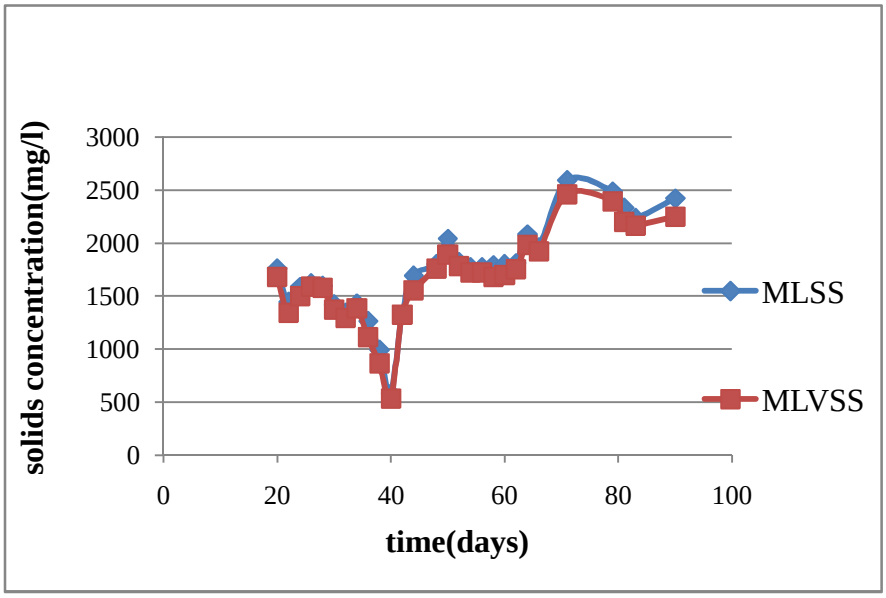


Figure A.2 Solids Concentration versus time graph for 5 (1) meq/L of Magnesium under deficient phosphorus concentration

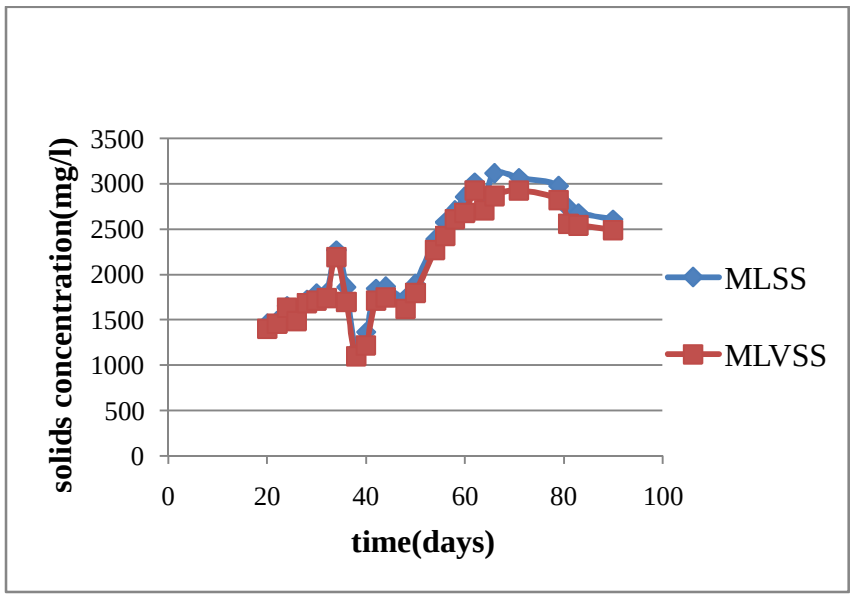


Figure A.3 Solids Concentration versus time graph for 5 (2) meq/L of Magnesium under deficient phosphorus concentration

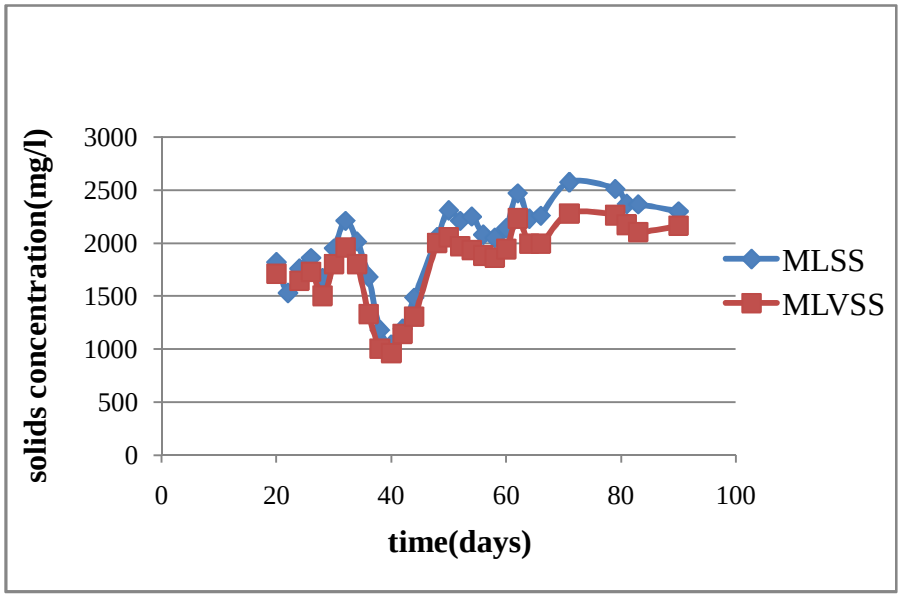


Figure A.4 Solids Concentration versus time graph for 15 (1)meq/l of Magnesium under deficient phosphorus concentration

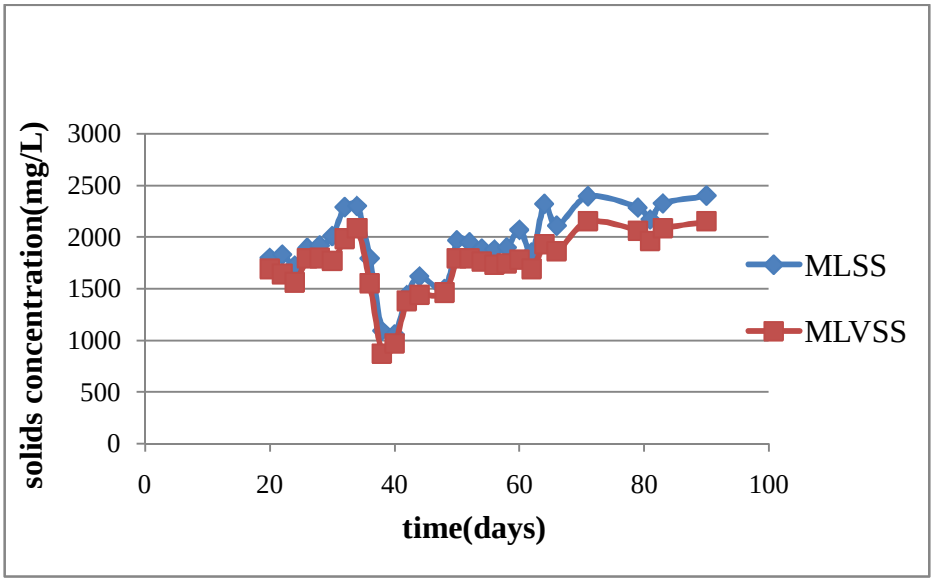


Figure A.5 Solids Concentration versus time graph for 15 (2) meq/l of Magnesium under deficient phosphorus concentration

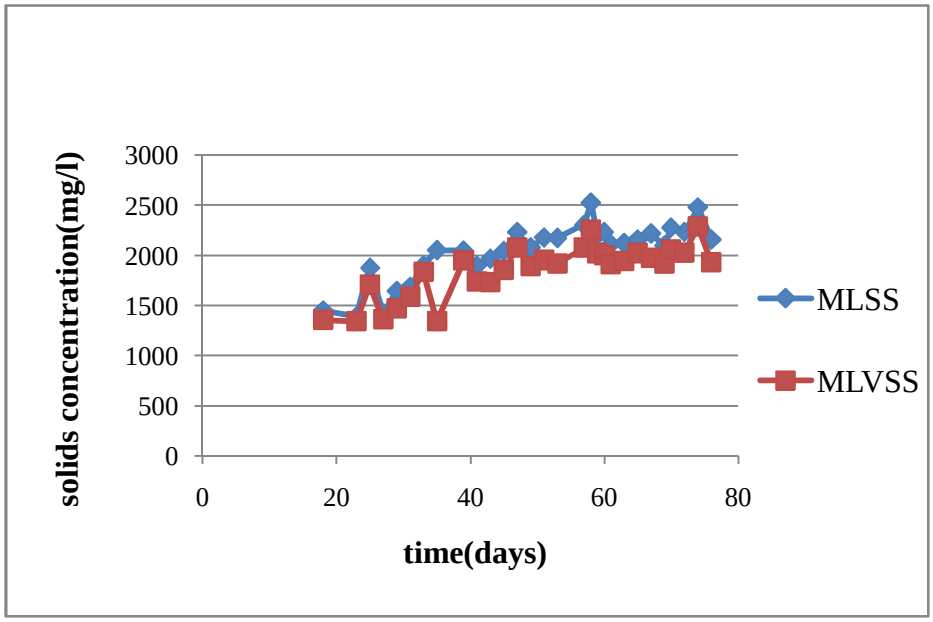


Figure A.6 Solids Concentration versus time graph for 0.5 (1) meq/l of Magnesium under sufficient phosphorus concentration

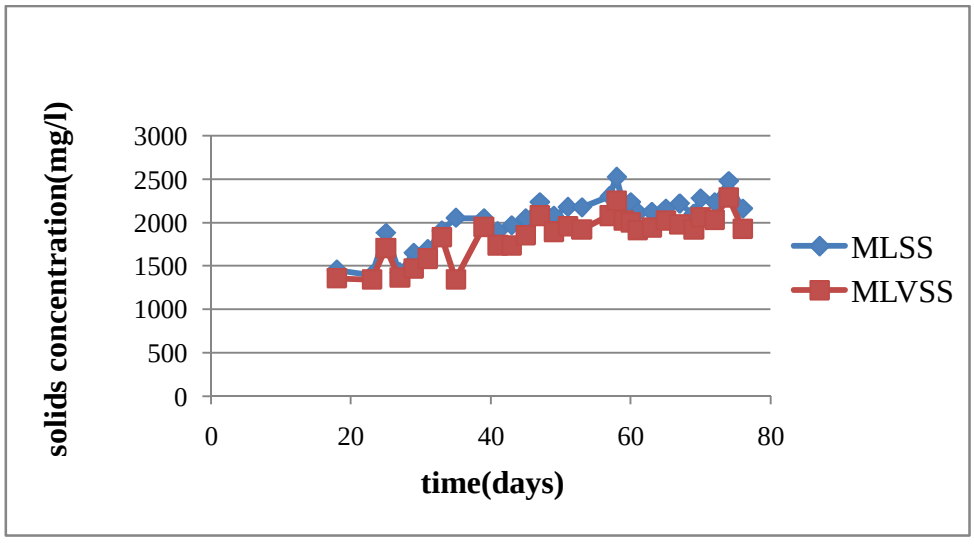


Figure A.7 Solids Concentration versus time graph for 0.5 (2) meq/l of Magnesium under sufficient phosphorus concentration

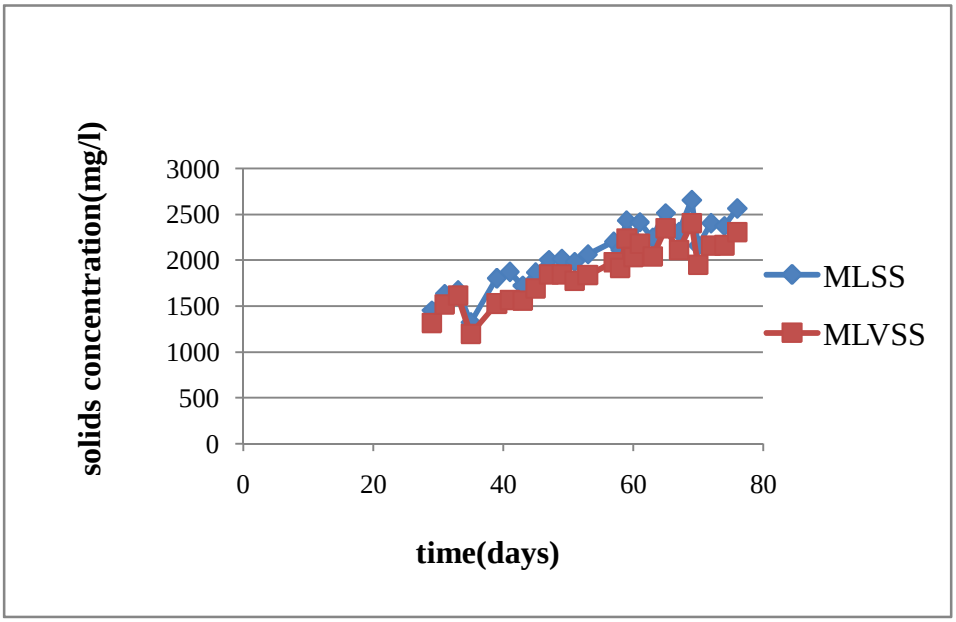


Figure A.8 Solids Concentration versus time graph for 5 (1) meq/l of Magnesium under sufficient phosphorus concentration

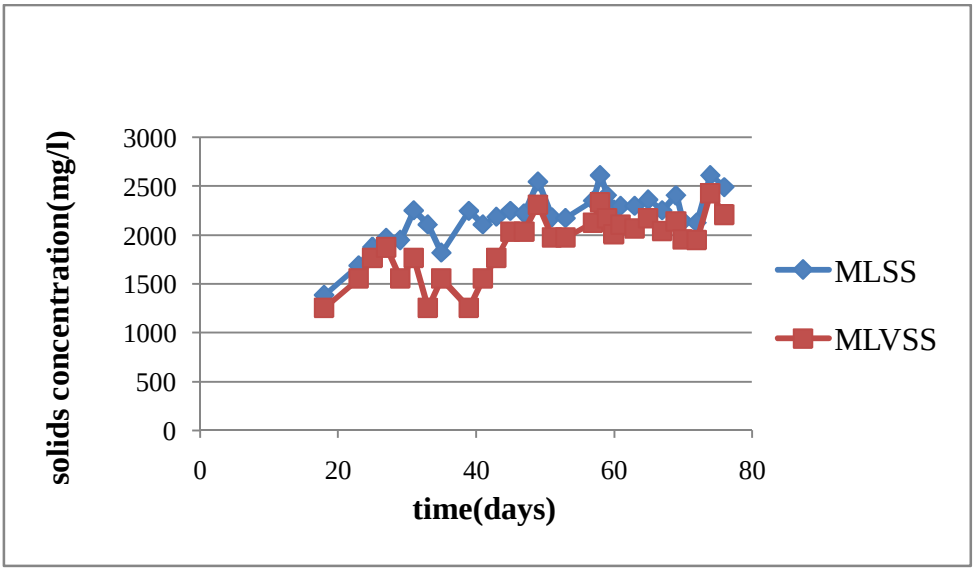


Figure A.9 Solids Concentration versus time graph for 5 (2) meq/l of Magnesium under sufficient phosphorus concentration

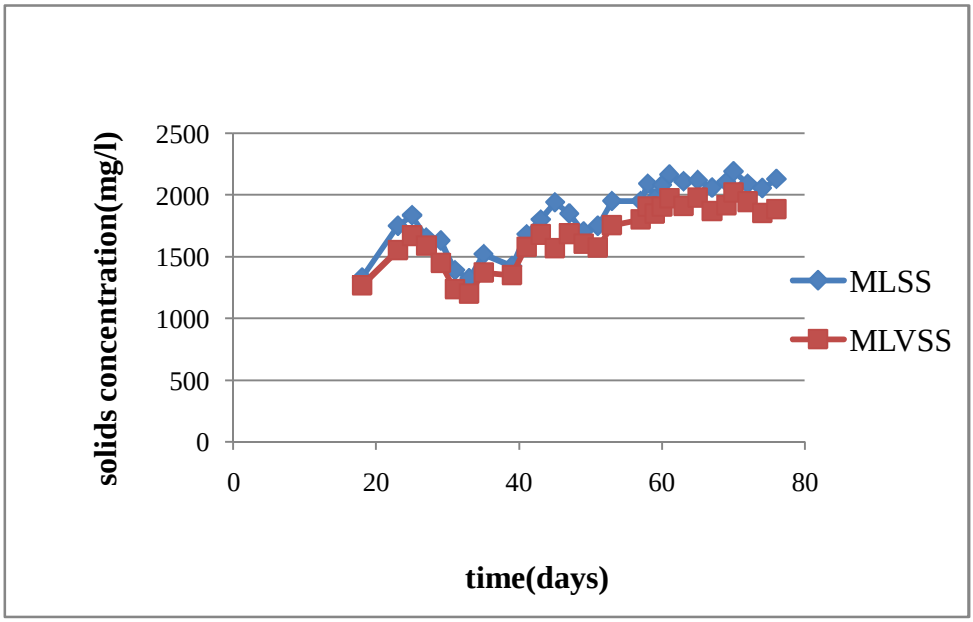


Figure A.10 Solids Concentration versus time graph for 15 (1) meq/l of Magnesium under sufficient phosphorus concentration

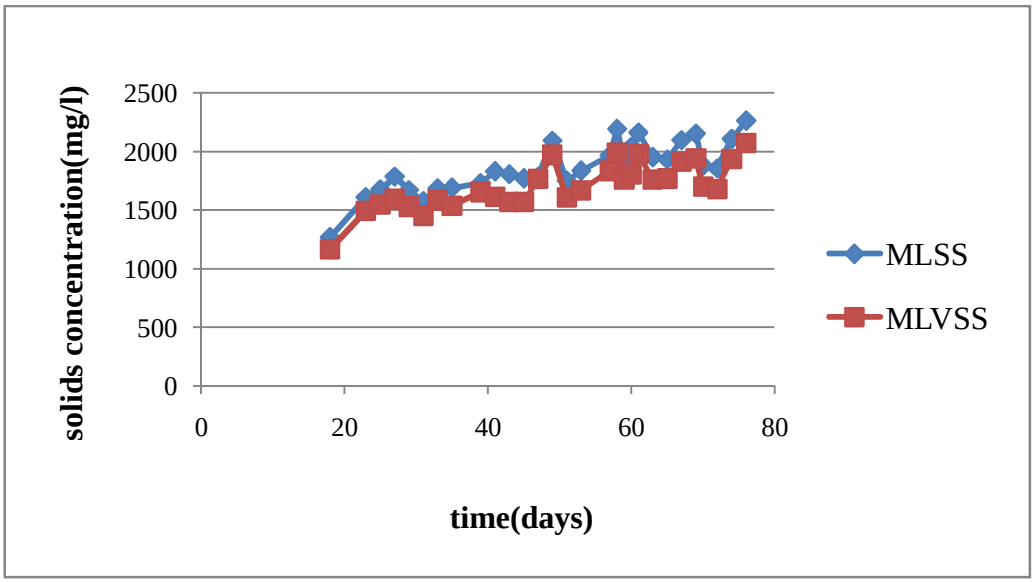


Figure A.11 Solids Concentration versus time graph for 15 (2) meq/l of Magnesium under sufficient phosphorus concentration

APPENDIX B

Calibration Curves for EPS Analyses

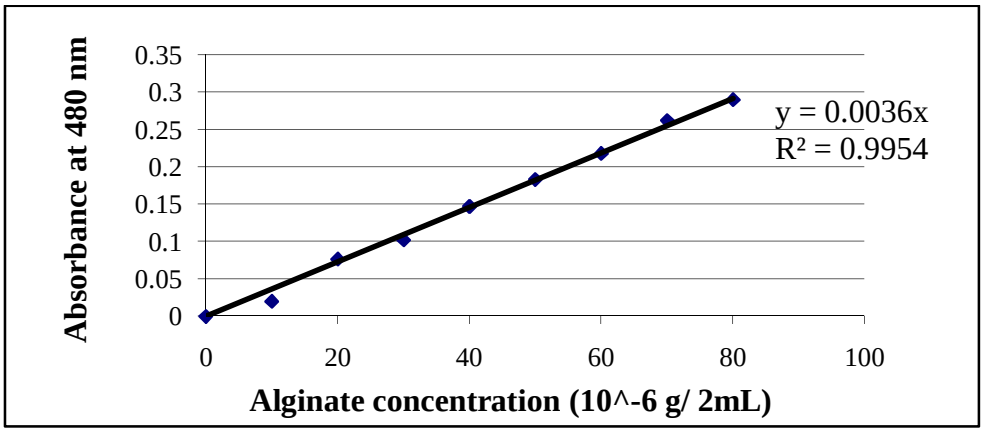


Figure B.1 Calibration curve for carbohydrate by using Dubois Method under deficient phosphorus concentration

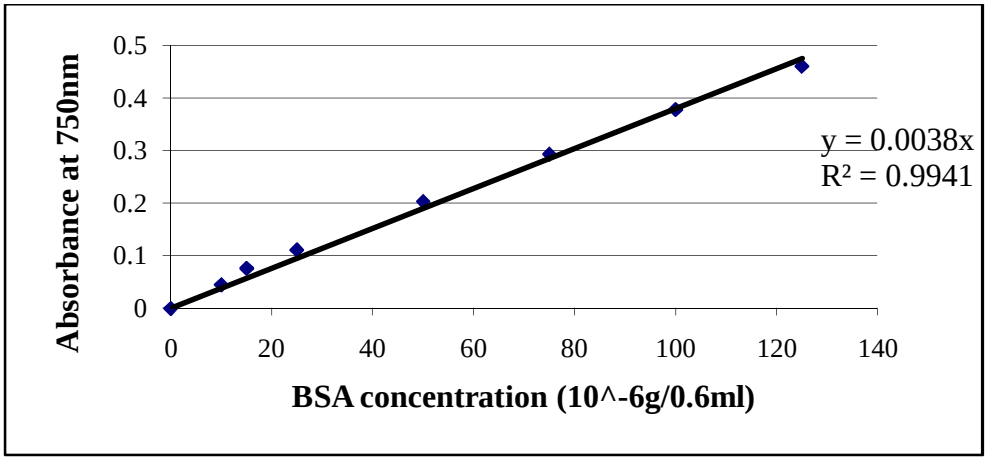


Figure B.2. Calibration curve for protein by using Lowry Method under deficient phosphorus concentra

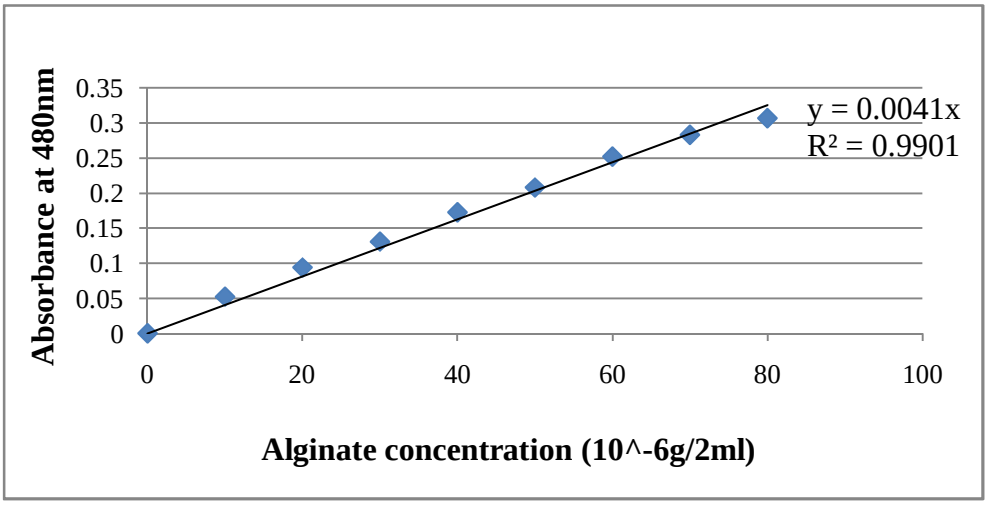


Figure B.3 Calibration curve for carbohydrate by using Dubois Method under sufficient phosphorus concentration

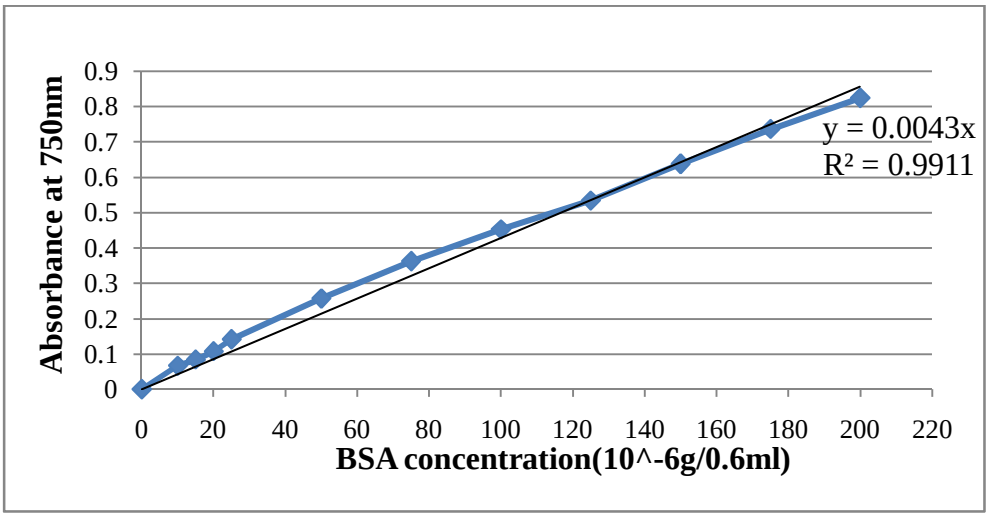
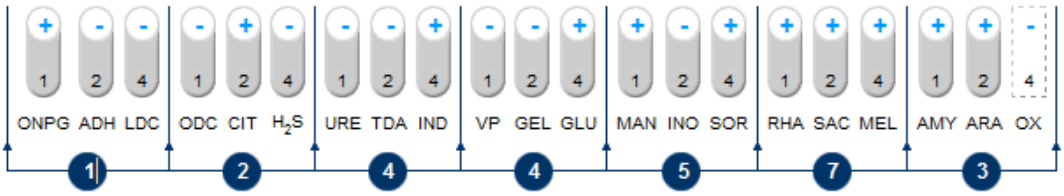


Figure B.4 Calibration curve for protein by using Lowry Method under sufficient phosphorus concentration

APPENDIX C

Api Test Results

API 20 E Results for First Set

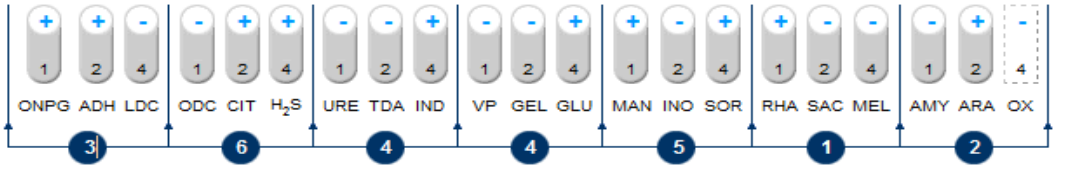


GOOD IDENTIFICATION TO THE GENUS	
Strip	API 20 E V4.1
Profile	1 2 4 4 5 7 3
Note	POSSIBILITY OF Erwinia spp

Significant taxa	% ID	T	Tests against			
Pantoea spp 2	70.4	0.97				
Pantoea spp 4	25.7	0.88				

Next taxon	% ID	T	Tests against			
Citrobacter koseri/farmeri	1.8	0.59	ODC 100%	CIT 25%		

Figure C.1 Test result for the specie isolated from 15 meq/L concentration

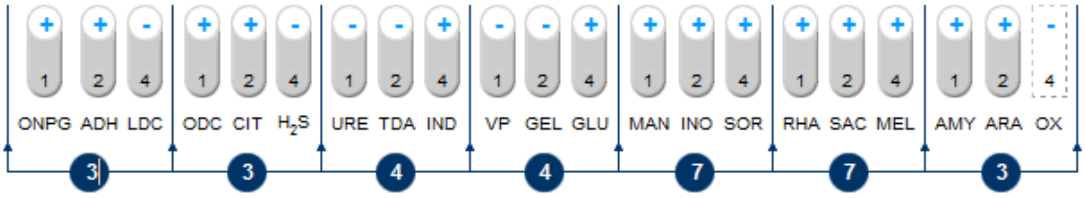


VERY GOOD IDENTIFICATION	
Strip	API 20 E V4.1
Profile	3 6 4 4 5 1 2
Note	

Significant taxa	% ID	T	Tests against			
Citrobacter youngae	99.8	0.67	IND 1%			

Next taxon	% ID	T	Tests against			
Citrobacter freundii	0.1	0.2	ADH 24%	IND 1%	SAC 99%	MEL 82%

Figure C.2 Test result for the specie isolated from 5 meq/L concentration



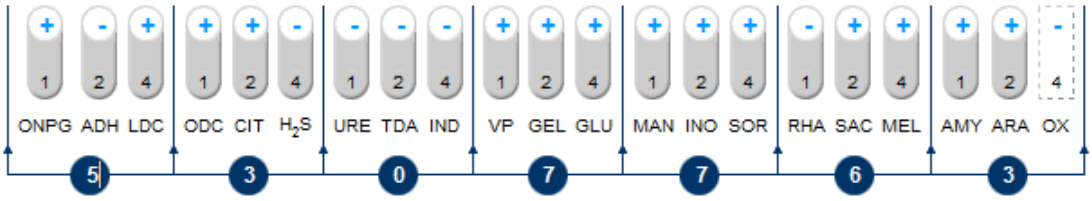
GOOD IDENTIFICATION	
Strip	API 20 E V4.1
Profile	3 3 4 4 7 7 3
Note	POSSIBILITY OF Enterobacter cloacae

Significant taxa	% ID	T	Tests against					
Enterobacter sakazakii	93.4	0.58	IND	25%	VP	91%	SOR	8%

Next taxon	% ID	T	Tests against					
Citrobacter koseri/farmeri	3.7	0.32	ADH	2%	CIT	25%	INO	1%

Complementary test(s)	YELLOW	ESC (HYD.)		
Enterobacter cloacae	0%	30%		
Enterobacter sakazakii	98%	100%		

Figure C.3 Test result for the specie isolated from 0.5 meq/L concentration



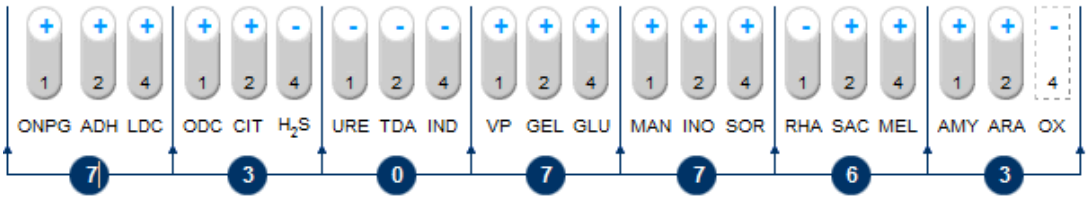
VERY GOOD IDENTIFICATION TO THE GENUS	
Strip	API 20 E V4.1
Profile	5 3 0 7 7 6 3
Note	

Significant taxa	% ID	T	Tests against					
Serratia liquefaciens	69.0	1.0						
Serratia marcescens	30.8	0.92	ARA	25%				

Next taxon	% ID	T	Tests against					
Serratia odorifera 1	0.1	0.43	IND	99%	RHA	99%		

Complementary test(s)	dXYLOSE	METHYL RED		
Serratia liquefaciens	100%	93%		
Serratia marcescens	7%	20%		

Figure C.4 Test result for the specie isolated from 15 meq/L concentration

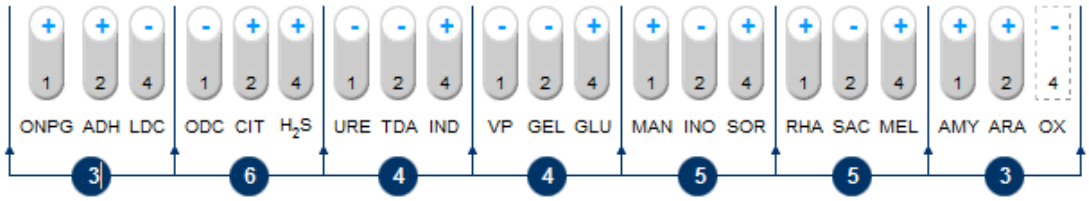


GOOD IDENTIFICATION	
Strip	API 20 E V4.1
Profile	7 3 0 7 7 6 3
Note	

Significant taxa	% ID	T	Tests against					
Serratia liquefaciens	96.0	0.67	ADH	1%				

Next taxon	% ID	T	Tests against					
Serratia marcescens	3.8	0.42	ADH	0%	ARA	25%		

Figure C.5 Test result for the specie isolated from 5 meq/L concentration



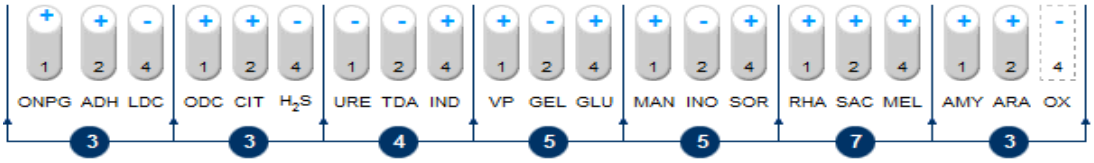
GOOD IDENTIFICATION	
Strip	API 20 E V4.1
Profile	3 6 4 4 5 5 3
Note	

Significant taxa	% ID	T	Tests against					
Citrobacter braakii	92.9	0.47	ODC	99%	IND	4%		

Next taxon	% ID	T	Tests against					
Citrobacter freundii	6.0	0.28	ADH	24%	IND	1%	SAC	99%

Figure C.6 Test result for the specie isolated from 15 meq/L concentration

API 20 E results for the Second Set



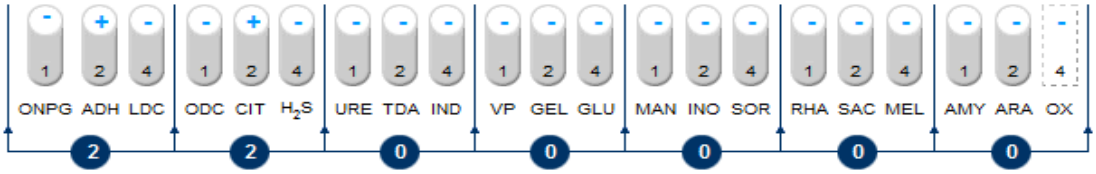
GOOD IDENTIFICATION	
Strip	API 20 E V4.1
Profile	3 3 4 5 5 7 3
Note	POSSIBILITY OF Enterobacter cloacae

Significant taxa	% ID	T	Tests against							
Enterobacter sakazakii	91.1	0.67	IND	25%	INO	75%	SOR	8%		

Next taxon	% ID	T	Tests against							
Enterobacter cloacae	8.5	0.5	IND	0%						

Complementary test(s)	YELLOW	ESC (HYD.)		
Enterobacter cloacae	0%	30%		
Enterobacter sakazakii	98%	100%		

Figure C.7 Test result for the specie isolated from 15 meq/L concentration



GOOD IDENTIFICATION TO THE GENUS	
Strip	API 20 E V4.1
Profile	2 2 0 0 0 0 0
Note	

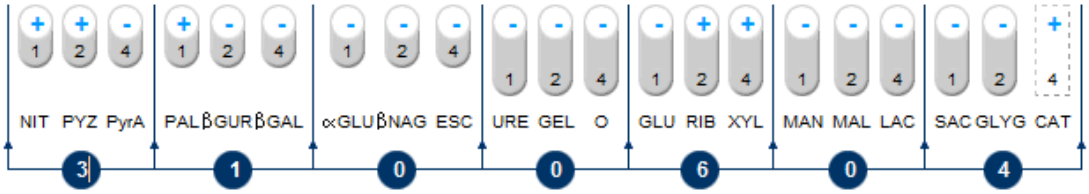
Significant taxa	% ID	T	Tests against							
Pseudomonas fluorescens/putida	44.5	0.72	OX	99%						
Pseudomonas aeruginosa	27.9	0.69	GEL	75%	OX	97%				
Pseudomonas luteola	20.0	0.63	ONPG	86%	GLU	84%	ARA	85%		

Next taxon	% ID	T	Tests against							
Pseudomonas oryzihabitans	3.9	0.5	ADH	0%						

Complementary test(s)	42°C	ESC (HYD.)	YELLOW	Tween 80
Pseudomonas luteola	98%	100%	98%	56%
Pseudomonas aeruginosa	98%	2%	2%	94%
Pseudomonas fluorescens	2%	0%	2%	+(-)
Pseudomonas putida	4%	0%	2%	-

Figure C.8 Test result for the specie isolated from 5 meq/L concentration

API CORYNE Results for the Second Set



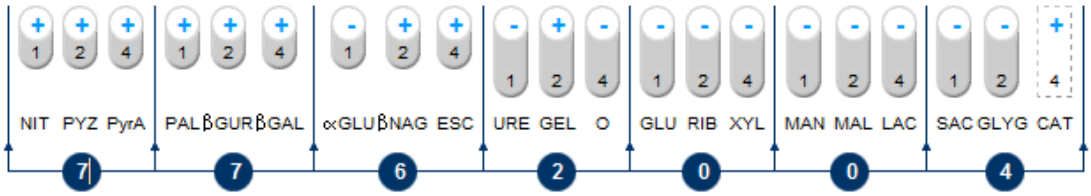
GOOD IDENTIFICATION	
Strip	API CORYNE V3.0
Profile	3 1 0 0 6 0 4
Note	

Significant taxa	% ID	T	Tests against					
Brevibacterium spp	94.9	0.49	NIT	25%	RIB	20%	XYL	7%

Next taxon	% ID	T	Tests against					
Corynebacterium jeikeium	2.6	0.0	NIT	3%	GLU	98%	XYL	0%

Complementary test(s)	42°C
Brevibacterium casei	-
Brevibacterium epidermidis	+(-)

Figure C.9 Test result for the specie isolated from 0.5 meq/L concentration

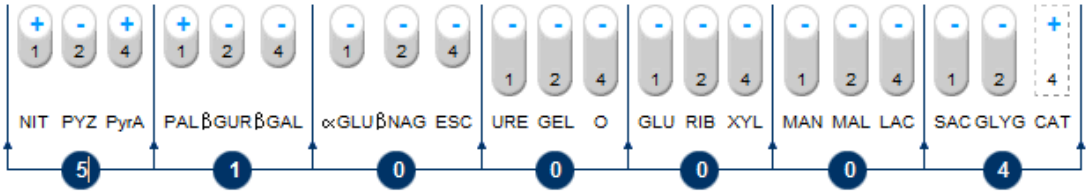


VERY GOOD IDENTIFICATION	
Strip	API CORYNE V3.0
Profile	7 7 6 2 0 0 4
Note	

Significant taxa	% ID	T	Tests against					
Arthrobacter spp	99.9	0.64	αGLU	75%	ßNAG	14%		

Next taxon	% ID	T	Tests against							
Brevibacterium spp	0.1	0.09	NIT	25%	ßGUR	0%	ßGAL	20%	ßNAG	20%
			ESC	20%						

Figure C.10 Test result for the specie isolated from 0.5 meq/L concentration



GOOD IDENTIFICATION	
Strip	API CORYNE V3.0
Profile	5 1 0 0 0 0 4
Note	POSSIBILITY OF Rhodococcus equi

Significant taxa	% ID	T	Tests against			
Corynebacterium propinquum	91.2	0.89				

Next taxon	% ID	T	Tests against					
Corynebacterium pseudodiphtheriticum	4.2	0.65	PYZ	93%	URE	92%		

Complementary test(s)	PINK	CAMP(S.au)		
Corynebacterium propinquum	-	-		
Rhodococcus equi	+	+(-)		

Figure C.11 Test result for the specie isolated from 0.5 meq/L concentration