ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

CHEMICAL CHARACTERIZATION OF EXOPOLYSACCHARIDES FROM FLOCCULAR AND AEROBIC GRANULAR SLUDGE RECEIVING SYNTHETIC/ INDUSTRIAL WASTEWASTERS

M.Sc. THESIS

Stanley Bortse SAM

Department of Environmental Engineering

Environmental Biotechnology Programme

SEPTEMBER 2014

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

CHEMICAL CHARACTERIZATION OF EXOPOLYSACCHARIDES FROM FLOCCULAR AND AEROBIC GRANULAR SLUDGE RECEIVING SYNTHETIC/ INDUSTRIAL WASTEWASTERS

M.Sc. THESIS

Stanley Bortse SAM
(501121811)

Department of Environmental Engineering

Environmental Biotechnology Programme

Thesis Advisor: Assoc. Prof. Dr. Ebru DÜLEKGÜRGEN

SEPTEMBER 2014

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

SENTETİK/ENDÜSTRİYEL ATIKSU İLE BESLENEN FLOKÜLER VE AEROBİK GRANÜLER ÇAMURDAN ELDE EDİLEN EKZOPOLİSAKKARİTLERİN KİMYASAL KARAKTERİZASYONU

YÜKSEK LİSANS TEZİ

Stanley Bortse SAM
(501121811)

Çevre Bilimler Ve Mühendisliği Anabilim Dalı

Çevre Bioteknolojisi Programı

Tez Danışmanı: Doç. Dr. Ebru DÜLEKGÜRGEN

EYLÜL 2014

Stanley Bortse SAM, a **M.Sc.** student of ITU **Institute of / Graduate School of Engineering and Technology** student ID **501121811**, successfully defended the **thesis/dissertation** entitled "**Chemical characterization of exopolysaccharides from floccular and aerobic granular sludge receiving synthetic/industrial wastewaters**", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor:	**Assoc. Prof. Dr. Ebru DÜLEKGÜRGEN** Istanbul Technical University	.....
Jury Members:	**Prof. Dr. Süleyman ÖVEZ** Istanbul Technical University	.....
	Assoc. Prof. Dr. Gülsüm YILMAZ Istanbul University	.....

Date of Submission: 01 September 2014
Date of Defense: 30 September 2014

To my family,

FOREWORD

I would like to express my profound gratitude to my supervisor Assoc. Prof Ebru Dülekgürgen; you have been a tremendous teacher and mentor for me. I owe you special thanks for your incessant encouragement and support in my research and most importantly for the discipline you have instilled in me as a researcher. Without you this work could not have been possible. I owe a debt of gratitude to the ITU BAP Support group for the financial assistance for this project.
My sincere thanks also go to Assoc. Prof Gülsüm Yilmaz for providing us with aerobic granules that were used to complete the characterisation experiments.
I would also like to acknowledge Türker Türken and Serkan Güçlü of ITU MEMTEK laboratories for their immense technical assistance. And the jury members, through whose critical reviews this work was successfully completed. A special thanks to my family, your prayers and love is what has sustained me thus far. To all others who contributed in diverse ways to the completion of this work, through constructive criticisms and encouragement, I say thank you.

September 2014

Stanley Bortse SAM
(Environmental Biotechnologist)

TABLE OF CONTENTS

Page

ABBREVIATIONS

BioWWT : Biological Wastewater Treatment
CAS : Conventional Floccular Activated Sludge
COD : Chemical Oxygen Demand
EGTA : Ethylene glycol tetra acetic acid
E-SEM : Environmental Scanning Electron Microscopy
EPS : Extracellular Polymeric Substances
ExoPS : Exopolysaccharides
FTIR : Fourier Transform Infra-Red spectroscopy
GAS : Aerobic Granular Activated Sludge
MALDI TOF : Matrix Assisted Laser Desorption/Ionisation-Time-Of-Flight Mass Spectroscopy
OLR : Organic Loading Rate
SBR : Sequencing Batch Reactor
TSS : Total suspended solids
UASB : Upflow Anaerobic Sludge Blanket
UV-VIS : Ultraviolet-Visible spectroscopy

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHEMICAL CHARACTERIZATION OF EXOPOLYSACCHARIDES FROM FLOCCULAR AND AEROBIC GRANULAR SLUDGE RECEIVING SYNTHETIC / INDUSTRIAL WASTEWASTERS

SUMMARY

Biological wastewater treatment (BioWWT) ensures efficient removal of pollutants from wastewater by removing the soluble organic components of the wastewater and incorporating it into microbial biomass which can then be separated from the wastewater. Granular Activated Sludge (GAS) application has several advantages over the conventional floccular activated Sludge (CAS) systems. One outstanding characteristic of GAS is the excellent settling properties of the granules. In BioWWT, efficient settling reduces the time and the volume required for the separation of the solid-liquid phase and the cost of operation. The enhanced settling properties of GAS are due to the compact/dense structure of the granules facilitated by the Extracellular Polymeric Substances (EPS), in other words "the microbial glue", which consist mainly of extracellular polysaccharides (ExoPS) and extracellular proteins (ExoPN), some humic substances, nucleic acids and non-cellular organic/inorganic materials. EPS acts as adhesives that bind microbial cells together into a colony. Larger colonies results in increased density, compactness and further aggregation and hence faster settling of the activated sludge.

In this thesis, the focus is on the extraction and characterization of the ExoPS produced by CAS and GAS. The ExoPS was extracted from GAS and CAS generated in a lab-scale bubble column SBR fed with synthetic and brewery wastewaters. The method described by Lin et al (2010) was used for ExoPS extractions. The gel forming characteristics of ExoPS was also tested by introducing the ExoPS into $CaCl_2$ solution in a 1:1 ratio. The experiments were carried out at the same time with commercial sodium alginate (Sigma) as a reference polysaccharide. Gels produced were then examined with E-SEM. From the results, ExoPS from both CAS and GAS produced hydrogels or gel-like structures in $CaCl_2$, similar to those produced by alginate. The ExoPS extracts were also examined with FTIR and the spectra obtained were very similar to that of the reference sodium alginate; confirming that the extracted substances were indeed alginate-like-polysaccharides. UV-Visible spectroscopy at the range of 190-800nm ruled out the possible contamination of the extracts with proteins. Running block fractionation experiments with GAS from a different source showed that the exoPS of the sludge was composed mainly of MG and GG blocks (50.7% and 45.8% respectively); the former providing flexibility, and the latter conferring the gel formation properties. It can be concluded from the results that CAS/GAS-associated ExoPS are alginate like exopolysaccharides and that the characteristics of GAS-exoPS, namely good gel forming ability, as well as housing homo-monomeric blocks providing flexibility and gelation, seem to confer on the compact structure and thus efficient settling of GAS making it a cost effective method of biological wastewater treatment.

SENTETİK / ENDÜSTRİYEL ATIKSU İLE BESLENEN FLOKÜLER VE AEROBİK GRANÜLER ÇAMURDAN ELDE EDİLEN EKZOPOLİSAKKARİTLERİN KİMYASAL KARAKTERİZASYONU

ÖZET

Atıksudan kaynaklanan farklı tipteki kirleticilerin verimli bir şekilde giderildiğinden emin olmak ve tabi ki çevreyi korumak amacıyla; ayrıca katı mevzuat nedeniyle atıksu arıtma teknolojilerindeki gelişimler gerekli hale gelmiştir. Biyolojik atıksu arıtma, atıksu arıtma prosesinin bir parçası olup; atıksudaki çözünebilir organik maddeleri giderir ve mikrobiyal biyokütle tarafından bünyesine alınmasını sağlar ki böylelikle atıksudan ayırarak temiz çıkış elde edilir. Granüler aktif çamur (GAÇ) uygulaması, çevre biyoteknoloji alanındaki en başarılı hikayelerden biri olarak tıksu arıtımında dikkati çekmiştir. Bu durum öncelikle sistemin klasik veya floküler aktif sistemlere göre birtakım avantajlarının olmasından kaynaklanır.

Aerobik granüler çamur (AGÇ), granül görünümünde olan ve kendi-sabitliyor. Hücresini (örneğin; alt tabaka bulunmaz) içeren bir tür biyofilmdir. Biyofilm tabakası, biyolojik atıksu arıtımında kullanılır, mikrobiyal yapıların biraraya gelerek çökmesi ile oluşmaktadır. Dahası, uluslarası su birliğine göre, 'aerobik granüler aktif çamurdaki granüllerin mikrobiyal orjinli agregat oldukları; hidrodinamik stres altında çökelmedikleri ve aktif çamur floklarından gözle görünür şekilde daha iyi çökeldikleri anlaşılmıştır'(de Kreuk et al, 2005). AGÇ'un önemli karakteristik özelliğine oluşan granüller sayesinde iyi çökelebilme özelliğini verebiliriz. Çökelebilirliğin tespitinde kullanılan kilit bir parametre olan çamur hacim indeksi (ÇHİ); aerobik granüler çamur sistemlerinde düşük (80ml/g) ve hatta daha da düşük, 20ml/g olabilmektedir. Bu durum biyolojik atıksu arıtma için önemlidir; bu sayede çökelme süresi ve katı-sıvı faz ayrımı için gereken hacim azalır; dolayısıyla işletme maliyetini düşer.

AGÇ'un iyi çökelebilme özellikleri; esas olarak hücre dışı polisakkaritleri (exoPS), hücre dışı proteinlerin (exoPN), bazı humik maddeler, nükleik asitler ve hücresel olmayan organik/inorganik maddeleri içeren egzopolimerik yapılardan – EPS kaynaklanır. EPS, mikrobiyal hücreleri biraraya getirerek koloni oluşturmalarını sağlayan yapıştırıcı gibi davranır. Koloniler büyüdükçe yoğunlukları artar ve bundan dolayı mikroorganizmalar hızlıca çökelir. Bira endüstrisi çıkış suyu şeker, çözünebilir nişasta, uçucu yağ asitleri vb. maddelerden oluşan yüksek konsantrasyonlarda biyobozunur organik madde içermektedir. Kimyasal oksijen ihtiyacı (KOİ) sonuç olarak nispeten yüksek bir BOİ/KOİ oranına, 0.6-0.7, sahiptir. Bira endüstrisi çıkış suyu yüksek miktarda organik madde içermesi, mikrobiyal kirlilik ve kimyasallar nedeniyle yatırım maliyeti düşük olan, ve ayrıca BOİ ile KOİ açısından 80-90 % arıtma verimine sahip olan biyolojik metotlara ihtiyaç duymaktadır.

Bu tezde, öncelikli olarak floküler aktif çamur ve granüler aktif çamurdan ekstrakte edilen hücre dışı polisakkaritlerin karakterize (fiziksel ve kimyasal) edilmeleri

amaçlanmıştır. İkincil olarak, egzopolisakkaritler ve alginat arasındaki muhtemel benzerliklerin araştırılması ve ayrıca egzopolisakkaritlerin kimyasal özellikleri ile granüler aktif çamur sistemlerindeki rolü arasındaki ilişkinin belirlenmesi amaçlanmıştır. Bu çalışmanın kapsamında iki farklı metot uygulanmıştır; katyon değişim reçinesi ve (CER-DOWEX, Frolund et al., 1996 tarafından) ve sentetik atıksu ve bira endüstirisi atıksuyu ile beslenen KAÇ ile GAÇ'dan hücre dışı polisakkaritlerin ekstrakte edilmesi için Na_2CO_3 (Lin et al., 2010) kullanımıdır.
Karakterizasyon açısından, hücre dışı polisakkaritlerin jel oluşturma kapasitesi $CaCl_2$ soüsyonundaki jelleşmesine bakılarak test edilmiştir. Eksrakte edilmiş olan hücre dışı polisakkaritlerin morfolijeleri ve kabaca bileşenleri sırasıyla SEM ve UV-VIS spektroskopisi ile incelenmiştir. Ayrıca kimyasal yapısını belirlemek için de FTIR analizi gerçekleştirilmiştir.

KAÇ ve GAÇ'deki hücre dışı polisakkaritler laboratuvar ölçekli oluşturulan ve sentetik, bira endüstrisi atıksuyu ile beslenmiş AKR'lerden elde edilmiştir. Söz konusu ekstraksiyon iki farklı metot ile gerçekleştirilmiştir; DOWEX (Frolund et al., 1996) ve Na_2CO_3 ile (Lin et al, (2010). DOWEX yönteminde egzopolimerik maddelerin ekstraksiyonu yapılırken; Na_2CO_3 yönteminde ise spesifik olarak alginat benzeri egzopolisakkaritlerin ekstraksiyonu yapılmaktadır. Hücre dışı polisakkaritlerin Na_2CO_3 yöntemi ile ekstraksiyonunda floküler ve aerobik granüler numunelerinde 2% verimle, hemen hemen eşit miktarlarda hücre dışı polisakkaritler elde edilmiştir. Hücre dışı polisakkaritlerin jel oluşturma kapasitelerini ölçmek adına 1:1 oranındaki $CaCl_2$ solüsyonuna bırakılmış ve bir gece boyunca oda sıcaklığında jelleşmenin oluşması için bekletilmiştir.Deneylerde aynı zamanda ticari olarak temin edilen sodyum alginat referans olarak kullanılmıştır. Sonuçlara göre, 2% alginat solüsyonunun1:1 oranındaki $CaCl_2$ ile mükemmel küresel hidrojel tanelerini oluşturduğu tespit edilmiştir.Yine de jel oluşturma kapasitesinin alginat konsantrasyonun azalması ile düştüğü belirlenmiştir. Na_2CO_3 yöntemi ile ekstrakte edilen hücre dışı polisakkaritlerde de alginatlardakine benzer jelleşme göstermiştir.Klasik aktif çamur sisteminden elde edilen hücre dışı polisakkaritler asetat ile beslendiklerinde; 1:1 oranındaki $CaCl_2$ ile hemen hemen küresel hidrojel taneler (alginattaki gibi mükemmel olmayan) oluşturmuştur.Oysa bira atıksuyu ile beslenen granüler aktif çamurdan elde edilen hücre dışı polisakkaritler hidrojeller yerine küçük topaklar oluşturabilmiştir. Oluşturulan jeller daha sonra elektron mikroskobu ile incelenmiştir. Klasik aktif çamurdan (sentetik atıksu ile beslenen) ve granüler aktif çamurdan (bira atıksuyu ile beslenen) elde edilen hücre dışı polisakkaritlerin morfolojik açıdan alginat ile karşılaştırılmaları sonucu klasik aktif çamurdan elde edilenin yapısal açıdan ince lif ağından oluşan alginat ile benzerliklerinin olduğu belirlenmiştir. Granüler aktif çamurdan elde edilen küçük topaklardaki hidrojeller ise bir dereceye kadar alginata benzer katlanmalar göstermiştir. Hücre dışı polisakkaritleri aynı zamanda FTIR ile de incelenmiş ve buradan çıkan sonuçlar ile hücre dışı polisakkaritler ile alginatlar arasındaki benzerlikler doğrulanmıştır. Hücre dışı polisakkaritlerin granül oluşumunun farklı evrelerinin spektrosu alginattaki fonksiyonel gruplara eş değer fonksiyonel gruplar içerdiklerini ortaya koymuştur.Alginik uronik asit için karakteristik olan manuronik asit ile guluronik asit kalıntılarına her iki sistemdeki hücre dışı polisakkaritlerinde rastlanmıştır. Bu doğrulama ekstrakte edilen maddelerin polisakkarit olduklarından emin olmak ve alginat ile benzer olduklarını göstermek için yapılmıştır.

FTIR sonuçları ayrıca hücre dışı polisakkaritlerin Ca^{2+}ile olan reaksiyonunu daha iyi kavramamızı sağladı. $CaCl_2$ solüsyonundaki hücre dışı polisakkaritlerin birleşmesini

sağlayan divalent katyonlar karboksil gruplarının varlığını göstermektedir. Ekstraktların proteinlerle olabilecek muhtemel kontaminasyonu ise 190-800 nm dalga boyundaki UV-Visible spektroskopi altında incelenmiştir. Na_2CO_3 yöntemi ile ekstrakte edilen hücre dışı polisakkaritler UV spektrosunda alginat ile benzerlikler göstermiştir. Her iki sistemde de tipik UV aralığında (120nm ve 190nm) piklere rastlanmıştır. Klasik aktif çamur numunlerinin yüksek konsantrasyonlarda alginat göre daha yoğun absorpsiyonlarının olduğu görülmüştür. 250-290 nm dalga boyundaki piklerin olmayışı ekstraskiyon metodunun özgünlüğünden kaynaklı olabilir ki DNA ve proteinlerle girişim yaptığı düşünülebilir. DOWEX metodu ile ekstrakte edilen egzopolimerik maddeler ve supernatant solüsyonundaki hücre dışı polisakkaritler de U-VIS spektroskopisinde incelenmiştir. Her ikisinde de alginat ve egzopolimerik maddelerde olduğu gibi geniş aralıklarda piklere rastlanmıştır. Fakat her ikisindeki absorbanslar alginat ile mukayese edildiğinde daha yüksektir. 240-290 nm dalga aralığında belirgin bir absorbans kaydedilmesi ile her ikisinde de protein ve nukleik asitlerin olduğu söylenebilir ki egzopolimerik maddelerin proteinler, karbonhidratlar, bazı humik maddeler, nukleik asitler, lipitler ve diğer maddelerden oluştuğu bilinmektedir. Bu durum ile egzopolimerik maddelere özgü olan her iki metot arasındaki farkları açıkça ortaya koymaktadır. Aynı zamanda egzopolimerik maddelerin ekstraksiyonunda metot seçiminin önemini vurgulamaktadır.
Granüler aktif çamurdaki egzopolimerik maddelerdeki guluronik ve mannuronik asit kalıntılarını (homo-monomeric [-GG-, MM-] ve hetero-monomeric [-MG-] blocks) belirlemek için blok fraksiyonlaşması gerçekleştirilmiştir. Egzopolimerik maddelerin %16.48'inin GG bloklarından; %18.24'sinin MG bloklarından oluştuğu bulunmuştur. Ayrıca GG blok fraksiyonunun GM fraksiyonun az olduğu; bu durumda GG blok konsantrasyonun daha da az olduğu durumlarda egzopolimerik maddelerden ekstrakte edilen ve oluşturulan hidrojelin yine oluşabileceği gösterilmiştir. Yüksek miktarlardaki MG blokları; oluşan jelin elastikiyetinin artmasını sağlayan mükemmel esnekliği sağlamaktadır. Jelin mükemmel elastikiyette olması aktif çamurun reaktördeki baskılara karşı daha dayanaklı olmasına yardımcı olmaktadır. Güçlü jellerin oluşması ve kararlı aktif çamur, GG ve MG bloklarının çamurun çökelebilme özelliğini açıkça artırmasıyla gözlemlenmektedir. Daha yoğun ve kararlı çamurun sistemden kaçması mümkün olmayacaktır.

Nem içeriği egzopolimerik maddelerin hidrojel olarak tanımlanebilirliği belirlemek içindir. Hidrojellerin 90%'dan fazlası sudur ve literatür verilerine göre kalsiyum ile birlikte egzopolimerik maddelerin nem içeriklerinin 93% oldukları belirlenmiştir. Ekstrakte edilen egzopolimerik maddelerin nem içeriği 95.8%'dir ki bu da net bir şekilde hidrojel olduklarını göstermektedir.
%0.2'lik alginatta geri kazanılan karbohıdrat, glukoz olarak yaklaşık %7.4'tur. Deşiken geri kazanım yüzdeleri ekstrakte edilen ekzopolimerik maddelerden elde edilmiştir. Örneğin, granüler ekzopolimerik maddeler için geri kazanım %7.8'dir. Diğer klasik aktıf çamur sistemlerindeki ekzopolimerik maddelerden %7.4'den az geri kazanım görülürken; kaçınnılmazbir şekilde floküler ekzopolimerik maddelerden 52 gün itibariyle %16.8 oranında kazanım kaydedilmiştir ki bu da algınatta geri kazanılanın iki katı kadardır. EPS-ilişkili egzopolisakkaritlerin alginat benzeri egzopolisakkaritler oldukları ve yüksek jel oluşturma kapasitesine sahip granüler aktif çamur egzopolimerik maddelerin daha iyi çökelme özellikleri ile düşük maliyetli biyolojik atıksu arıtma sağlayacakları söylenebilir.

1. INTRODUCTION

1.1 Significance of the study

Granular Activated Sludge (GAS) is a kind of biofilm consisting of self-immobilised cell (i.e. without substratum) and bearing the appearance of granule (Goa et al, 2011). It is formed through an aggregated growth of the microbial consortium for biological wastewater treatment. At the first session of the aerobic granular sludge seminars held by the International Water Association (IWA) in Munich-Germany in 2005, a precise definition of aerobic granular sludge was given as consisting of aggregates of microbial origin which do not coagulate under reduced hydrodynamic shear and which settle significantly faster than activated sludge flocs (de Kreuk et al 2005). The application of aerobic granular sludge technology has become a subject of attraction because of the numerous advantages that this system has over the conventional activated sludge. Of prime importance in aerobic granular systems is the settling property of the granules which is higher than that of conventional activated sludge systems. The sludge volume index (SVI) which is a key parameter in the measurement of settling ability is lower (80ml/g) and may be as low as 20ml/g in aerobic granular sludge systems (Zheng et al, 2005). The enhanced settling properties of granular aerobic sludge are a function of the exopolymeric substances which act as adhesives to bind the bacteria to form a colony. Smaller granules and aggregates are also trapped and connected by these exopolymeric substances to form larger colonies.

Bacterial exopolysaccharides are integral components of the complex exopolymeric substances secreted by the microorganisms to form a slimy, 3D matrix in which the bacteria are embedded. According to Lin et al (2010), exopolysaccharides contributes immensely to the structure of aerobic granular sludge as well as providing morphological and structural support for biofilms. In adverse environmental conditions, the exopolysaccharides provide the cells with a protective layer against the harsh environment and also serve as a nutrient and energy source

for the sustenance of the microorganism (Wang et al., 2006). The aggregation of microorganisms in aerobic granular sludge resulting in increased settling velocities has been largely associated with the gelling effect of the exopolysaccharide content (Seviour et al 2009). Due to the perceived function played by exopolysaccharides in the settling properties of GAS, its extraction and characterisation has therefore become paramount to understand its functions in aerobic granular sludge technology and hence the effect on the efficiency of biological wastewater treatment.

Recent works, notably by Lin et al (2008) have focussed on the extraction of some exopolysaccharides similar to alginates and having the gel-forming properties and other characteristics like alginates. These had been extracted from aerobic granules fed with synthetic wastewater in a lab-scale reactor. The question of whether such exopolysaccharides can be obtained in aerobic granules treating different types of wastewater and providing the same granular stability still remains unanswered. The significance of Exopolysaccharides in matrix formation of GAS and its contribution to granular stability makes it a subject worthy of research.

1.1 Aims and Scope of the study

In view of the above mentioned significance, the objective of this work is to:

1. To physically and chemically characterise exopolysaccharides (ExoPS) extracted from Conventional Floccular (CAS) and Aerobic Granular Activated Sludge (GAS).
2. To evaluate the possible similarities between the extracted exoPS and Alginate.
3. To establish the relationship between the chemical characteristics of ExoPS and its role in granular activated sludge systems.

The scope of this work involved the application of two different methods, cation exchange resin (CER-DOWEX, introduced by Frolund et al., 1996) and Na_2CO_3 (Lin et al., 2010) for the extraction of EPS/ExoPS from both CAS and GAS which are being fed with synthetic wastewater and brewery effluent. In terms of characterisation, the gel forming capacity of the ExoPS was tested through gelation in $CaCl_2$ solution. The morphology and a rough composition of the extracted ExoPS was analysed with SEM and UV-VIS Spectroscopy respectively. To identify the chemical structure of the ExoPS, FTIR analysis was also conducted.

2. LITERATURE REVIEW

2.1 Wastewater Treatment (WWT)

WWT has become a crucial issue due to diminishing water resources coupled to high cost of waste water disposal. To achieve the stringent regulatory limits associated with effluent discharge, wastewater must be treatment before disposal. Municipal wastewater emanates from different sources such as sanitary facilities, residential areas, commercial and industrial facilities, institutions and surface runoffs. These sources of wastewater vary widely in their composition, consisting of inorganic and organic components in either dissolved or suspended state. Wastewater thus serves as a potential environmental and health hazard and thus requires appropriate treatment before discharging into the environment.

Wastewater treatment systems are conventionally divided into three stages; primary, secondary and tertiary stages to ensure efficient removal of the different types of contaminants. The primary stage and a preliminary stage (which precedes the primary stage) are treatment stages where physical separation of suspended solids and floating objects occur. A large amount of the TSS (about 50-70%) is removed by gravity separation in the primary clarifier with a resultant BOD decrease about 25-50% (Culpla and Ali, 2012).

Secondary treatment is a biological WWT process which ensures the removal of dissolved organic matter from the wastewater. The basic principle of biological WWT is to convert dissolved organic compounds into insoluble forms that can be separated. Hence BioWWT involves the selection of microbial populations with characteristics such as a proper metabolic activity in order to utilise the organic matter in the wastewater and also generate a stable biomass that can be separated from the effluent wastewater (Wilderer et al, 2001). The activities of microorganisms as well as secondary settling processes through gravity settling result in about 90% removal of organic matter in BioWWT.

The most widely used BioWWT technology is the Activated Sludge System which was developed in England by Arden and Lockett in 1914.

2.2 Aerobic Activated Sludge

Aerobic activated sludge is basically a biological process that utilises microorganisms to stabilize the dissolved and particulate organic content of wastewater. In the process, the organic contaminants serve as carbon and energy sources for the growth and reproduction of the microbial population. Hence, the organic matter is converted to cell mass and removed from the wastewater through gravity settling. In a conventional activated sludge system, part of the settled sludge is recycled into the in-coming wastewater to maintain a high concentration of biomass in the reactor and part is also wasted to ensure a balance of biomass in the reactor.

Engineered biological wastewater treatment systems are replicas of natural systems providing a favourable artificial environment for the microorganisms. The biomass retained in such systems exists as suspended growth forms or in the attached growth form as in the trickling filters or rotating biological contactors (Gebara, 1999). However some new treatment technologies such as the fluidized bed system employs a combination of both suspended and attached biomass in a hybrid growth system. The suspended growth of microorganisms is the most common method that exists in activated sludge system. In this system, the microorganisms exist as flocs which are recycled back into the reactor through settling. For years, conventional activated sludge system has served as an efficient process for biological wastewater treatment.

In spite of its efficiency, the process has some inherent flaws and notably amongst them is the slow and difficult settling of the biological flocs. The difficulty in settling suggests that the conventional activated sludge system can only operate at a low volumetric loading rate to avoid exceeding the flux capacity of the settling tank (Gao et al, 2011). The conventional activated sludge system also requires a large surface area for treatment and biomass separation units. The development of a system, similar to the conventional activated sludge system with relatively small settling tanks but with higher organic loading capacity and enhanced settling was thus apparent. Aerobic granulation accommodates the various processes occurring in several tanks in the conventional activated sludge system in a single granule.

2.3 Aerobic granulation technology

The efficiency of wastewater treatment systems is a function of a good removal rate of carbon, nitrogen and phosphorus to meet discharging standards as well as a good solid-liquid separation (settling properties) of the sludge. According to Bindhu and Madhu (2013), the settling properties of a discrete particle in a solution depend on the shape of the particle and this is evident in the Stokes law equation.

$$Vs = \frac{((Pp-Pf))d^2}{18u} \quad \textbf{(2.1)}$$

d = diameter of the particle

ρp and ρf =density of the particle and media respectively

μ = viscosity of the media

From this principle, the settling velocity of the particle can be increased by either an increase in the difference in densities between the particle and the fluid or an increase in the radius of the particle or by reducing the dynamic viscosity of the fluid. This suggests that increasing the size of the particle through aggregation of the microorganisms increases the radius of the particle (granule) hence increasing the settling velocity of the granule.

Granular sludge is a self-immobilised microbial sludge consortium which has a granular appearance, a high density and high treatment efficiencies in biological wastewater treatment. This dense consortium is made of different species of bacteria and consists of millions of organisms per gram of biomass (Liu and Tay, 2004). Tay and Liu (2002) describes it as a biofilm formed through an aggregated growth mechanism without a substratum.

The consensus on the description of microbial granules is; "*granules making up GAS are to be understood as aggregates of microbial origin, which do not coagulate under reduced hydrodynamic shear, and which settle significantly faster than activated sludge flocs.*" (de kreuk et al., 2005). The history of Granular sludge dates back to the late 1970s when it was observed in Upflow anaerobic sludge blanket reactor treating industrial wastewater (Lettinga, 1980). Though discovered in anaerobic systems, certain drawbacks such as long start up periods, relatively high operating temperatures, unsuitability for low strength organic wastewater and low efficiency in nutrient removal in the anaerobic process led to the development of

Aerobic granular sludge which has gained popularity in organic and nutrient removal (Dulekgurgen et al., 2003, de Kreuk et al., 2004, Adav and Lee., 2008).

Sequencing batch reactors which are modified CAS reactors have proved to become the reactor of choice for the cultivation of aerobic granules. The increasing popularity of GAS is due to some advantages that the process possesses over the conventional activated sludge system. Due to the increase in size and density of the granules, this process has good settling properties reaching between 50-90m/h (Gao et al., 2011). The structure of the granule and concentration of the biomass in this process also offers the resistance to shock loading. According to Beun et al (1999), another advantage of granular aerobic sludge process is its ability to carry out simultaneously nitrification and denitrification due to the presence of both aerobic and anoxic conditions in the granule.

2.3.1 Formation of aerobic granules

Aerobic granules are considered as a dense and compact aggregate of microbes with a granular appearance. According to Calleja (1984), 'it is defined as the gathering of cells to form a fairly stable, contigious multicellular association under physiological conditions'. Liu and Tay in 2004 observed that the formation of aerobic granules is actually a stepwise process occurring through some form of aggregation from activated seed sludge to the final granule. In an experiment carried out in a sequencing batch reactor, it was observed that activated seed sludge with very loose and irregular structure and consisting mainly of filamentous microbial population developed into mature granules, compact with clear round outer edges.

Due to the electrostatic forces of repulsion and the hydration interactions among bacteria, self-adhesion of the microorganisms is nearly an impossibility and they prefer to grow in suspension rather than in flocs, biofilms or granules (Beun et al 1999). Hence granule formation is induced by the appropriate environmental conditions progressing from activated seed sludge to compact aggregates and then to the fully defined granule. The formation of granules is influenced by a number of factors such as the composition of substrate, organic loading rate, hydrodynamic shear force, settling time, hydraulic retention time, dissolved oxygen, pH and temperature, etc.

Examples of aerobic granules formed in lab- or pilot scale SBRs fed with different wastewaters (synthetic ww, brewery effluent, domestic ww) are given in Figure 2.1.

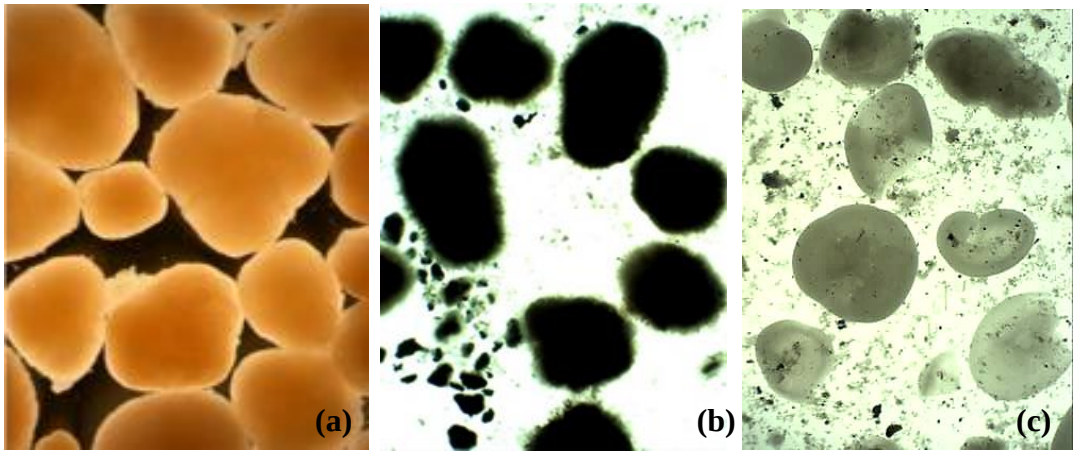

Figure 2.1: Aerobic granules grown on (a) synthetic wastewater/acetate (Dulekgurgen, 2006), (b) brewery wastewater (Dülekgürgen and Karahan-Özgün, 2012), (c) domestic wastewater (Yılmaz et al., 2014).

2.3.2 Factors affecting aerobic granulation

2.3.2.1 Composition of substrate

The carbon source in wastewater treatment is very essential to the development of aerobic and anaerobic granules. The composition of the microbial species as well as the microstructure of the granule is directly dependent on the choice of substrate or the carbon source fed to the system. Formation of granules has been observed in systems with different kinds of substrate. For example, substrates like acetate, glucose phenols, ethanol, molasses, sucrose and synthetic wastewater have all been used in the production of granules (Adav and Lee, 2008). It is now clear that acetate-fed reactors seem to generate compact granules with that granular appearance and clear round outer edges. This is supported by the presence of dominant non-filamentous bacteria. A less compact and much fluffy type of granule with a predominant filamentous bacteria population were reported by liu and Tay in 2004 using glucose as the substrate .

2.3.2.2 Organic loading rate

The dependency of granule formation on organic loading rates seems to vary in terms of the process being employed. In anaerobic systems such as the Upflow anaerobic sludge blanket (UASB), the formation of granules is directly related to the organic loading rate (OLR). That is, high OLR increases granule formation. This is however

not the case in aerobic granulation where the formation of granules is independent of the OLR. Aerobic granules have occurred in a wide range of OLR mostly from 2.5 - 15kgCOD/m^3day (Moy et al 2002, Lin et al 2003). Despite the insignificant role played by OLR on aerobic granulation, some physical parameters are dependent on the OLR. The mean size of aerobic granule is subject to the rate of organic loading and increases with increase in OLR. Unlike the morphology of the granule which responds insignificantly to the OLR, the physical strength of the granule is indirectly related to the OLR.

2.3.2.3 Hydrodynamic shear force

Hydrodynamic shear force is a microscopic shear force that occurs due to the motion of different layer of the fluid at different velocities. In activated sludge systems, hydrodynamic shear force is an example of a physical stress condition induced by aeration of the bioreactor. According to Dulekgurgen et al, (2008), aerobic granulation has a close association with the hydrodynamic conditions of the reactor. The application of relatively high hydrodynamic stress achieved by increasing the superficial upflow air velocity contributes to initiation, the formation and stability of aerobic and anaerobic granules. The formation of granules is however subjected to a threshold shear force value above which the morphology of the granule is transformed into a regular, rounder and compact form. Microbial populations also have an effect on the morphology of the granule formed and superficial air upflow velocity acts as a major hydrodynamic shear force exerted on microbial population. Low superficial air velocity leads to an increase in filamentous microorganisms which results in poor settling. On the other hand, high superficial air velocity promotes granules with dominant non filamentous bacteria which result in better settling.

Hydrodynamic stress is also known to have a close association with the production of extracellular polysaccharides which intend promotes the stability of aerobic granules. Increase in upflow air velocity results in a concomitant increase in the hydrodynamic shear force which according to Ohashi and Harada (1994) is the driving force for the production of Extracellular polysaccharides. In Wastewater treatment, the application of a relatively high hydrodynamic shear force leading to a surge in extracellular

production, coupled to high granule density and low SVI ensure efficient solid-liquid separation and hence high effluent quality.

2.3.2.4 Settling time

The settling time is the period within which the biomass is allowed to settle before the effluent is withdrawn and it is a major hydraulic selection pressure on the microbial population in sequencing batch reactors. It is a factor that determines the rate of solid-liquid separation in the bioreactor. Microorganisms with good settling properties settle in a short time whereas those with poor settleability remain suspended and are washed out with the effluent (Qin et al 2004). There is evidence that under stressful conditions, bacteria are able to alter their surface hydrophobicity. Studies have shown that short settling times in SBRs seem to facilitate aerobic granulation. Short settling as a hydraulic selection pressure triggers a metabolic response in the microorganisms that stimulate the production of extracellular polysaccharides and enhance the surface hydrophobicity of the cell (Adav and Lee 2008, Liu et al 2004 and Qin et al, 2004). This is an adaptation that promotes microbial association through self-aggregation and prevents cells from being washed out from the reactor. According to Qin et al (2004), the settling time required for a successful aerobic granulation would not be more than 5 minutes.

2.3.2.5 Aerobic Starvation

The aerobic starvation stage of SBRs is known to consist of two important phases: a degradation phase where the substrate supplied is depleted to the minimum and an aerobic starvation stage where there is no substrate available for utilisation by the microbial population. Tay et al in (2001) reported that under this starvation conditions, the microorganisms become more hydrophobic and tend to aggregate leading to the formation of stronger and denser granules. This assertion is also supported by the work of Bossier and Verstraete in 1996. However, starvation does not always favour granulation as long starvation periods weaken the stability of the granules (Wang et al 2006). This suggests that though starvation may contribute to hydrophobicity, it cannot be proposed as a prerequisite for granulation (Adav and Lee, 2008).

2.3.2.6 Hydraulic retention time

The hydraulic retention time can be explained as the average residence time of the wastewater in the aeration tank. In SBRs, there is a correlation between the HRT and the frequency with which solids are discharged with the effluent through withdrawal. In this reactor, high washout frequencies (short cycle) has the advantage of reducing or eliminating the growth of suspended solids by means of the constant withdrawal. In spite of this, operation of the SBR at very short cycling times results in loss of the active biomass. At very high washout frequencies, the entire sludge blanket may be lost through hydraulic washout resulting in failure of microbial granulation.

It is thus apparent that the SBR should be operated at a short HRT to prevent the growth of suspended solids but also long enough to ensure microbial growth and aggregation. This was exemplified by Tay et al in 2002 in their investigation on the effect of hydraulic selection pressure on the development of nitrifying granules. It was observed that granulation of nitrifying sludge occurred at an optimum cycle time between 6-12hours whiles granulation was absent at higher and lower cycle times. As in the case of settling time, a short cycle time also enhances microbial activity, increases polysaccharide production and cell hydrophobicity leading to formation of granules.

2.3.2.7 Feed composition

Besides the effect of particular substrates on granulation, the presence of positive divalent ions such as Ca^{2+}, Mg^{2+}, Fe^{2+} and Fe^{3+} also contribute significantly to the formation of granules. Mahoney et al in 1987 concluded that these ions can bind to negatively charged groups present on bacterial surface and extracellular polysaccharides to form microbial nuclei. Ca^{2+} ions thus act as a bridge to promote aggregation of the microbial population. Jiang et al (2003) also reported that the addition of Ca^{2+} accelerated aerobic granulation. It was observed in an experiment that Ca^{2+}-induced granulation occurs faster (16 days) as compared to granulation in cultures without Ca^{2+} (32days). The Ca^{2+}-induced granules also exhibited better settling and strength characteristics and very high polysaccharide content. Since polysaccharides play a key role in maintaining the structural integrity of microbial aggregates through the formation of strong and sticky non-deformable polymeric gel-

like matrix, it is apparent that Ca^{2+}-induced granules with more polysaccharide content will exhibit better granule characteristics.

The microbial growth rate is also affected by the pH of the medium. McSwain et al in 2004 reported that oxidation at high OLR produced sufficient CO_2 which reduces the pH in an unbufferd solution. In an experiment with fungi, it was observed that at low pH, fungi grow well and may contribute significantly to the initial granulation because they release protons thereby decreasing the pH further in the reactor. Though granule sizes of 7.0m and 4.8m have been observed under pH 4.0 and 8.0 respectively, the effect of pH on aerobic granulation has not been addressed.

2.4 Characteristics of aerobic granules

2.4.1 Morphology

The shape and size of the sludge obtained in waste water treatment are important physical parameters that are affected by a number of operational conditions. Aerobic granular sludge as its name suggest consist of granules with a clearly defined outlines and nearly or elliptical shape (Gao et al., 2011) unlike floccular. Granular size is also a crucial parameter in the physical characterisation of aerobic granules. The sizes of the granules formed dictate the settling ability, density and intensity. The average diameter of aerobic granules is between 0.2-5mm and that can be attributed to a balance between growth and abrasive detachment due to relatively strong dynamic shear force in aerobic reactors (Liu and Tay 2002). However the optimum size of aerobic granules in terms of mass transfer have been proposed by li et al 2005 to be 0.5mm. Particles less than 4mm in size exhibit better settling abilities unlike larger particles (greater than 4mm). Apparently, the increase in diameter caused the decline in the settling properties of the granules (Toh et al., 2003). The size of the granule is however a function of the operational conditions employed. For example long starvation periods and high shear conditions generate smaller sized granules whereas a high OLR results in the formation of larger granules (Gao et al 2011).

Dulekgurgen et al. (2008a) conducted a study to determine the influence of shear forces coming from different sources on macro-scale morphology of aerobic GAS maintained in a lab-scale anaerobic/aerobic SBR fed with propionate as the sole carbon source. They reported that it was not possible to produce GAS when the shear in the system was too high because of continuous mechanical mixing. After

decreasing this component of the shearing forces, they observed formation of the granules but those were covered by filamentous-outgrowths, resulting in poor granule morphology (shape factor; 0.36) and poor settling properties (SVI; 417 mL/g). Further monitoring of the system showed that the number of filaments decreased in time, granulation proceeded quickly, GAS concentration increased (2100-4300 mg MLSS/L), settling properties improved (SVI of 130-90 mL/g), granules became smoother (shape factor; 0.66) and bigger (3.31-2.42 mm). Based on their observations, they suggested that air-supply needed to provide desired shearing effect can be decreased by applying mechanical mixing, but the balance should consider the results of different trajectories on macro-structural features of granules so as to avoid process instability (Dulekgurgen et al., 2008a).

2.4.2 Porosity

The activity of aerobic granules also depends on the porosity of pore size of the aerobic granules. Functions such as the transportation of substrate into the granule and the utilization of dissolved oxygen are subject to the pore size of the granule. Blockage of the pores in granules prevents the uptake of substrate and dissolved oxygen in the media. Dissolved oxygen concentrations thus increases. Aerobic granules have relatively low porosity compared to floccular sludge. Typical porosity values ranges 0.68-093 for aerobic granules and about 0.95 for floccular sludge (Zheng et al, 2007). Porosity of the size of the granule is also related to the size of the granule. That is, porosity increases with an increase in the size of the granules.

2.4.3 Settleability

The separation of solids from liquid in the settling tank is an important process in wastewater treatment that actually determines the efficiency of the process. This is dependent on the settling properties of the granules. Aerobic granules compared to conventional activated sludge have lower SVI which indicates a higher tendency of solids (granules) to become concentrated during settling. A more concentrated sludge will thus settle faster and ensure a clear solid-liquid separation. Settling velocities of aerobic granules have been observed to be in the range of 30-70m/h which is about 3 times higher than that of floccular sludge (8 – 10m/h) (liu et al 2004). An improvement in the settling properties of aerobic granules ensures a high solid retention in the reactor and ultimately improves the performance and stability of the

reactor. Another advantage of the aerobic granules is the fact that high settling velocities allows for the applications of relatively high hydraulic loads without any significant biomass washout. A high concentration of retained biomass ensures faster degradation of pollutant and relatively compact reactors.

2.4.4 Cell Surface hydrophobicity

Aerobic granulation is a cell to cell self-immobilisation process and so it is apparent that the cell surface hydrophobicity plays a significant role in the aggregation of the cells. The cell surface hydrophobicity has certain correlation with the excess Gibbs free energy where its increase results in a decrease in excess Gibbs energy leading to aggregate of cells. Zheng et al (2005) reported that the cell surface hydrophobicity of aerobic granules is much higher than that of floccular. Mean contact angle values were 35^{o} and 46.3^{o} respectively for floccular sludge and aerobic granules. The granule structure as well as the operating conditions employed is the main factors the influence the cell surface hydrophobicity of the granule. In terms of structure, the outer portions of the granule exhibit higher hydrophobicity than the core. The microbial composition of the granule also determines the degree of hydrophobicity induced. Bacteria-dominated granules are seen to have a greater cell surface hydrophobicity than fungal-dominated granules due to the hydrophobicity of the filamentous forms (Zheng et al 2006). Chemical composition of medium also dictates the degree of hydrophobicity of the granules.

2.5 Extracellular polymeric substances EPS

EPS is an organic metabolic macromolecular polymer that accumulates on the surface of the bacteria. It is a general property of microorganisms living in natural environments and its secretion is observed in both prokaryotic and eukaryotic organisms. Bacteria EPS constitutes a 3-D matrix in which the bacteria are embedded and its secretion is influenced by specific environmental conditions. EPS mainly consist of proteins, carbohydrates, some humic acids, nucleic acids, lipids and other materials (Frolund et al 1996) and it offers a number of functions to the microorganism. The retention of exoenzymes near the cell surface and the building up of organic matter are typical functions of EPS (Fowler et al., 1988). The most common function of EPS is to aid in the formation of a gel-like network that keeps

bacteria cells in a biofilm or attached to a surface. Under some critical conditions however, it is utilised by bacteria due to the biodegradability of the majority of its polysaccharides and proteins. Bacterial EPS is responsible for forming microbial colonies and holding colonies, cell and other particles together. It also promotes the cell to cell recognition and aggregation thus protecting cells against adverse environmental conditions such as turbulence, dehydration and biocides (Wingender et al, 1999).

In granular sludge, EPS which is a sticky hydrated matrix is known to alter the physico-chemical characteristics of the cellular surface. It migrates from different sources and this is demonstrated by its chemical heterogeneity and differences in physiological characteristics. It consists of biodegradable and non-biodegradable components with the biodegradable parts maintained at the core of the granule. The non-biodegradable components occupy the exterior portions of the granule and contribute to the structural stability of the aerobic granule. The composition of EPS can be due to bacteria secretion, lysed cellular components from raptured cell structure, digested substances and materials adsorbed from the environment such as from wastewater. These substances are released at the beginning of the starvation process or as a product of cell growth (Wingender et al., 1999). EPS mainly consist of proteins and polysaccharides which are mainly the products of metabolism. However, the organic compounds absorbed from wastewater can increase the polysaccharide, protein and lipids component of the EPS matrix.

The distribution of proteins and carbohydrates in the EPS has been found to be closely linked to the type of feed supplied. Chen et al (2007) observed that in acetate and phenol–fed reactors, β-D-glucopyranose polysaccharides and proteins concentrated in the core of the granule with the α-D-glucopyranose occupying in the outer shell. However, in the phenol-fed reactors, only the proteins were found in the core whereas the α and β-D glucopyranose accumulated in the outer layers.

Comparatively, the concentration of EPS in GAS far outweighs that in floccular sludge in literature. This notwithstanding, the relationship between granule formation and EPS concentration is still a subject of debate due to contrasting results reported in some studies. The chemical composition and structure of EPS from different sources are somehow different due to the fact that the polymers are produced by different organisms under different nutritional and hydrodynamic conditions (Huber et al, 1999). According to di laconi et al, (2006) proteins are the major component of

EPS and their concentrations seem to increase more than polysaccharides during granulation. The protein content of EPS is therefore recognised to be a major contributing factor in the structural stability of the granule. Other studies have also indicated the dominance of polysaccharide content which supports the contribution of exopolysaccharides to the structural strength of granular activated sludge. Ultimately, EPS contributes to granulation in GAS through its effects on factors such as cell surface hydrophobicity, cell surface charge and bridging properties.

2.5.1 Cell surface hydrophobicity and EPS

Cell surface hydrophobicity contributes to a decrease in excess of Gibbs energy on the surface of cell according to thermodynamic theory, and thus promotes cell-to-cell aggregation. Since granulation is a known cell-to-cell self-immobilisation process, liu et al (2003) suggested that hydrophobicity may play a significant role in granulation through enhanced coagulation as a result of reduced Gibbs free energy. Proteins which have been suggested to make up the dominant component of EPS, with its associated amino acids are more hydrophobic and insoluble. This property of the EPS generates a hydrophilic core and hydrophobic outer shell that facilitates the formation of granules through cell-to-cell binding.

In their study, Dulekgurgen et al. (2008b) investigated the relationship between formation and maintenance of GAS in an acetate-fed lab-scale anaerobic/aerobic SBR and the granulation parameters, namely the %cell surface hydrophobicity and extracellular polymeric substances (EPS). They reported that both % cell surface hydrophobicity and EPS production increased as granulation proceeded, and then decreased when the granules were disintegrated. They also reported that their results pointed to a direct correlation between %hydrophobicity and EPS-composition expressed in terms of the ratio between the extracellular proteins and polysaccharides (ExoPN/ExoPS) (Dulekgurgen et al., 2008b).

2.5.2 Cell surface charge

According to fundamental laws of electrostatics, like charges repel while unlike charges attract each other. Under neutral pH conditions, bacterial cell surfaces are negatively charged and so there is an electrostatic force of repulsion that prevents the aggregation of cells. Cell surface charge therefore plays a key role in the aggregation and stability of bacterial flocs or granules (Goa et al., 2011). The problem of

electrostatic repulsion is subdued by EPS which is known to influence the flexibility of the cell surface, reducing the cell surface charge and thus promoting aggregation (Tsuneda, 2003). This function of EPS is primarily due to its ratio of exoproteins to exopolysaccharides. A higher ratio of proteins to carbohydrates produces a more hydrophobic and less negatively charged cell surface that reduces the electrostatic force of repulsion between cells (Zhang et al, 2007).

2.5.3 Bridging functions

The polymers making up EPS play essential roles in the cohesion and adhesion between bacterial cells and also between cells and substrata. The exopolysaccharides in EPS are known to serve as adhesives that bridge cells to form a three dimensional structure which interact with more cells and with particulate matter. This promotes aggregation of small molecules into larger aggregates. This function of exopolysaccharides in EPS is enhanced by high hydrodynamic shear force which stimulates the production of more ExoPS and hence increased bridging function. It is apparent that development and stability of GAS is associated with hydrodynamic shear force-enhanced production of ExoPS.

2.6 Alginate

Alginates are linear anionic unbranched polysaccharides which is made up of two uronic acid repeating monosaccharide residues; α-L-guluronic acid and β-D-mannuronic acid joined by an α -1, 4 linkage (Christensen,1999). It is obtained mostly from marine brown algae and the composition of its monomers varies widely depending on the algae spices and the season of harvest (Draget and Taylor, 2011). Alginates have been determined to be a block copolymer without a definite or regular repeating unit. The structure of alginates can thus consist of stretches of single M and G blocks only or an alternating MG block structure which is mostly dominant (Figure 2.2). In spite of the limited knowledge on the monomeric composition and distribution, the chemical and physical properties of alginates are dependent on the proportions, distribution and length of these monomeric units (Seviour et al., 2012). The gel forming capacity which is the most important physical property of alginate (Draget and Taylor, 2011) is provided by the GG blocks whiles the MM and MG blocks contribute to the flexibility of the polymer chain (Seviour et al., 2012).

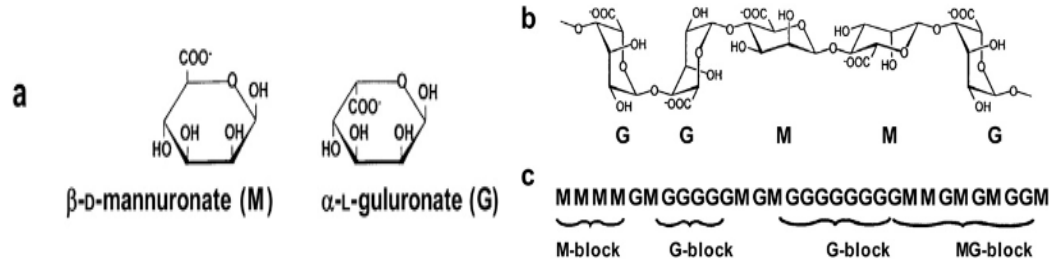

Figure 2.2: Structure of alginate: (a) alginate monomers (b) chain conformation (c) block distribution (Draget and Taylor, 2011).

Hydrogels formed by naturally produced biopolymers or synthetic polymeric substances have a wide application range including serving as scaffolds for tissue engineering, vehicles for drug delivery, actuators for optics and fluidics, and model extracellular matrices for biological studies (Sun et al., 2012 and the references therein). Concomitantly, application of alginates are based on the fact that alginates can absorb water and form very viscous aqueous solutions –in other words "hydrogels"-, they have the ability to form films of sodium alginate on surfaces and uphold significant gel-forming properties when introduced into solutions including divalent/multivalent cations (Fig 2.3). Carboxylates from alginic acidic have the ability to bind with cations which results in a gel-like structure (Lattner et al., 2003).

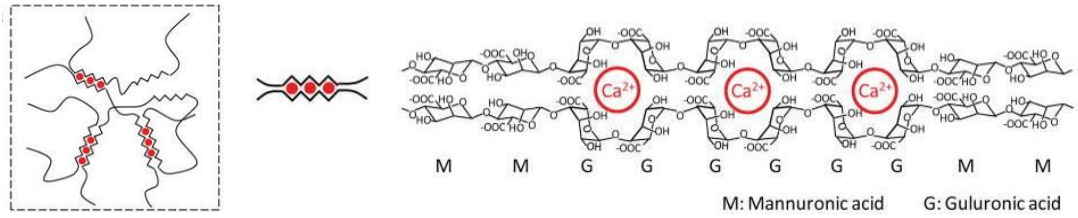

Figure 2.3: Schematic presentation of the chemical structure of and crosslinking in alginate hydrogels: GG blocks at individual polymer fragments align and form an egg-box structure, then crosslink with each other through Ca^{+2} (Sun et al., 2012).

The importance and application of alginate in bio-flocculation of activated sludge was suggested by Bruus et al. (1992) due to the outcome of their experiments where the addition of Na^{+}, K^{+}, Mg^{2+}, and EGTA resulted in the simultaneous extraction of Ca^{2+} with an increase in solution turbidity and a decrease in sludge filterability. The addition of Cu^{2+} however improved the filterability of sludge. It was also observed in the experiment that cation selectivity for $Cu^{2+}$and Ca^{2+}over other cations is a typical characteristic of alginate polysaccharides (Sutherland, 1999).The water absorption capacity of alginates makes is a suitable material as an additive in dehydrated

products. This has been employed in the manufacturing of paper and textiles. It is also used in the food industry for thickening drinks, ice cream, soups and jellies and also as a gelling agent in cosmetics. Alginates have also been widely used in medical and pharmaceutical industries such as the production of drugs and in dentistry. For instance Calcium alginate is used in medical products, such as burn dressings. The advantage in this application is that alginate associated dressings can be removed with less pain than conventional dressings. In biotechnology and medicine the application of alginates are based on biological effects of the alginate molecule, its molecular building blocks, or the characteristic temperature independent gel-forming capacity from solutions in the presence of multivalent cations such as Ca^{2+}. Alginates are therefore highly suitable for the immobilization of biocatalysts such as enzymes and living cells (Draget and Taylor, 2011).

2.7 Brewery wastewater

The brewery industry is an essential economic component of every country with beer actually being the fifth most consumed beverage in the world coming after tea, carbonates, milk and coffee (Fillaudeau et al, 2006). The production of beer involves the steeping of a starch source mainly cereal grains in water and then fermenting with yeast. Associated with the brewing process is the packaging of the finished product and these 2 processes constitutes the main sections of the brewery industry generating large volumes of highly polluted effluent. The brewing process involves the use of large volumes of water. Furthermore, processes such as cleaning of tanks, bottles, machines, and floors also generate high quantities of polluted water which are discharged together with production wastewater as brewery effluent. Beer brewing generates large waste effluents with an estimation of 3–10 L of waste effluent per 1 L of beer (Simate et al, 2011). Brewery effluents contain high amounts of organic matter mainly excess yeast and spent grains from mashing which are the by-products generated during the beer production. These are generated through fermentation and filtering and has high biochemical oxygen demand. Effluents from these processes are however low in volume, contributing about 3% of the total wastewater volume but about 97% to the BOD (Simate et al, 2011). Processes such as bottle washing results in a large wastewater volume, but contributes insignificantly to the total amounts of organics discharged in the brewery effluent.

The discharge of high organic content effluent into water bodies poses serious risk to the environment because of the high oxygen concentrations involved in the degradation. In receiving waters, the bacteria oxidize the organic matter by consuming oxygen from the water. However, the rate of oxygen utilisation by the bacteria is faster than the rate at which oxygen dissolves into the water from the atmosphere. This may lead to anaerobic conditions in the water body with adverse effect on the aquatic life. Hence treatment of brewery effluent is very essential to prevent severe pollution problems.

2.7.1 Brewery wastewater composition

Brewery effluent contains high concentrations of biodegradable organic matter consisting of sugars, soluble starch, volatile fatty acids etc. The chemical oxygen demand (COD) is therefore very high and subsequently with a relatively high BOD/COD ratio of 0.6-0.7 (Driessen and Vereijken, 2003). Organic acids resulting from fermentation reduces the pH of brewery effluent. However, depending on the type of chemical used for cleaning and sanitising, the pH can vary widely within the range of 2-12. Typical chemicals employed are caustic soda, phosphoric acid and nitric acid. Nitrogen concentrations of brewery effluents depends on the quantity of yeast present in the effluent whiles phosphorus is contributed by the type of chemical used during handling of the raw material. Phosphorus-containing cleaning agents and sanitizers increase effluent concentrations. The temperature of brewery wastewater ranges from 25 °C to 38 °C (Goldammer, 2008).

Table 2.1: Typical characteristics of brewery wastewater[a] (Driessen and Vereijken 2003, Simate et al., 2011) and some values reported in Turkey[b] (Dülekgürgen and Karahan-Özgün, 2012).

Parameter	Value[a]	Value[b]
Total COD (mg/l)	2000-6000	4459-5673
Filtered COD (mg/l)		4192-4740
BOD(mg/l)	1200-3600	-
TSS(mg/l)	200-1000	360-760
Temperature °C	18-40	-
pH	3-12	5.2-5.6
Nitrogen (mg/l)	25-80	21-97
Phosphorus (mg/l)	10-50	48-57

2.7.2 Treatment of Brewery effluents

Treatment of brewery effluent may involve a combination of physical, chemical and biological methods. Biological treatment processes however have some inherent advantages over both the physical and chemical processes. Apart from the low investment cost, biological treatment also has high treatment efficiency in terms of BOD and COD removal ranging from 80-90% (Simate et al, 2011). It is apparent that brewery effluent which contains high amounts of organic matter, microbial contaminant and chemicals requires biological methods of treatment. Biological treatment processes can however be employed after the effluent has undergone physical and chemical pre-treatment to remove some of the coarse solid matter. Both aerobic and anaerobic methods of wastewater treatment have been applied in the treatment of brewery effluent but aerobic treatment has been generally applied successfully. In recent times the focus is on anaerobic systems because the production of biogas which is used as fuel to meet the high investment costs for a combined heat and power unit (Simate et al, 2011).

3. HYPOTHESIS

Current knowledge about GAS still falls short of fully explaining its formation, structure and function. Exopolysaccharides (ExoPS) are key component of GAS that have been linked to the structural support of the granule. To understand the formation of the granule and its morphology, extraction and characterization of the ExoPS has become necessary. Based on reviews of other works on ExoPS reported in literature, this work is based on the following hypothesis:

1. ExoPS are also present in Aerobic Granular Sludge treating Brewery wastewater
2. Floccular and Aerobic Granular Sludge ExoPS are Alginate-like exopolysaccharides
3. If so, then hydrogel formation capacity and special chemical composition - providing flexibility and gelation and thus aggregation- of those extracellular polymers confer on the structural properties of activated sludge, especially on that of dense, compact, durable and excellent settling properties of GAS.

According to Lin et al (2010), ExoPS were extracted from GAS treating municipal wastewater. Considering that a sequencing batch reactor mode of operation was employed in the cultivation of the granules, the first hypothesis emerged: that with similar operational conditions, ExoPS could also be extracted from GAS fed with synthetic wastewater and treating industrial wastewater.

The second hypothesis is also based on the results of Lin et al (2010) who concluded that exopolysaccharides extracted from GAS cultivated in a pilot plant treating municipal sewage were indeed like alginates. Through chemical, spectroscopic and electrophoresis characterisation of the extracted ALE, it was concluded that the chemical properties of ALE clearly match that of alginate and thus contribute to the hydrophobic strong and compact structure of GAS.

In this study, the gel-forming capacity of the extracted ExoPS and FTIR analysis would be used to evaluate the similarities between ExoPS and alginate. Further

characterisation will involve Scanning electron microscopy to determine the morphology of the hydrogel beads and UV spectroscopy.

The third hypothesis is based on the conclusions of Leal et al (2008) and of Lin et al (2010). According to these works, the block fractions of Alginate-like exopolysaccharides; heteropolymeric MG blocks, polymeric MM blocks and polymeric GG blocks give much information on the function of the granule. The different amounts of the block fraction observed led to the conclusion that the gel forming capacity and other functions of the ExoPS is related to the ratio GG: GM: MM blocks. Leal et al 2008 observed that the gel forming capacity depends on the presence of GG blocks. The MM and MG functions however contribute to the flexibility of the polymer chain. To confirm this relationship, the property-function relation will be investigated through block fractionation experiments of the extracted ExoPS.

4. MATERIALS AND METHODS

4.1 Lab-scale SBR Set-up and Operation

Floccular and granular sludge used for the experiment were obtained from a lab scale bubble column SBR operated with both aerobic and anaerobic sections and with a total cycling time of 4hours (Fig. 4.1). The reactor was operated in 3 different phases with different feeding regimes and operation parameter (Table 4.1). Filling volume was 1.0L at phase-1 and 1.5L for the second and third phases and the corresponding working height-to-diameter ratio (H/D) was 7.9 and 9.7. The first 2 phases constituted an optimization process for the development of granules. In the first phase (day1-day46), the system was fed with acetate with adjustment in operation parameters to achieve a total cycling time of 4hrs. The 2nd phase (day 46-day 58) also involved acetate feeding and a cycling time of 4hrs with different parameters. The last phase (day58-day199) involved feeding with real WW (brewery WW). Feeding was done from the base of the reactor, slowly and under anaerobic conditions. CAS and GAS were obtained at different stages in the reactor operation for ExoPS extraction (Table 4.1).

Figure 4.1: (a) Column SBR set-up (b) Granules in the column SBR.

Table 4.1: Stages and conditions of Column SBR used for granule formation.

PHASES	Time (days)	Cycle Configuration (min)*								
		T_f	T_{N2}	T_{anaer}	T_{aer}	T_s	T_w	T_{N2}	T_i	T_c
Phase 1										
Acetate/**CAS**	1-46	60	10	70	108	2	60			240
Phase 2										
Acetate/**CAS**	46-58	90	2	92	133	1.5	4	1.5	4	240
Phase 3										
Brewery ww/CAS	58-79	90	2	92	133	1.5	4	1.5	4	240
Brewery ww/GAS	80-199	90	2	92	133	1.5	4	1.5	4	240

*Tf-feeding time, TN2-nitrogen purging time, Tanaer – anaerobic period, Taer – time for aeration, Ts- settling time, Tw- withdrawal time, Ti – idle time, Tc –total cycle time. All values are in minutes.

4.2 Pilot-scale SBRs (R1 and R2)

To determine the distribution of guluronic and mannuronic acid residues (homo-monomeric [-GG-, MM-] and hetero-monomeric [-MG-] blocks) in granular aerobic sludge ExoPS, GAS samples were obtained from two pilot-scale SBRs (28L) operated at Istanbul University. Reactor 2(R2) was fed with raw wastewater from a mixture of food court wastewater and domestic wastewater. Characteristics of the raw wastewater included a COD of 1127mg/l, MLSS of 721mg/l and MLVSS of 616mg/l. NH_4-N and PO4-P were recorded as 44 mg/l and 8.3 mg/l whilst the TKN value was 74mg/L. Reactor 1 (R1) was fed with a mixture of the settled wastewater consisting of a COD of 900 mg/L, MLSS of 203mg/L and MLVSS of 162mg/L. NH_4-N and PO_4-P values for the settled wastewater were 44mg/L and 8.4mg/L respectively and the TKN was 67mg/L. Effluent characteristics of R1 were 87% COD removal, 46% NH_4-N removal and 4% PO_4-P removal. Removal efficiencies of R2 were 86%, 59% and 3.3% for COD, HH_4-H and PO_4-P respectively. R2 GAS samples(23.07.14) used for the extraction of ExoPS and further characterisation had MLSS of 6480mg/L, MLVSS of 6100mg/L, SVI_5 of 160 and SVI_{30} of 150. All of the above mentioned values, as well as the GAS samples, were generously provided by Assoc. Prof. Gülsüm Yılmaz from Istanbul University.

4.3 Extraction of Exopolysaccharides/EPS

The extractions were carried out with two different methods using DOWEX (Frolund et al., 1996) and Na_2CO_3. DOWEX was used to extract the total extracellular polymeric substances (EPS) and Na_2CO_3 was used to extract specifically the Alginate–like exopolysaccharides (ALE). Detailed information on the samples used for EPS/exoPS extractions and the all different types of experiments carried out for their characterisation in are listed Table 4.2.

EPS extraction from floccular and granular sludge samples by DOWEX method were previously carried out by Assoc. Prof. Ebru Dülekgürgen within the framework of a TUBITAK project (Dülekgürgen and Karahan-Özgün, 2012). For that, sludge samples collected from the lab-scale SBR were subjected to extraction in accordance to the procedure proposed by Frolund et al. (1996) with minor modifications (Dulekgurgen et al., 2008b). EPS extracts and supernatant samples obtained from those extractions and stored at -20oC were used in this study to characterize and compare them with the exoPS extracts obtained from sludge samples from the same lab-scale SBR.

Extraction of exoPS with Na_2CO_3 was done following the procedure outlined by Lin et al. (2010). For that, MLSS samples stored at -20^oC were thawed gradually in ice and then under room temperature to obtain a liquid solution of the sample. The samples were homogenized in a vortex mixture before an accurate volume equivalent to 0.5gMLSS was measured into each of two separate falcon tubes. The samples were centrifuged at 9000rpm for 20minutes after balancing the weight on a weighing balance. The biomass obtained was extracted in 80ml of 0.2M Na_2CO_3 at 80^oC (in a water bath) for 1hr. The samples were then centrifuged again at 9000rpm for 20minutes to obtain the supernatant containing the ExoPS. The pellets were thus discarded. The pH of the supernatant solutions was adjusted to 2 using 0.1M HCL. Each sample was then divided into two equal parts to ensure effective centrifugation after which centrifugation was carried out at 9000rpm for 30minutes. The precipitates obtained after centrifugation were washed with di-deionised water until a pH close to 7 was reached. The pellets were then dissolved in 5ml of 0.1MNaOH. The ALE in the supernatant was precipitated with 20ml of cold absolute ethanol to obtain a final concentration of 80% (v/v). The samples were subjected to gentle

shaking and allowed to form precipitate overnight. The resultant solution was centrifuged at 9000rpm for 30 minutes to obtain the Exopolysaccharides. The Exopolysaccharides were washed with cold absolute ethanol and lyophilised.

Table 4.2: List of samples used in ExoPS/EPS extractions and the down-stream applications employed for ExoPS/EPS characterisation.

Time (days)	MLSS (mg/l)	MLVSS (mg/l)	SVI1.5 (ml/g)	SVI30 (ml/g)	Vol. extract (mL)	Vol. Supernat (mL)	Extraction method	Gelation and E-SEM	FTIR	UV-VIS	Total carbs	Block Fractionation
-12	18420	9200	n.a	30	40	n.a	**Na_2CO_3**	√	√		√	
35	15290	10420	48	18	40	n.a	**Na_2CO_3**	√	√	√	√	
46	14600	9870	49	21	2	20	**DOWEX**	√	√	√		
49	6910	4460	111	X	15	n.a	**Na_2CO_3**	√	√	√	√	
52	7260	4980	55	n.a	27.5	n.a	**Na_2CO_3**	√	√	√	√	
64	3090	2070	169	X	1	15	**DOWEX**	√				
68	3124	3267	n.a	n.a	25	n.a	**Na_2CO_3**					
73	4390	3450	142	n.a	n.a	n.a	**DOWEX**	√				
99	8610	5590	10	n.a	15	n.a	**Na_2CO_3**	√	√			
101	7690	4570	12	n.a	n.a	n.a	**DOWEX**	√				
109	4110	3060	28	n.a	7.5	20	**DOWEX**	√		√		
113	6460	4400	26	n.a	15	n.a	**Na_2CO_3**	√	√			
115	4640	3360	36	n.a	10	35	**DOWEX**					
136	5030	3540	15	n.a	20	n.a	**Na_2CO_3**	√	√			
142	2990	1830	24	n.a	20	n.a	**Na_2CO_3**	√	√			
150	7220	3640	14	n.a	17,5	n.a	**Na_2CO_3**	√	√			
192	5540	2340	0	n.a	40	n.a	**Na_2CO_3**	√	√			
199	5810	4490	62	n.a	90	n.a	**Na_2CO_3**	√	√	√	√	
213	n.a	n.a	n.a	n.a	50	n.a	**Na_2CO_3**					
R2*	6480	6100	150	150	2800	>200	**Na_2CO_3**	√	√	√		√
R1*	5110	4600	74	74	800x	>200	**Na_2CO_3**	√	√	√		√

*R2 and R1: Those GAS samples were generously provided by Assoc. Prof. Gülsüm Yılmaz (İstanbul University); from pilot-scale SBRs fed with raw and settled domestic ww, respectively.

4.4 Characterisation of Exopolysaccharides (ExoPS)

4.4.1 UV-VIS Spectroscopic analyses

UV-visible spectroscopy analysis was conducted by recording spectra of the aqueous EPS samples and dissolved exoPS extracts in 190-800 nm wavelength range (UV-Vis Spectrophotometer, Lambda25, Perkin Elmer). Spectrum of sodium alginate was also recorded for comparison.

4.4.2 Total carbohydrate determination

The total carbohydrate in the extracted ExoPS was estimated by the Anthron method which is relatively less sensitive to interferences from other cellular component (Gerhardt et al., 1994). D-(+)-glucose anhydrous (Sigma) was used as standard for calibration. 75% H_2SO_4 solution was prepared a day before the experiment by putting 100ml of di-deionised water in a 500ml volumetric flask with a magnetic stirrer. The flask was then put on an ice bath and placed on a stirring plate. 390ml of 95-97% concentration reagent grade H_2SO_4 was measured into the volumetric flask. The acid was added slowly and carefully and allowed to cool to room temperature under the fume hood. The volume was then adjusted to 500ml with di-deionised water. 250ml Anthron solution was prepared freshly on the day of measurement by weighing 0.5g of Anthron into a small beaker containing about 5ml of absolute ethanol. By rinsing the weighing boat with about 5ml absolute ethanol, the Anthron was dissolved in 10ml absolute ethanol and transferred into a 250ml volumetric flask containing 75% H_2SO_4. The Anthron- H_2SO_4 solution was stirred for complete mixing and the volume adjusted to 250ml. The solutions (Anthron, H_2SO_4) were chilled whiles the samples were thawed and heating blocks set to 100^{o}C.

4.4.2.1 Preparation of glucose dilutions

0.01g of glucose was added to 100ml volumetric flask containing di-deionised water. The solution was stirred to dissolve glucose and the volume adjusted to 100ml. A serial dilution of glucose was prepared from 0mg/l to 100mg/l from which a standard curve was obtained for calibration.

4.4.2.2 Digestion and UV-VIS reading

Standards and samples preparation was done in triplicates and kept on ice. 1ml of each standard/samples was transferred to a COD vial after mixing and placed on ice. 2ml of the already chilled 75% H_2SO_4 solution was added to each tube. The mixture was capped and vortexed to mix. 4ml of already chilled Anthron solution was also added to each tube and vortexed to mix. The COD tubes were placed on the heating blocks to boil for 20-25mins at 100^{o}C. After heating, the tubes were cooled to room temperature whiles the UV-VIS spectrophotometer was turned on to warm up and stabilize. Sufficient amount of the digested samples were transferred to semi micro cuvettes and the absorbance read on the UV-VIS Spectrophotometer (Perkin Elmer, Lambda25) at 578nm. The calibration curve was prepared from the absorbance reading of the standards and the total carbohydrates of the samples in mg glucose/L were calculated from the curve. All reactions were run in triplicate.

4.4.3 Gel formation Capacity of ExoPS extracted from CAS and GAS

To determine the gel forming capacity of ExoPS, the extracted ExoPS was extruded in of $CaCl_2$ solutions in a 1:1 ratio. A commercially available sodium alginate (A2158, Sigma, from brown algae) was also included in the experiments as the reference polysaccharide to enable comparative evaluations.

Design

1. Preparation of 2%,1%, 0.1%, 0.01% sodium alginate solutions
2. Preparation of 0.01% extracted ALE solutions
3. Preparation of ruthenium red stain
4. Staining of sodium alginate and extracted ALE samples
5. Hydrogel bead formation

4.4.3.1 Preparation of ruthenium red solution

0.5ml of di-deionized water was pipetted into a 2.2 mL eppendoff tube and 1.8mg of ruthenium red dye was carefully weighed and added into the tube. 1.3 mL of di-deionized water was added to dissolve the dye in a total volume of 1.8ml. The solution was vortexed for complete dissolution.

4.4.3.2 Staining the hydrogels

To make hydrogel formation visible in the CaCl2 solution, alginate and the extracted exoPS were stained with 1% ruthenium red dye solution. For 2% alginate, 1.95ml of the sample was mixed with 0.05ml ruthenium red stain. 1.90ml of 0.2% exoPS solution was mixed with 1.0ml ruthenium red stain. Supernatant solutions were also treated in a similar fashion; mixing 1.85ml sample with 0.05ml ruthenium red stain.

4.4.3.3 Hydrogel formation

For alginate standard, 0.05ml ruthenium red dye was added to 1.95ml of the alginate. The solution was vortexed very well and transferred into a syringe fixed to a needle. $CaCl_2$ solution with the required percentage concentration was put into a petri dish and the samples carefully extruded through the $CaCl_2$ solution from beneath the surface. Gelation of the extracted ExoPS was also carried out in the same manner with 1.90ml ExoPS solution stained with 1ml ruthenium red dye. Supernatants solutions obtained before extraction were also examined by staining 1.85ml supernatant with 0.05ml ruthenium red dye before extrusion in $CaCl_2$ solution.

Figure 4.2: Preparation of ExoPS and EPS hydrogels in $CaCl_2$ solution.

4.4.4 Scanning Electron microscopy (E-SEM)

The hydrogel beads from sodium alginate and the hydrogels/gel-like structures produced by the EPS/exoPS extracts were examined by E-SEM (Quanta FEG 250 Scanning Electron Microscope). Prior to observation, the samples were dried under a vacuum.

4.4.5 Moisture content

The moisture content of the Ca-ExoPS hydrogels was determined by the gravimetric method through drying the samples at 105^{o}C and recording the loss in weight. For that, 0.45um filters to be used in filtering the samples were dried in an oven at 105^{o}C for 30minutes and cooled to room temperature in and desiccator for 30minutes. The filters were weighed and the weight recorded. The wet samples were filtered through the previously weighed filter by the help of a syringe and needle and the weight of the samples and filter were recorded. The filters together with the samples were dried in an oven for 1 hour at 105^{o}C after which the dried samples were cooled to room temperature in a desiccator for 30mimutes. The dried samples were reweighed to determine the mass of water lost through drying. Moisture content was determined as Percentage moisture and was determined on wet weight basis by the formula.

$$\% \text{ Moisture} = \frac{\text{weight of water}}{\text{wet weight of sample}} * 100 \quad \textbf{(4.1)}$$

Weight of water = wet weight of sample – dry weight of sample

4.4.6 FT-IR spectroscopy

The chemical composition of the lyophilised ExoPS samples and commercially available sodium alginate were investigated by obtaining the FTIR (Fourier Transform Infrared) spectra in the 4000-400 cm^{-1} wavenumber region (Spectrum 100, Perkin Elmer). Data derivation was performed with Microsoft excel 2010. Table 4.3 indicates assigned functional groups of FTIR spectra obtained from literature and against which the observed spectra in this study is compared.

Table 4.3: Examples form the literature on FTIR spectra of GAS-exoPS extracts: wavenumbers and the corresponding assigned functional groups.

Wavenumber (cm^{-1})	**Assigned Functional Groups**	**Reference**
> 3000	O-H stretching vibrations	Lin et al. (2010), Seviour et al. (2012)
2927	Weak C-H stretching	Lin et al. (2010), Seviour et al. (2012)
1000-1200	C-O-C, C-O vibrations	Lin et al. (2010), Seviour et al. (2012)
1654	O-C-O asymmetric stretching	Lin et al. (2010)
1400	O-C-O symmetric stretching	Lin et al. (2010)
1539	C=C pyranose ring	Lin et al. (2010)
1240	O-Acetyl ester	Lin et al. (2010)
1400/1053	Existence of proteins and additional carbohydrates	Lin et al. (2010)
3400-3500, 1560-1630, 1020-1360	Indicate the presence of b-glucosamine and N-acetyl-b-galactosamine residues	Seviour et al. (2012)
1539	Pyranose ring	Lin et al. (2010)
820	C1-H deformation vibrations of **mannuronic** acid residues	Lin et al. (2010), Seviour et al. (2012)
902	Anomeric C-H deformation vibrations of L-**guluronic** acid residues	Lin et al. (2010), Seviour et al. (2012)

4.4.7 Blocks fractionation

The ExoPS extracted from the GAS samples taken from the pilot-scale SBRs treating domestic wastewater were fractionated according to a method described also by Lin et al. (2010). 250mg of ExoPS was dissolved into 9 ml di-deionized water. 1ml of 3.0 M HCl was added to the ExoPS solution and heated at 100C for 1hour. After cooling, the mixture was centrifuged at 9000rpm for 30minutes to obtain a supernatant solution which was neutralized with 1.0 M NaOH. The resultant solution was poured into 80 ml of ethanol to produce a precipitate. The precipitate was dissolved in di-deionized water and freeze-dried to produce the first fraction (F1). The insoluble pellets were dissolved in 5ml 1.0 M NaOH and the pH was adjusted to 2.85 by the addition of 1.0 M HCl. The solution was centrifuged at 9000rpm for 30 minutes and the supernatant fraction was neutralized with 1.0 M NaOH followed by precipitation by the addition of 40ml ethanol to a final concentration of 80% (Vol/Vol), the precipitate was obtained by centrifugation and freeze dried to obtain

Fraction 2. The insoluble pellets were further dissolved by neutralization with 5ml 1.0 M NaOH and precipitated by ethanol. The precipitate was obtained through centrifugation and subsequently freeze-dried to obtain fraction 3(F3).

5. RESULTS AND DISCUSSIONS

According to Lin et al. (2010), GAS is a complex microbial consortium, consisting of different kinds of exopolysaccharides of which some are neutral, polyanionic and polycationic. Due to the interaction of these exopolysaccharides with proteins, lipids metal ions as well as other exopolysaccharides, extraction of all the exopolysaccharides is somehow not feasible. However, specific methods directed at specific exopolysaccharides can be employed to extract these ExoPS based on their chemical properties. Some ExoPS similar to alginate (ALE) have been extracted from GAS treating municipal wastewater. In this work, a similar method was employed to extract such ALE in floccular sludge fed with synthetic wastewater and then GAS treating brewery wastewater. The ability to extract these specific ExoPS gave way for further investigation into its chemical characteristics and role in GAS.

5.1 Yield of Exopolysaccharide Extraction

The yield of the ExoPS extraction method was calculated in this study as the ratio between the mass of the lyophilized exoPS extract to the mass of volatile suspended solids of the biomass sample (mg exoPS/g VSS), and the extraction efficiency as % mg exoPS/mg VSS. From the results provided in Table 5.1 on the outcome of the extraction of ExoPS by the Na_2CO_3, it is apparent that ExoPS-enriching extraction efficiency of GAS samples was slightly higher than those recorded for CAS samples. GAS samples had an extraction efficiency ranging from 2.2% to 6.5% with an average of 4.3% mg exoPS/mg VSS. CAS samples on the other hand had extraction efficiencies varying between 1.5% and 3.8% resulting in an average of 2.3% mg exoPS/mg VSS. Average extraction efficiency for all samples was 3.5% and the average yield was 34.6 mg exoPS/g VSS. However, Lin et al. (2010) reported a Yield value of 160 mg ALE/g biomass (VSS ratio) from the extraction of ALE from granular biomass sample Clearly, comparing the yield values obtained in this study with that reported by Lin et al. (2010) shows that the ExoPS-enriching extraction performed in this study was less efficient. Nonetheless, the method was successful in

extracting the exoPS both from floccular and from granular sludge samples in this study. Moreover, efficiencies varied within a narrow range (1.5-6.5%) indicating the consistency in the extraction procedure.

Table 5.1: Information on and data from exoPS extraction experiments by Na_2CO_3 method: biomass, feed, concentrations, yields and % extraction efficiencies.

Biomass and Feed	**Time (days)**	VSS (mg/L)	Centrifuged volume (mL)	VSS in centrif. volume (mg/mL)	Mass after freeze-drying (mg)	**Yield (mg exoPS/ g VSS)**	**% Efficiency (mg exoPS/ mg VSS)**
CAS+Acetate	d-12	9200	43.5	400	7.6	19.0	1.9%
CAS+Acetate	d35	10420	38.4	400	9.1	22.7	2.3%
CAS+Acetate	d46	9870	15	148	5.6	37.8	3.8%
CAS+Acetate	d49	4460	15	66	1.5	22.4	2.2%
CAS+Acetate	d52	4980	80	398	8.2	20.6	2.1%
CAS+Brewery ww	d68	2376	25	59	0.9	15.2	1.5%
GAS+Brewery ww	d99	5590	15	83	4.2	50.1	5.0%
GAS+Brewery ww	d113	4400	15	66	3.5	53.0	5.3%
GAS+Brewery ww	d136	3540	20	70	4.6	65.0	6.5%
GAS+Brewery ww	d142	1830	20	36	0.9	24.6	2.5%
GAS+Brewery ww	d150	3640	17.5	63	4.1	64.4	6.4%
GAS+Brewery ww	d192	2340	40	93	2.1	22.4	2.2%
GAS+Brewery ww	d199	4490	80	359	8.5	23.7	2.4%
GAS+Brewery ww	d213	[a]n.a.	50		0.9	[a]n.a.	[a]n.a.
GAS+Domestic ww (raw)	R2	6100	2800	17080	1523	89.1	8.9
GAS+Domestic ww (settled)	R1	4600	800	3680	116.5	31.7	3.2%
GAS+Municipal ww	*Lin et al.*	*[b]8000* -				*160*	*11.2%*

[a]n.a.; data not available. [b] mg/L TSS values reported by Line et al. (2010).

5.2 UV-VIS Spectroscopy

UV–VIS spectroscopic analysis of exopolysaccharides is characterized by a maximum absorption in the wavelength area of 200–230 nm. This is mainly due to n–σ* and/or π–π* transitions, which signify the existence of functional groups like amine, carboxyl, carbonyl and ester (Yun & Park, 2003). Another common feature is a shoulder around 260–280 nm which is attributed to the presence of π–π* electron transitions in aromatic and poly-aromatic compounds found mostly in conjugated molecules including proteins (Jia et al., 2007). In figure 5.1, exoPS extracted with Na_2CO_3 and DOWEX showed a similar UV spectrum with that of alginate. In figure 5.1(a), ExoPS from CAS fed with acetate displayed distinct peaks like alginate, specifically between 210nm and 190nm wavelength range. This is in agreement with the results obtained by Lin et al (2010) for alginate-like exopolysaccharides extracted with the same method. Higher concentrations of d-12, d35 and d49 samples however resulted in higher intensities of absorption compared to alginate. The specificity of the extraction method also resulted in the absence of peaks around 250-290nm region which could be the interference of DNA and proteins.

ExoPS (extracted with Na_2CO_3) from GAS fed with brewery wastewater also demonstrated a similar spectrum with alginate. A broad absorbance peak was seen within the 210-190nm range. Figure 5.1(b) illustrates the comparison of GAS ExoPS and alginate. As it is clearly seen in the figure, the higher concentration of GAS ExoPS (51.4mg/L) translates into a peak of greater intensity than that of alginate. The shoulder around 260–280nm which are indicative of proteins or nucleic acid interference is clearly absent in both alginate and GAS ExoPS.

A comparison of the Na_2CO_3-extracted ExoPS from CAS and GAS also revealed similar UV spectra. In figure 5.1(c), CAS samples; day49 and day52 demonstrated peaks similar to that of GAS samples (d199). Peaks were observed in the UV range, specifically in the 210-190nm range like that of alginate.

As exhibited in figure 5.1(d) exoPS and EPS present in the supernatant samples also displayed peaks in the UV region. With a 1:100 dilution, peaks similar to that of the reference alginate were seen as shown. The slight elevation of the supernatant bands within 240 to 290nm region is likely to be the interference of proteins, nucleic acids and humic acids which may be present in the supernatant.

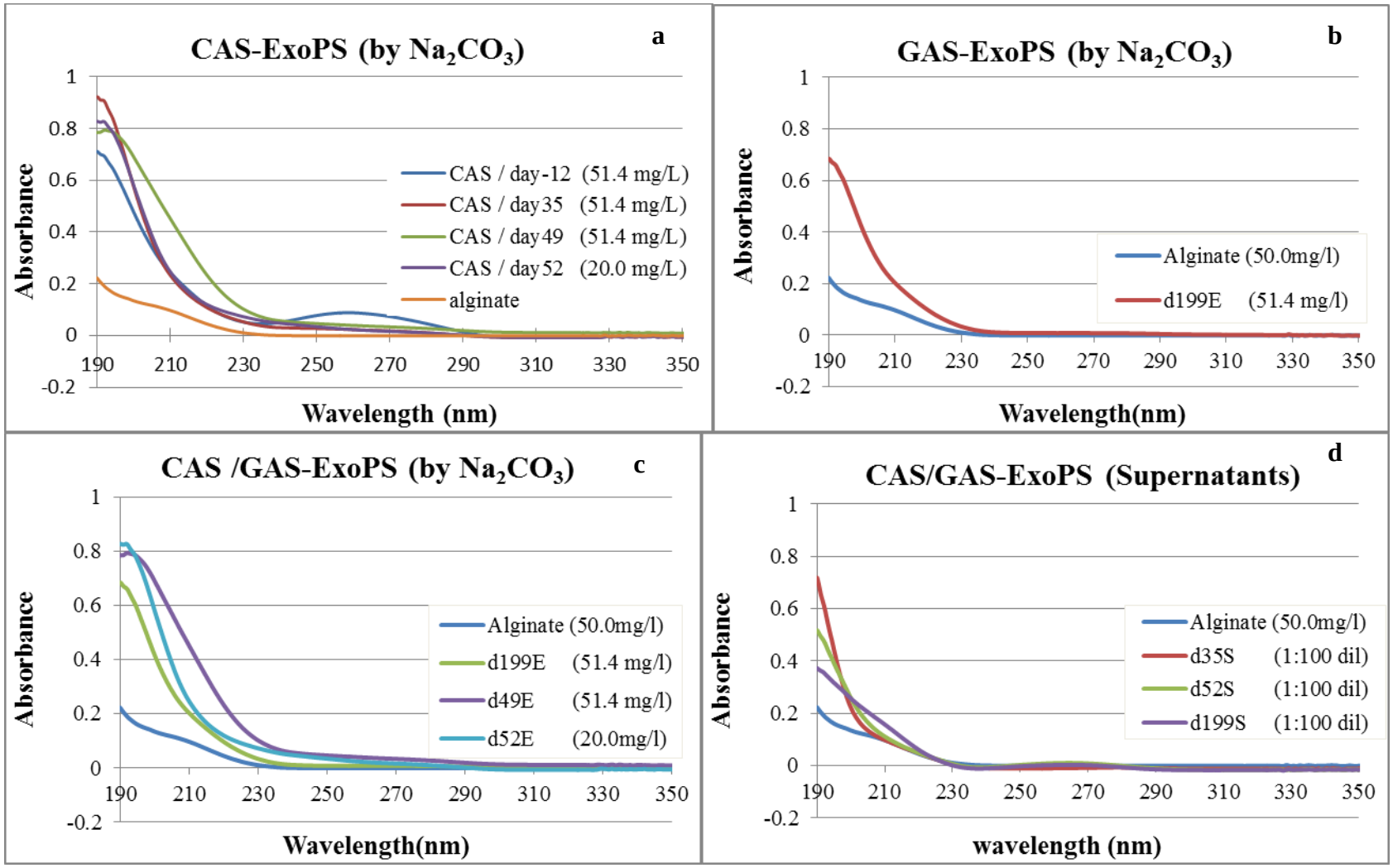

Figure 5.1: Magnified (at 190-350 nm range) UV-VIS spectra of exoPS extracts from (a, c) CAS, from (b, c) GAS, and from (d) supernatants (extraction by Na_2CO_3 method). Floccular sludge (CAS): days -12, 35, 49, 52). Granular sludge (GAS): day199.

Figure 5.2 shows the spectra of EPS extract obtained with the DOWEX method and EPS in the supernatant solution. Similar peaks were observed for both the extracts and the supernatant. However, the absorbance for the EPS in both extracts and supernatant were higher as compared to that of alginate. Since EPS mainly consist of proteins, carbohydrates, some humic acids, nucleic acids, lipids and other materials, the occurrence of a shoulder between 240-290nm for both the extract and supernatant signifies the presence of the proteins and nucleic acids.

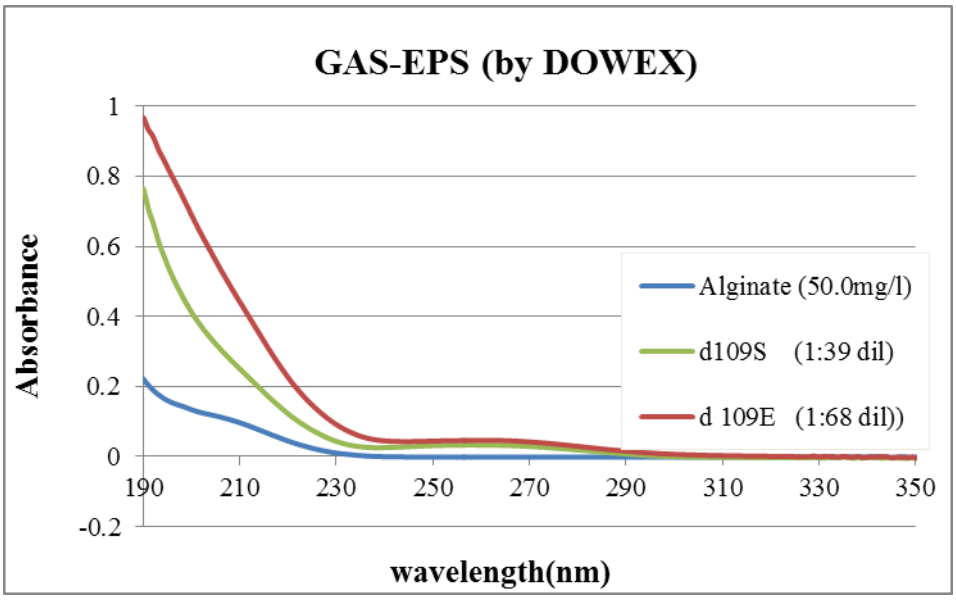

Figure 5.2: Magnified (at 190-350 nm range) UV-VIS spectra of GAS-EPS extract and supernatants (DOWEX extraction).

The presence of the shoulders also demonstrates the differences between the DOWEX extraction method and the Na_2CO_3 method, which is specific for exopolysaccharides.

Figures 5.3(a) shows a comparison of the spectra displayed by EPS extracted with DOWEX (d109E) and ExoPS extracted with Na_2CO_3 (d199E). Both extracts demonstrated peaks similar to that of alginate. DOWEX extract d109E with a dilution of 1: 68 generated the highest peak. 51.4mg/l ExoPS of d199E also showed a peak with higher absorbance than that of the reference alginate. The difference between the 2 methods is demonstrated by the occurrence of the shoulder in the DOWEX-extracted sample.

Similar to figure 5.3(a), the supernatant samples of EPS and ExoPS displayed peaks in the UV range similar to alginate (figure 5.3(b)). 1:39 dilution of DOWEX-extracted EPS (d109E) displayed a peak with higher absorbance than the 1:100

dilutions Na_2CO_3-extracted ExoPS. Both EPS and ExoPS however showed higher absorbance than 50mg/l of alginate. From the UV-VIS analysis, EPS and ExoPS demonstrated similar properties. This gives an indication that the majority of the EPS content may consist of polysaccharides as suggested by Gao et al (2011). As can be seen in figures 5.2 and 5.3(a, b), a slight but broad spike in the EPS graph (d109E/S) indicates the presence of components such as proteins, nucleic acids and humic substances.

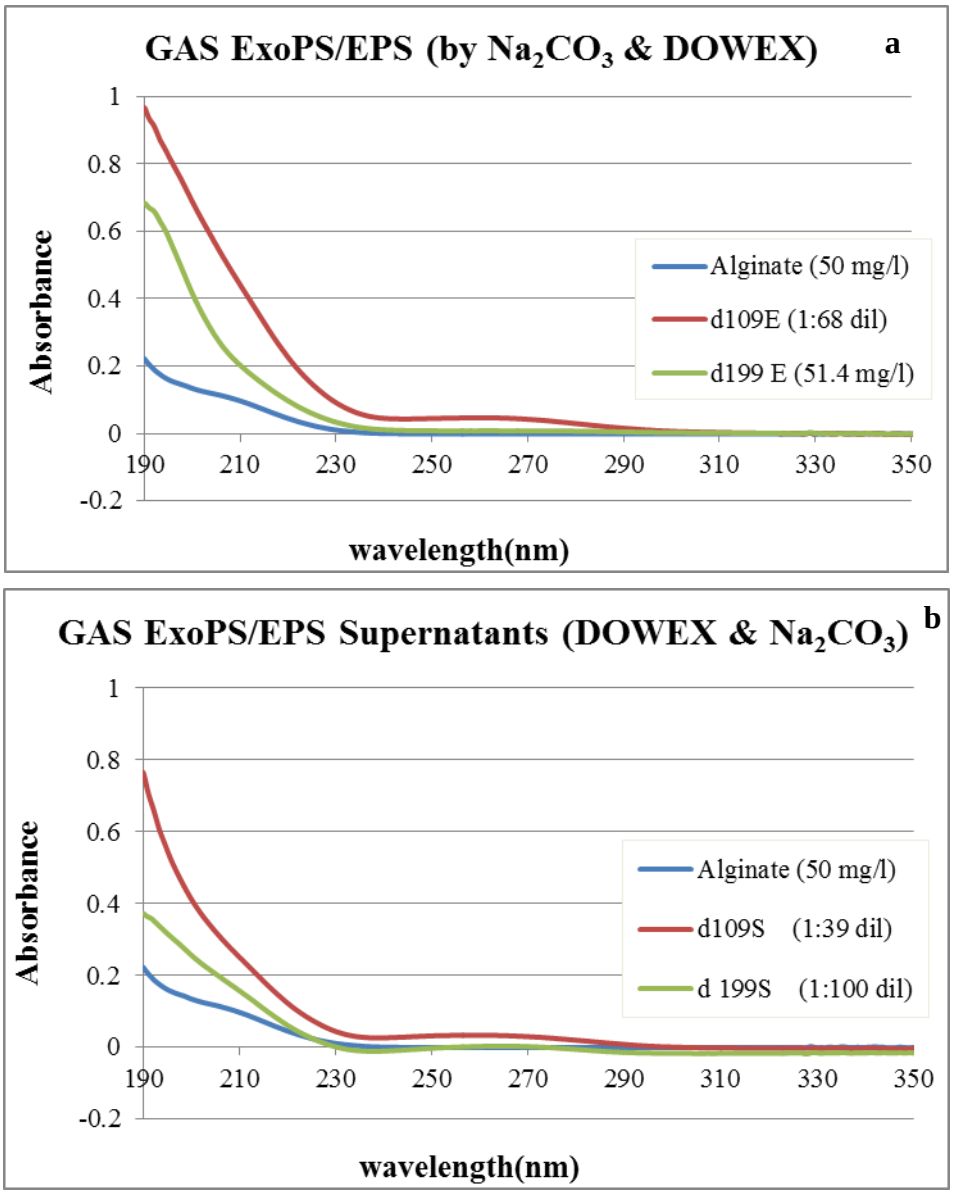

Figure 5.3: Magnified (at 190-350 nm range) UV-VIS spectra of (a) GAS exoPS/EPS extracts and (b) GAS exoPS/EPS in Supernatants (DOWEX & Na_2CO_3).

5.3 Total carbohydrate content of the extracted ExoPS

To measure the total carbohydrate content in terms of glucose, D-glucose anhydrous was used as reference to obtain a standard curve against which the total carbohydrate (measured as glucose) is determined. A standard curve of the different dilutions of glucose is displayed in a linear relationship with a slope of 0.0047 and R^2 of 0.9949 as seen in figure 5.4. With a good linear relationship, the total carbohydrate in both the reference alginate and extracted ExoPS is calculated in table 5.2. Results of the total carbohydrates analysis corroborate earlier results concerning the similarities of both GAS/CAS ExoPS and alginate. The percentage carbohydrate recovery in terms of glucose in 0.2% alginate was about 7.4%. Variable percentage recoveries were obtained for the extracted ExoPS. For instance, granular ExoPS had a percentage recovery of 7.8%. It is also evident in the results that apart from day52 of the floccular ExoPS samples that recorded a high recovery of 16.8%, which is more than twice the percentage recovery of carbohydrates in alginate, the percentage recoveries of carbohydrates in other CAS ExoPS were less than 7.4%. The carbohydrate content of CAS day 35 calculated in terms of alginate was 508mg alginate/g exoPS. This is quite similar to the value of 486.2mg alginate/g ALE reported by Lin et al (2010). The virtually equal levels of sugar in granular exoPS and alginate may suggest the similarities in their properties as demonstrated by other characterization methods such as the FTIR and SEM images. Although carbohydrate measurements were generally low, carbohydrate determination is dependent on the type of standard employed. The use of D-glucose as a standard may underestimate the sugar content by about 62% (Lin et al 2010). This suggests that the actual carbohydrate concentrations in the extracted exoPS and alginate may be in the range of 70-79% for CAS, GAS and alginate.

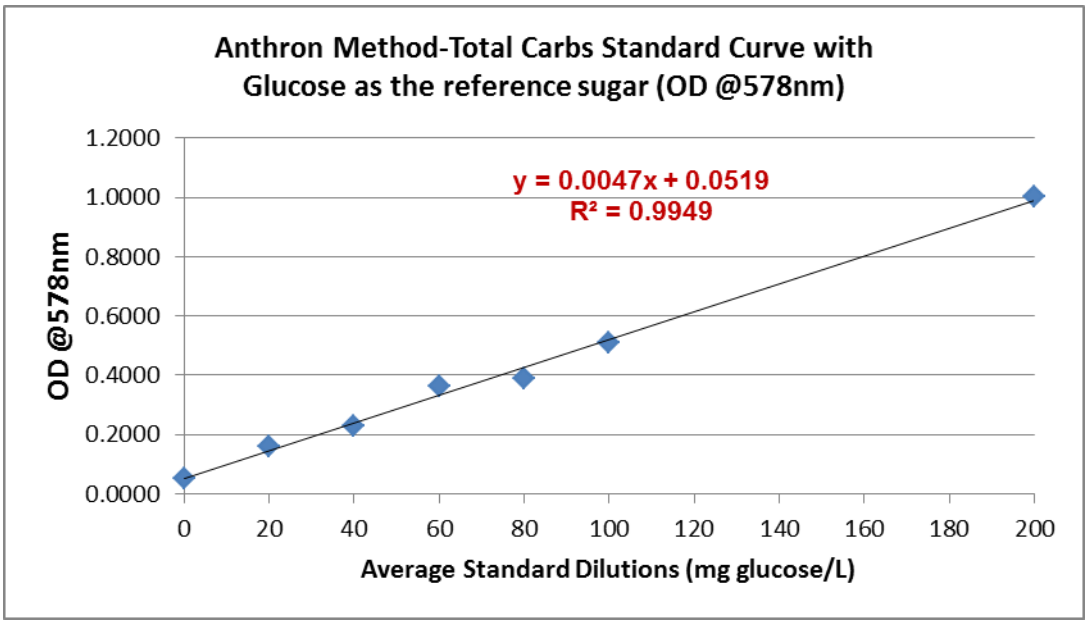

Figure 5.4: Standard curve for total carbohydrates with glucose.

Table 5.2: Total carbohydrate content of alginate/extracted exoPS and the efficiency of recovery by the Anthron method.

Sample ID	Sample Concentration	Carbs content as glucose (mg/L)	% Recovery of Anthron Method	Glucose equivalence; mg glucose/g sample
Sodium alginate				
2% alginate	2%; 20 g/L	1443	7.2%	72.2
1% alginate	1%; 10 g/L	736	7.4%	73.6
0.2% alginate	0.2%; 2 g/L	151	7.6 %	75.5
			Average 7.4%	73.8 mg glucose/g alginate
Lyophilized exoPS extracts after Na_2CO_3 extraction				
day-12	0.2%; 2 g/L	90	4.5	45 mg glucose/g exoPS
day35	0.2%; 2 g/L	75	3.7	37.5 mg glucose/g exoPS
day52	0.2%; 2 g/L	335	16.8	167.5 mg glucose/g exoPS
day199	0.2%; 2 g/L	156	7.8	78 mg glucose/g exoPS
Initial supernatants before Na_2CO_3 extraction				
day-12	-	71	-	-
day35	-	31	-	-
day52	-	69	-	-
day199	-	60	-	-

5.4 Gel formation capacity of alginate and exoPS/EPS extracts

Alginate is a sugar polymer produced naturally by some organisms (e.g., Brown seaweed) and consists of uronic acids residues. The chemical structure of alginate mainly consist of guluronic acid (G) and mannuronic acid (M) monomers which come together to form polymeric chains and are randomly distributed in homo-polymeric and hetero-polymeric blocks (GG, MM and MG blocks respectively). The physical and chemical properties of alginate molecules are determined by the length and distribution of the blocks relative to each other. This is due to the fact that these polymeric blocks have different geometric configurations that dictate the properties of the alginate. For instance, when two GG blocks are aligned side by side, diamond-shaped holes are formed which are able to accommodate divalent cations resulting in cross-linking of the polymers.

Alginate with this chemical structure is capable of forming hydrogels with divalent cations especially in solutions where Ca^{2+} ions are available. The cations induce a cross-linking of the alginate polymers by acting as bridges in an egg-box structure. In general, the GG blocks provide the gelling property, while the MM and MG blocks provide flexibility to the chain (Lin et al., 2010, 2013, Sun et al. (2012). Details of the egg-box structure and the crosslinking with Ca^{2+} are shown in Figure 5.5. Some functional groups that can be identified within the alginate molecule are as follows: CH, -COO, OH, CC, -COC- and -CO- groups.

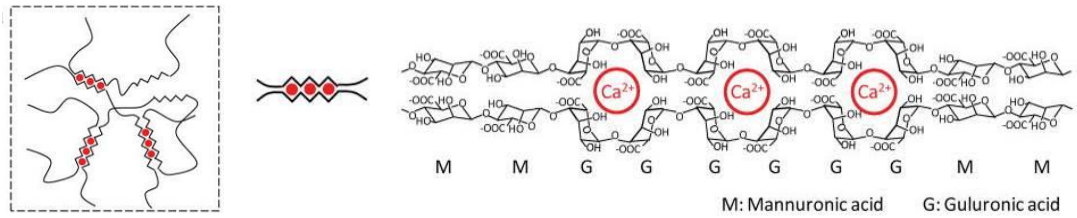

Figure 5.5: Alginate hydrogels: GG blocks at individual polymer fragments align and form an egg-box structure then crosslink with Ca^{+2} (Sun et al., 2012).

Results from the gel forming experiments indicate that the extracted ExoPS as well as EPS could form hydrogels in $CaCl_2$ solution. Gels from commercially obtained alginates were used as a benchmark for comparison with the gel forming ability of the extracted ExoPS and EPS. The gel forming capacity was tested for ExoPS from samples obtained from different phases of the reactor and with Na_2CO_3 and DOWEX extraction methods. The effect of solution concentration was studied with alginate to test whether it is possible to obtain alginate gels with less concentrated solutions. In doing so, a series of different alginate solutions were prepared and introduced to the corresponding $CaCl_2$ solutions (1:1 ratio). From the results, 2% alginate solution formed perfect spherical hydrogel beads in 2% $CaCl_2$ (Fig 5.6a). However, gel forming capacity decreased with decreasing concentration of the alginate as seen in figure 5.6. Concentrations of 0.1% alginate in 0.1% $CaCl_2$ yielded loose and diluted yet still distinguishable aggregates rather than spherical compact gel beads (Figure 5.6b). However, very low concentrations as 0.01% alginate solution could not generate detectable hydrogels.

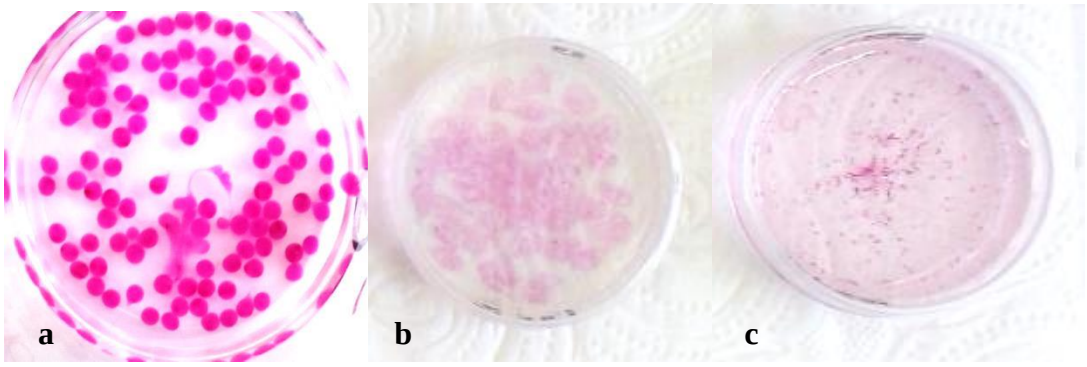

Figure 5.6: Hydrogel formation by alginate (a) 2% alginate: 2% $CaCl_2$, (b) 0.1% alginate: 0.1% $CaCl_2$ (c) 0.01% alginate: 0.01% $CaCl_2$.

ExoPS extracted with the Na_2CO_3 method also demonstrated similar results with alginate in terms of gelation in $CaCl_2$. 0.2% solution of ExoPS from CAS obtained under acetate feeding formed nearly spherical and compact hydrogel beads in a 1:1 ratio with $CaCl_2$. However ExoPS obtained from GAS under brewery wastewater feeding generated very loose and dilute but apparent gels as seen in Figure 5.7 (c). The formation of loose gels could be attributed to the decreasing concentration of ExoPS (d150). As was evident in the reactions of alginate with $CaCl_2$, the decreasing concentration here (1%) was somehow less sufficient in forming compact hydrogels as compared to 2% alginate solutions.

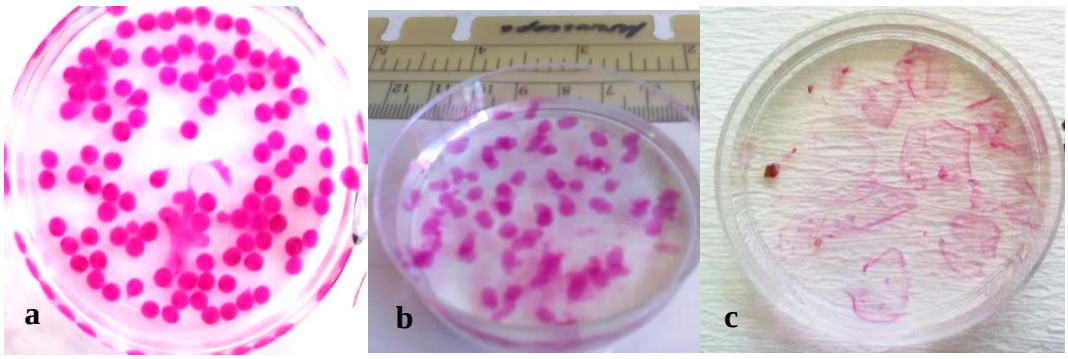

Figure 5.7: Hydrogel formation of (a) 2% alginate: 2% $CaCl_2$ (b) day35: 0.2% CAS ExoPS: 0.2% $CaCl_2$ (c) day150: 1% GAS ExoPS: 1% $CaCl_2$.

AS in the case of extracted ExoPS, EPS extracted by the DOWEX procedure also generated positive results. In figure 5.8, the EPS from CAS obtained under brewery effluent feeding (a), generated gel-like substances which were more dispersed compared to that obtained for GAS (b). The gel-like substances formed in this case (GAS treating brewery wastewater) were more compact and exhibited better gel-forming capacity than CAS. Although perfect spherical hydrogel beads could not be seen, the nature of the gels observed could be due to the low concentration of EPS.

Juxtaposing 0.0014% concentration with 2% alginate solution, it is apparent that the concentration was too low to form good beads in a 1:1 ratio with $CaCl_2$.

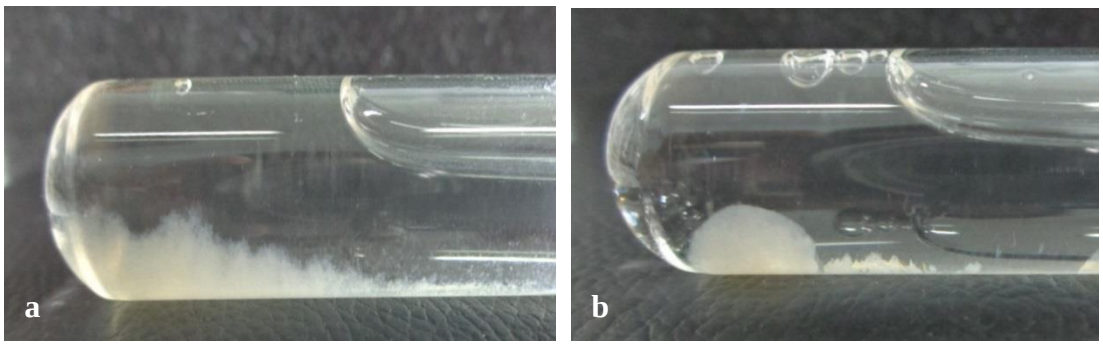

Figure 5.8: Hydrogel formation of (a) day 64: CAS-EPS and (b) day109: GAS-EPS in 2% $CaCl_2$.

Gel formation capacity of the ExoPS in the supernatants of the selected samples in $CaCl_2$ was less effective. Very tiny pellets of a gel-like substance were observed due to the low concentrations of ExoPS. Such gels were however observed under SEM for characterisation of the surface structure.

However, extracted ExoPS of granular samples from pilot scale reactors R1, figure: 5.9 (c, d) and R2, figure: 5.9 (a, b) treating settled and raw domestic wastewater respectively, demonstrated the gel forming capacity with divalent cations similar to alginates. The experiment showed nearly spherical hydrogel beads of 2% ExoPS solution in 2% $CaCl_2$ as can be seen in figure 5.10 and 5.11. The perfect hydrogel formation of R1 and R2 exoPS confirms the observation made by Lin et al (2010) and further gives a clue of why aerobic GAS systems perform better than CAS systems in biomass aggregation, compaction, and settling. Such property also helps to understand the GAS systems that have already been developed into a cost-effective wastewater treatment technology (de Bruin et al., 2004).

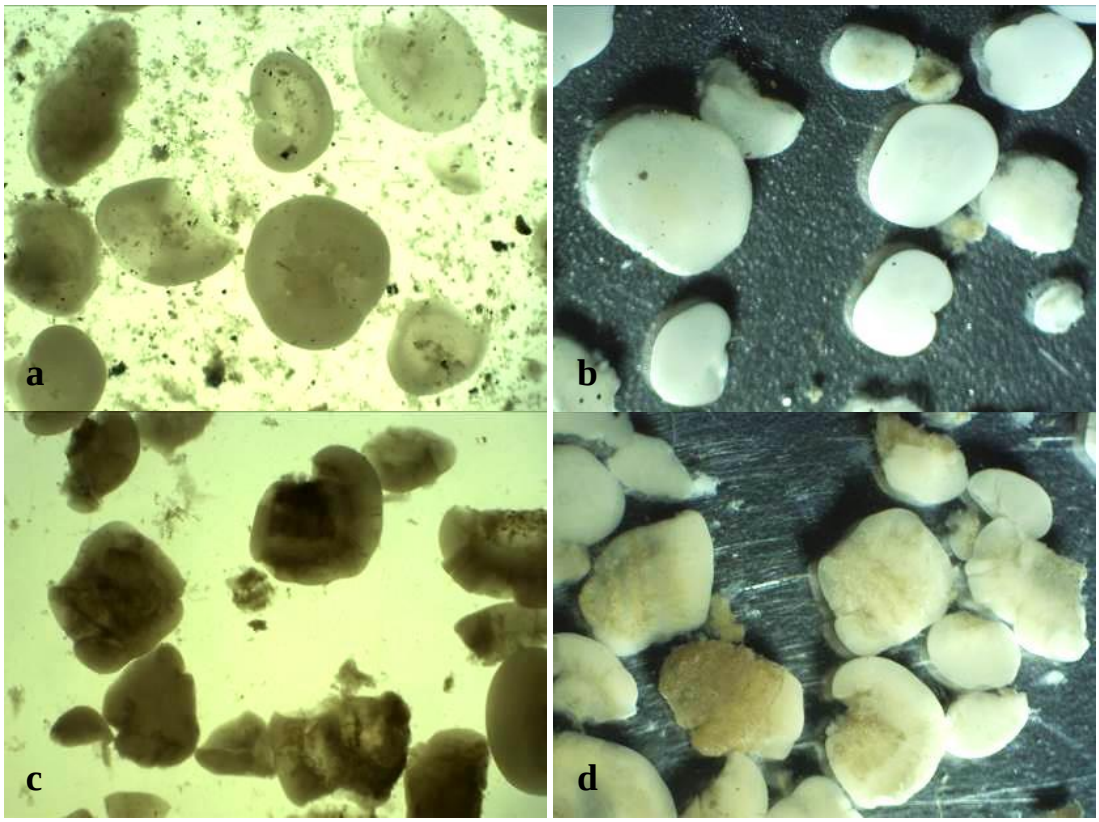

Figure 5.9: Granular sludge samples collected from (a, b) R2 (fed with raw wastewater) and (c, d) R1 (fed with settled sewage). (8x magnification).

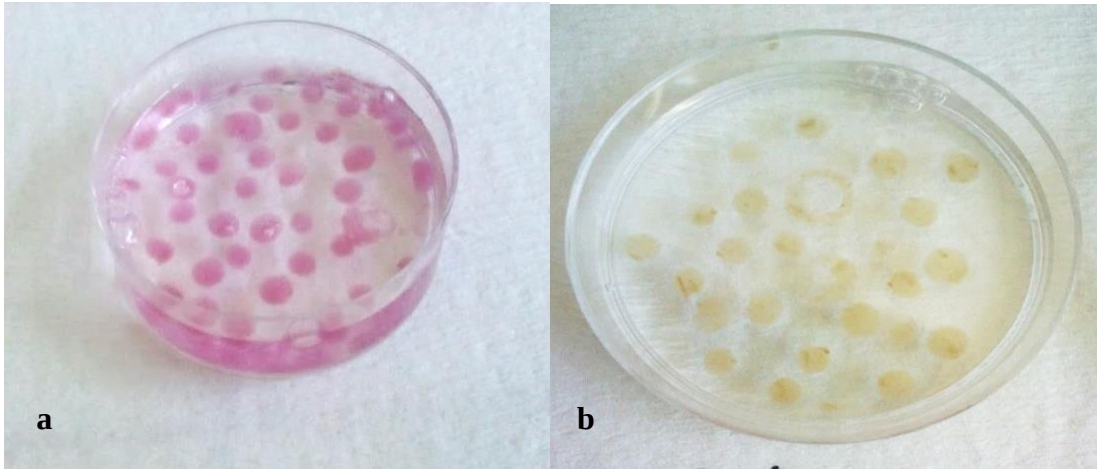

Figure 5.10: Hydrogel formation of (a) 2% R2 GAS-ExoPS: 2% $CaCl_2$ and (b) 2% R1 GAS-ExoPS: 2% $CaCl_2$, with and without ruthenium red staining, respectively.

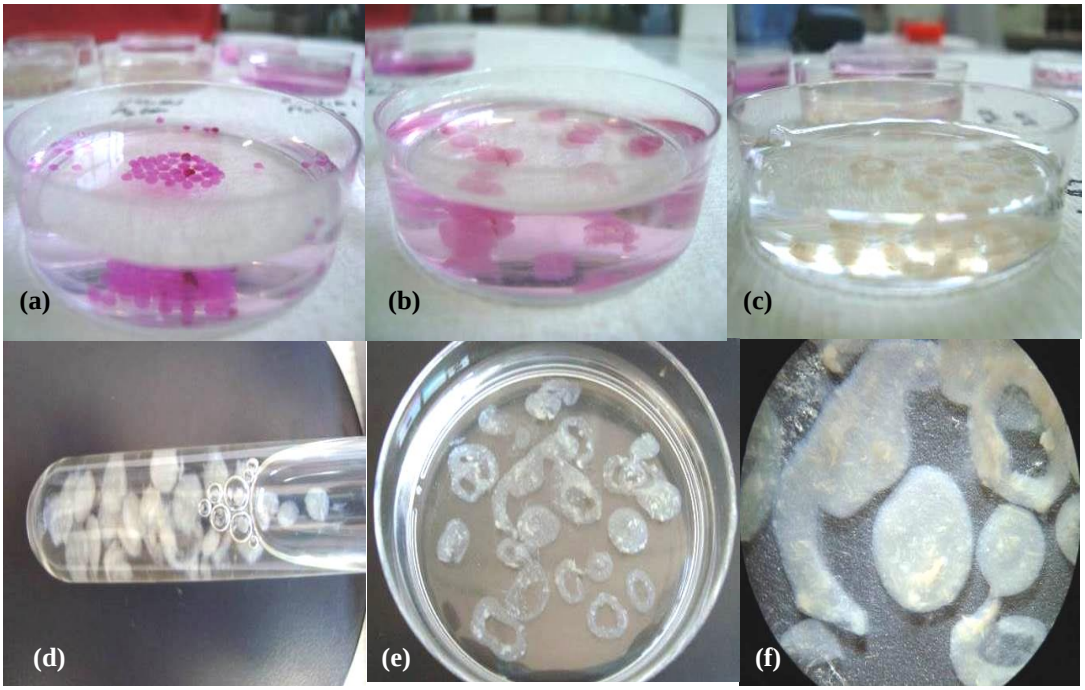

Figure 5.11: Results of hydrogel formation in $CaCl_2$: **(a)** alginate hydrogel (2%; w/v), **(b)** R1-ExoPS hydrogel (1%; w/v), **(c-e)** R1-ExoPS hydrogel (1%; w/v), **(f)** R1-ExoPS hydrogel; 8x magnifications.

5.5 Hydrogel Morphologies as revealed by E-SEM applications

A microscopic analysis of the surface structure of the generated hydrogel beads and gel-like substances from the extracted ExoPS and EPS of both CAS and GAS indicated similarities with the reference sodium alginate. The uronic acid units of alginate contain COO^- groups which are able to form precipitate with ruthenium red dyes (Lin et al., 2010). Hence the ability of the hydrogel beads obtained from ExoPS to be stained with ruthenium red is an indication of the presence of COO^- groups. SEM photos were obtained at both macro scale level to obtain the general surface structure of the samples and also the micro-scale levels for details in establishing the similarities between the extracted exoPS and the reference alginate. The results at both levels indicated similarities between the surface structure of the extracted ExoPS and alginate. As depicted in figure 5.12 (a, b), the surface structure of alginate demonstrates a continuous folded appearance at 500X magnification with some white patches likely to be the bonding regions with the ruthenium red dye. Much detail is revealed at 4000X magnification which shows a sponge-like interconnected network of fibres due to crosslinking with Ca^{2+} ions.

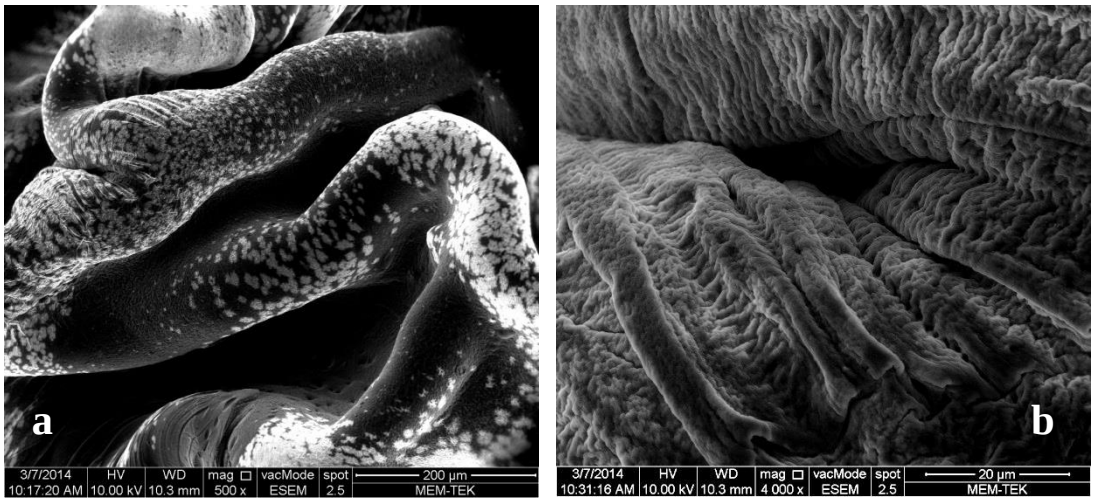

Figure 5.12: SEM images showing surface-structure of 2% alginate hydrogel (a) x500 (b) x4000.

A comparison of the morphology of ExoPS extracted from CAS (with acetate feeding) and GAS (with brewery effluent) with alginate indicate that CAS demonstrated more similar structural properties to alginate. At 4000X magnification, CAS ExoPS shows a more interconnected network of fine fibres like that of alginate as illustrated in figure 5.13(a). The small pellets of hydrogels produced from GAS also illustrated some degree of folding as seen in alginate. Although perfect spherical beads could not be obtained for GAS ExoPS, it is evident from figure 5.8b that there is some crosslinking between the ExoPS and Ca^{2+} ions. It is apparent from the SEM analysis that form ExoPS from CAS and CAS extracts are very similar to alginate in terms of their surface-structural properties.

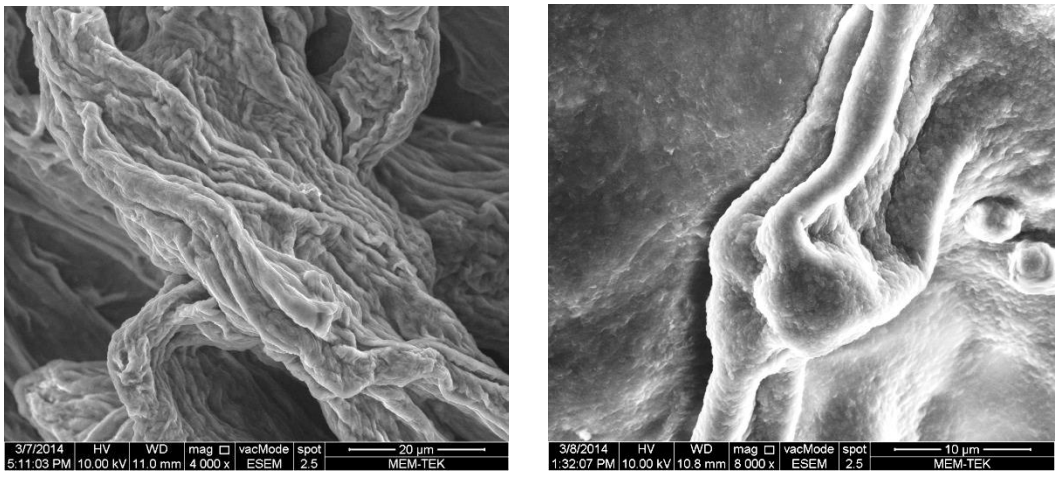

Figure 5.13: SEM images showing surface structure of ExoPS hydrogels (a) CAS d52 acetate x4000 (b) GAS d199- brewery wastewater x4000.

Similarities between alginates and exoPS were also demonstrated at the micro-scale level. Further magnification of the hydrogel surface at x4000 produced quite similar images in both cases. As seen in figure: 5.14, the course surface depicted in alginate is also prominent in ExoPS extracted from aerobic GAS. The folded structure which may occur during crosslinking with divalent cations is also observed for both alginate and GAS exoPS as seen in Figure 5.14 (c, d).

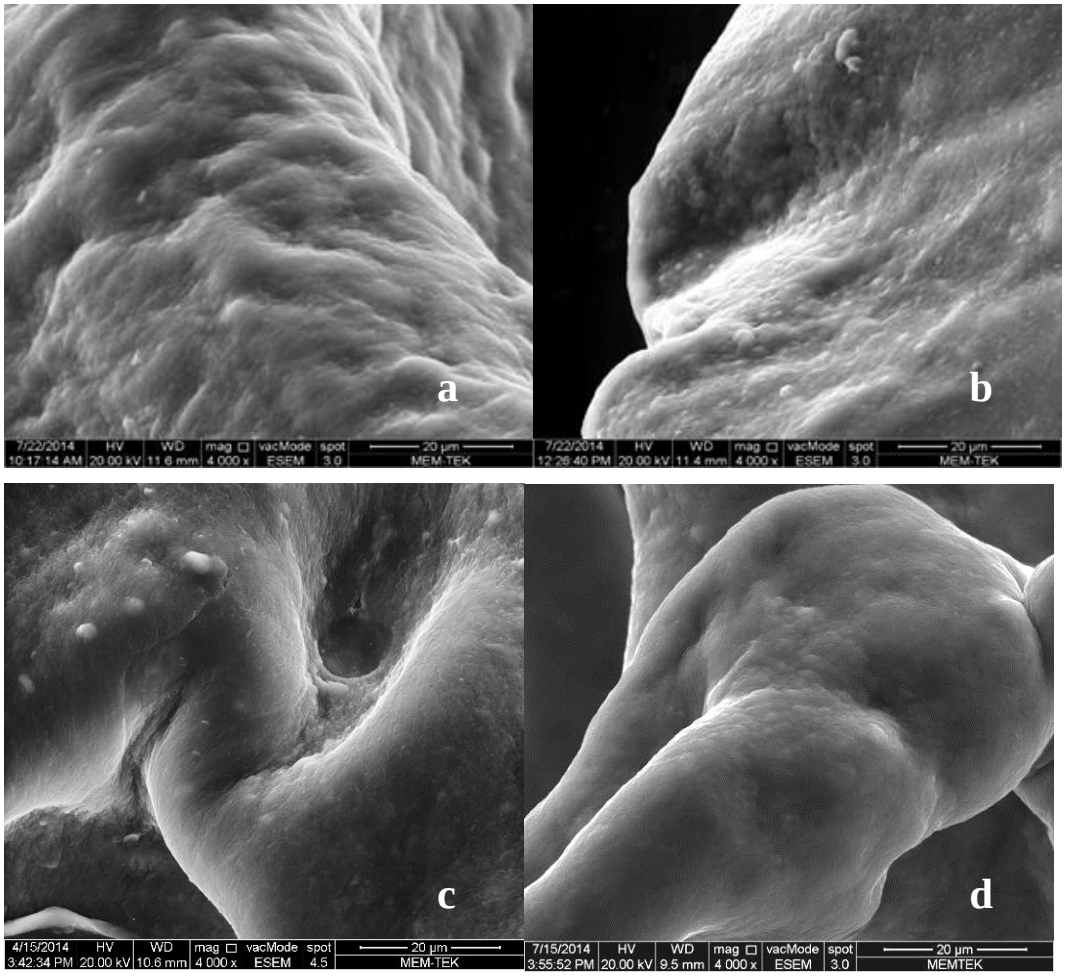

Figure 5.14: SEM images showing surface structure of hydrogels (a, c) 1% alginate (4000x), (b) 1% GAS-exoPS (day150, 4000x), (d) 1% GAS-exoPS (R1, 4000x).

The remnants of ExoPS in the supernatant solutions also behaved similarly to the extracted ExoPS. These ExoPS formed very tiny pellets of gel-like substances in a $CaCl_2$ solution. SEM images of these pellets showed a dense micro structure unlike the cross-linked structure seen in alginate. SEM photos in figure 5.15 show the surface structure of the Ca-ExoPS complex formed in both CAS and GAS supernatants.

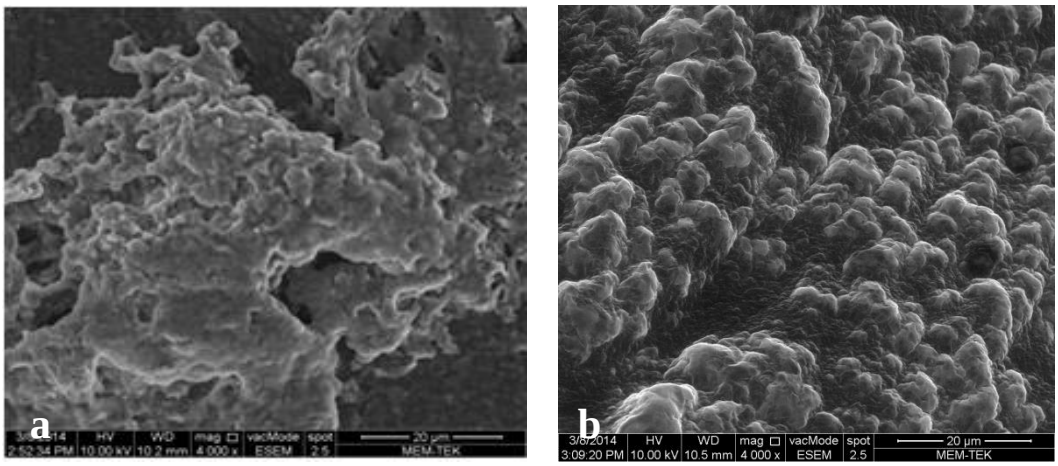

Figure 5.15: SEM images showing surface structure of gel-like structures formed by the supernatants collected before extraction: (a) CAS d52-acetate (4000x) (b) GAS d199- brewery wastewater. (4000x).

The gel-like substances obtained from gelation of EPS extracted by the DOWEX method did not reveal the interconnected network of fibres in SEM as seen for the alginate and extracts from Na_2CO_3 extraction. Likewise the outcome of the gelation experiments, the low concentrations of EPS in the extract also resulted in a more dispersed structure under SEM. Similarly, the dense structure of supernatant ExoPS could also have been due to low concentrations. Drying of the sample prior to observation is a factor that results in collapsing of the sample (Saarai et al, 2013) especially for smaller samples. Hence, it is possible that the structure of ExoPS in small pellets of hydrogel may be susceptible to some level of damage due to drying and thus generate less desirable images under SEM. As is depicted in figure 5.16, the structure of the supernatant EPS/ExoPS might have suffered from damage due to drying.

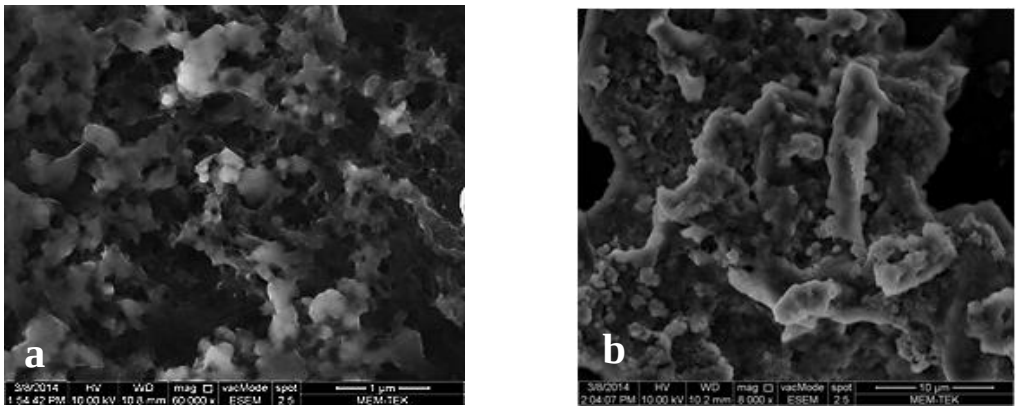

Figure 5.16: SEM images showing surface structure of gel-like structures formed by supernatants collected before extraction with DOWEX (a) with Na_2CO_3 (b): (a) GAS-supernatant (day109), (b) GAS-supernatant (d199).

In spite of the less prominent micro structure of the supernatants hydrogels, the similarities in the structure obtained for CAS and GAS (d52 and d199) extracts and that of alginate clearly indicate that the extracted ExoPS is like alginate.

5.6 Moisture content of ExoPS hydrogels

Hydrogels, like all other polymer gels are insoluble network of polymer chains formed through covalent or non-covalent cross-links. Hydrogels have the ability to absorb and dissipate water or other biological fluids. The crosslinking in such polymer and its ability to absorb water is related to the presence of hydrophilic polymers in the network. Alginate gels are rich in hydrophilic polymers, consisting of more than 90% water and thus share similar properties with hydrogels. According to Lin et al (2013), the moisture content Ca-ALE gels is around 93%. In this study, as indicated in table 5.3, the moisture content of the extracted ExoPS shows an average of 95.8% which is a clear indication that indeed they are hydrogels. Compared to alginate, the moisture content of ExoPS seems to be higher than alginate and this can be attributed to a much less crosslinking with Ca^{2+} which produces a loose network that is able to trap more water than in alginate. Hydrogel from granular sludge treating brewery wastewater had the highest water content of 99%.

Table 5.3: Moisture content of sodium alginate and ExoPS hydrogels.

Hydrogels formed by;	**Concentration (w:v)**	**% moisture content**
Sodium alginate	2%	94
CAS-exoPS (day-12)	0.2%	94
CAS-exoPS (day35)	0.2%	98
CAS-exoPS (day49)	0.2%	95
CAS-exoPS (day52)	0.2%	93
GAS-exoPS (day199)	0.2%	99
GAS-exoPS (pilot-scale SBR, settled sewage)	2%	96
CAS-ALE (Lin et al., 2012)	n.a	93
GAS-ALE (Lin et al., 2012)	2%	93

5.7 FTIR analysis

5.7.1 FTIR spectra of the exoPS extracted from CAS and GAS from lab-scale SBR fed with acetate or brewery wastewater

FTIR is a technique that has been used extensively in providing more information on the chemical composition of compounds. Results from this analysis further strengthened the similarities between the extracted ExoPS and alginate. The spectra of ExoPS from different stages of granule development all indicate characteristic functional groups synonymous with the functional groups in alginate. For the reference alginate, as seen in figure 5.17a, a characteristic broad stretching peak of hydroxyl group as well as a weak C-H stretching peak of methyl groups which are typical polysaccharide bands were observed at 3258cm^{-1} and 2914 cm^{-1}. According to Bramhacharri et al (2007), the presence of carbohydrates is indicated by peaks within 1000-1200 cm^{-1} wavelength which are specific for the C-O-C and C-O functional groups. This assertion is confirmed by the display of peaks at 1027cm^{-1} for alginate. Signals of mannuronic and guluronic acid residues were observed at 882cm^{-1} and 942cm^{-l} respectively. The peaks observed at wavelengths 1597cm^{-1} and 1407cm^{-1} have been suggested to be the asymmetric and symmetric stretching vibrations of O-C-O associated with carboxylic acid salts (Lin et al 2010, 2013, Silverstein et al 1991). This functional group is proposed to be specific to ionic bonding (Wong et al., 2002).

The spectrum of floccular sludge fed with synthetic wastewater (acetate-day49) seen in figure 5.17b showed the core polysaccharide bands at 3273cm^{-1}, 2973cm^{-1} and 1046cm^{-1}. As compared to alginate however, the band at 3273cm^{-1} corresponding to OH stretching becomes slightly narrower with a higher intensity of absorption. This could be as a result of an increase in carbohydrate concentration. The signature peaks of mannuronic and guluronic acid residues slightly shifted towards higher wavelengths at 897cm^{-1} and 964cm^{-1} respectively. Asymmetric and symmetric stretching of carboxylate O-C-O vibrations was observed at 1643cm^{-1} and 1429cm^{-1}. Some peaks specific for the extracted ExoPS but that could not be identified with any functional group were also seen at wavelengths 1530cm^{-1}.

Besides CAS fed with acetate, ExoPS was obtained from and intermediate stage of the reactor where CAS is fed with brewery wastewater. FTIR analysis of this sample

also indicates similarities with the reference alginate used. From figure 5.17c, CAS treating brewery wastewater (day 68) also demonstrated peaks at 3281cm^{-1}, 2981cm^{-1} and 1012cm^{-1} which signify the presence of polysaccharides.

A distinct peak of the mannuronic acid groups was also evident at 836cm^{-1}. However the signature peak of guluronic acids which appeared at a wavelength of 948cm-^{1} in alginate was not clearly seen. It is possible that this band could have been shielded or mixed with the intense band at 1012cm^{-1} (specific for the C-O-C and C-O stretching vibrations) in a similar fashion suggested by Lin et al (2010) about the absence of amide III, II, I and A vibrational modes of proteins which were supposedly obscured by bands of O-acetyl group, C=C in pyranose ring, -COO, and -OH in similar wavelength ranges.

FTIR of granular aerobic sludge obtained during brewery wastewater feeding also demonstrated similar results like CAS and alginate; illustrating the presence of polysaccharide bands at 3273cm^{-1}, 2943cm^{-1} and 1046cm^{-1}. The signature peaks of mannuronic and guluronic acid residues were observed at 879cm^{-1} and 902cm^{-1} respectively. Asymmetric and symmetric stretching of carboxylate O-C-O vibrations were also observed at 1643cm^{-1} and 1429cm^{-1}. A less distinct peak was also seen at wavelengths 1237cm^{-1} as shown in figure 5.17d. Unlike alginates, GAS displayed a broader band at 3273cm^{-1} and 2943cm^{-1} with fairly low absorption intensities. On the contrary, as is depicted clearly in the spectrum, the band corresponding to symmetric stretching of carboxylate O-C-O vibrations showed high absorption intensity (more O-C-O groups) which may signify increased ability of GAS to interact with other polymers or ions hence increasing rate of aggregation, compaction and settling. The difference in the quantities of functional groups depicted in the spectra can probably be due to the different sources of the polysaccharides. In spite of these differences, the chemical composition remains nearly the same thus exhibiting similar physical and chemical characteristics.

Aqueous samples of EPS and ExoPS analysed with FTIR also generated spectra quite different from that of the solid samples but with some absorption peaks of significance. For GAS EPS (extracted by DOWEX) spectra illustrated in figure 5.18a, a single polysaccharide-indicative band at 3323cm^{-1} and an asymmetric stretching O-C-O vibration at 1634cm^{-1} were observed. The supernatant EPS of these samples (figure 5.18b) also generated similar bands at 3320cm^{-1} and 1634cm^{-1}. The

absence of distinct peaks representing symmetric O-C-O stretching vibrations which are mainly the interacting groups in polysaccharides may explain why extracts from the DOWEX procedure formed weak gels in $CaCl_2$ solutions.

Aqueous samples of GAS ExoPS extracted with Na_2CO_3 and their supernatants also generated few distinct bands when analysed with FTIR. As displayed in figures 5.18c and 5.18d, the presence of polysaccharides was indicated by only a single broad band at 3321cm^{-1} for the extract and 3323cm^{-1} for the supernatant. Asymmetric stretching O-C-O vibrations were observed at 1634cm^{-1} for both the extracted exoPS and their supernatant.

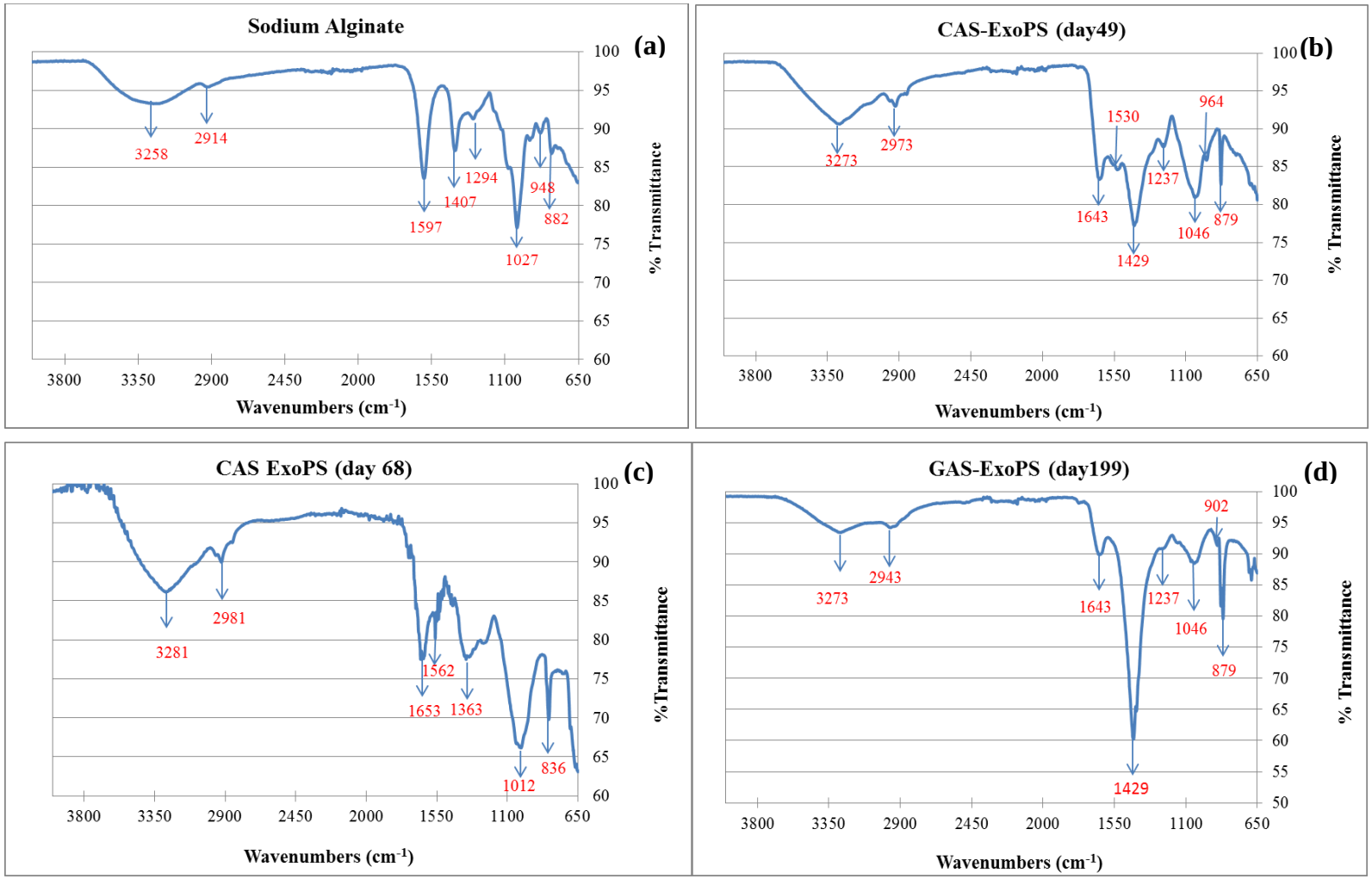

Figure 5.17: FTIR spectra of (a) sodium alginate (b) CAS ExoPS extract-day 49 (c) CAS ExoPS extract-day 68 (d) GAS ExoPS extract- day 199.

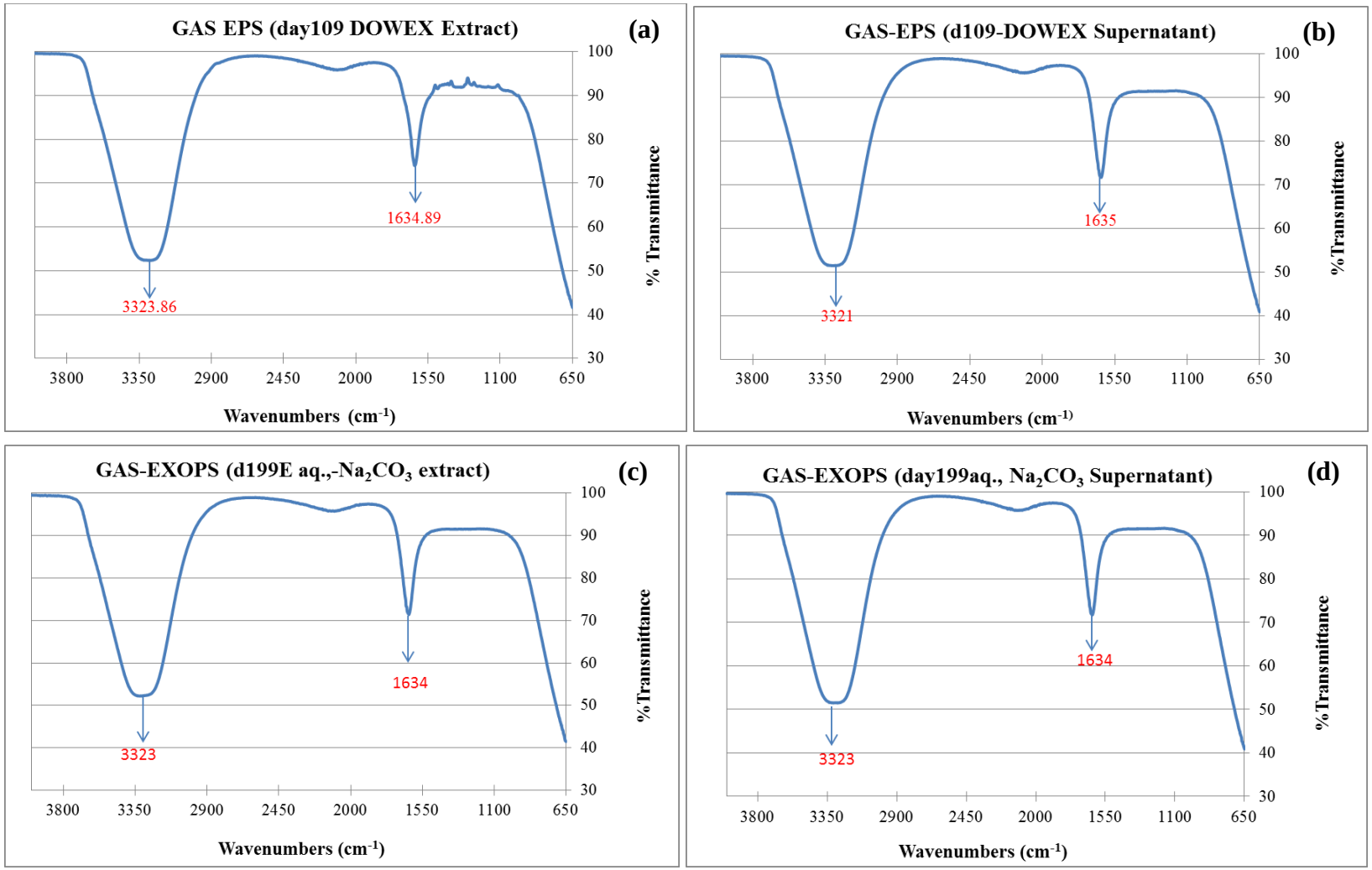

Figure 5.18: FTIR spectra of (a) GAS EPS- day109 Extracts (b) GAS EPS-(day 109 supernatant) (c) GAS ExoPS (day199 aq., extracted with Na_2CO_3) (d) GAS ExoPS (day 199 aq., supernatant).

Table 5.4: Summary of some of the FTIR spectroscopy results obtained for the exoPS/EPS extracted from the CAS and GAS collected from the lab-scale SBR in comparison with alginate and with some examples from the literature.

References/ **Samples**	**Wavenumbers (cm^{-1}) and assigned functional groups (A to I)***	**A**	**B**	**C**	**D**	**E**	**F**[a]	**H**	**I**
Leal et al. (2008)[a]	**Sea-weed pure culture alginic acid**	>3000 peak at 3428	2927	1616	1415		1125, 1091, 1035	**949**	**888 / 820**
Lin et al. (2010) [b,c]	**GAS-exoPS (municipal ww)**	>3000	2927	1654	1400	1240	1000-1200	**902**[b]	**820**[c]
This study	**Alginate (A2158, Sigma)**	>3000 peak at 3258	2914	1597	1407	1294	1027	**948**	**882**
This study	**CAS-exoPS (day49, acetate)**	>3000 peak at 3273	2973	1643	1429	1237	1046	**964**	**879**
This study	**GAS-exoPS (day199, brewery ww)**	>3000 peak at 3273	2943	1643	1429	1237	1046	**902**	**879**
This study	**GAS-EPS (day109, brewery ww) (*DOWEX extract)***	>3000 peak at 3324	-	1635	-	-	-	-	-
***Assigned functional groups (A to I)**	A: O-H stretching vibrations, B: C-H stretching vibrations, C: O-C-O asymmetric stretching, D: C-OH deformation vibrations and/or O-C-O symmetric stretching vibrations, E: O-acetyl ester, F: C-O-C and/or C-O vibrations, **H:** C-O stretching vibrations of **guluronic** acid residues, **I:** C1-H deformation vibrations of **mannuronic** acid residues / Characteristic peak for mannuronic acid								

[a] Values reported by Leal et al. (2008) for C-O stretching of pyranose rings, C-O and C-C stretching vibrations of pyranose rings, and C-O stretching vibration of uronic acid, respectively. [b, c] Second derivative values reported by Lin et al. (2010) for anomeric C-H deformation vibrations of L-guluronic acid residues and for C1-H deformation vibrations of mannuronic acid residues, respectively.

Aqueous samples of CAS ExoPS extracted with Na_2CO_3 and their supernatants also generated similar results as that of GAS. The typical polysaccharides identity was shown by a single band at 3320cm^{-1} and 3319 cm^{-1} for the extract and supernatant respectively (figures 5.19a and 5.19b) Asymmetric stretching O-C-O vibrations were observed at 1634cm^{-1} for both the extracted exoPS and their supernatants. In almost all aqueous samples, peaks were observed at nearly the same wavelength and with identical intensities. The similarities in the spectra produced by all the aqueous samples are indicative of a probable defect in the method of analysis in relation to the nature of the sample.

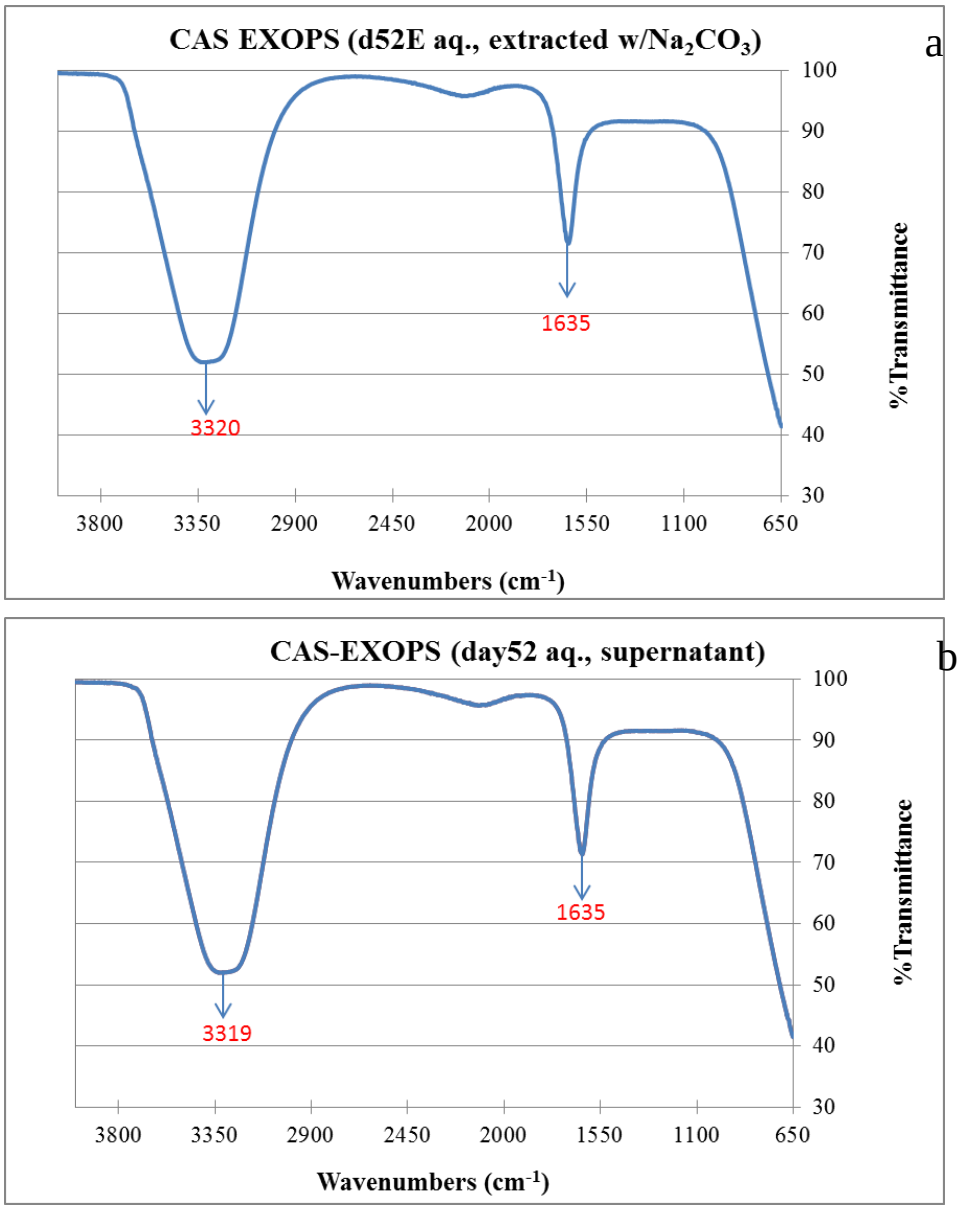

Figure 5.19: (a) CAS ExoPS (day 52 aq., extracted/Na_2CO_3) **(b)** CAS ExoPS (day 52Extracts).

Activated sludge consists of a complex array of microorganisms producing different kinds of exopolysaccharides. However, due to the high level of resemblance between the extracted ExoPS and Alginate, it can be inferred that the bulk of the aerobic granular sludge consist of alginate-like exopolysaccharides. EPS extracted with the DOWEX method also showed some resemblance with alginate in terms of gelation but its FTIR results only displayed 2 distinct peaks representing the hydroxyl groups and O-C-O asymmetric stretching vibrations of carbohydrates. This could be due to the aqueous nature of the samples which might have inhibited the display of less distinct bands. Furthermore, the FTIR results give more insight to the reaction of ExoPS with Ca^{2+}. The evidence of carboxyl groups which may serve as binding sites

for divalent cations (Bramhacharri et al 2007) supports the formation of gels by ExoPS in $CaCl_2$ solution.

5.7.2 FTIR spectra of the exoPS extracted from GAS from pilot-scale SBRs treating domestic wastewater

To understand the role of ExoPS in the function of granular sludge technology and the similarity with alginate, special attention was paid to ExoPS from GAS obtained from R1 and R2 fed with settled domestic wastewater and raw wastewater respectively. Figure 5.20b depicts the FTIR spectrum of R1 GAS ExoPS which is similar to the spectrum of alginate. Clearly, this is a polysaccharide with its identifying bands at 3281cm^{-1}, 2922cm^{-1} and 1051cm^{-1}. The bands within the range of 3500cm^{-1} -1000 cm^{-1} were almost the same as alginate. In the anomeric region, mannuronic and guluronic acids residues which are the signature peaks of alginates are indicated by bands at 839cm^{-1} and 959cm^{-1} respectively.

The spectrum of GAS ExoPS treating the raw wastewater (figure 5.20a) also displayed bands characteristic of alginate. Although very distinct peaks could not be generated in the 3500-1700 cm^{-1} wavelength region, the distinguishing peaks of polysaccharide; 3254 cm^{-1}, 2969 cm^{-1} and 1041 cm^{-1} were however not obscured. A peak at 958 cm^{-1} which was not distinctly resolved but visible, clearly represents the guluronic acid residues of uronic acids. Signals of mannuronic acid residues which were seen in alginate at 882 cm^{-1} was also present at a reduced wavenumber of 878 cm^{-1} in R2 ExoPS. The existence of the characteristic peaks of uronic acids (mannuronic acid and guluronic acids) gives evidence that the extracted ExoPS from R1 and R2 are like alginates.

Though similarities exist between the extracted ExoPS and alginate, one exception was that in alginate's spectrum, the peak for the O-C-O asymmetric stretching vibrations was at 1597 cm^{-1}. This was attributed to using a particular type of commercially available sodium alginate (A2158, Sigma) having a particular chemistry and exhibiting probable slight differences with the exoPS compared, as also highlighted by Leal et al (2008).

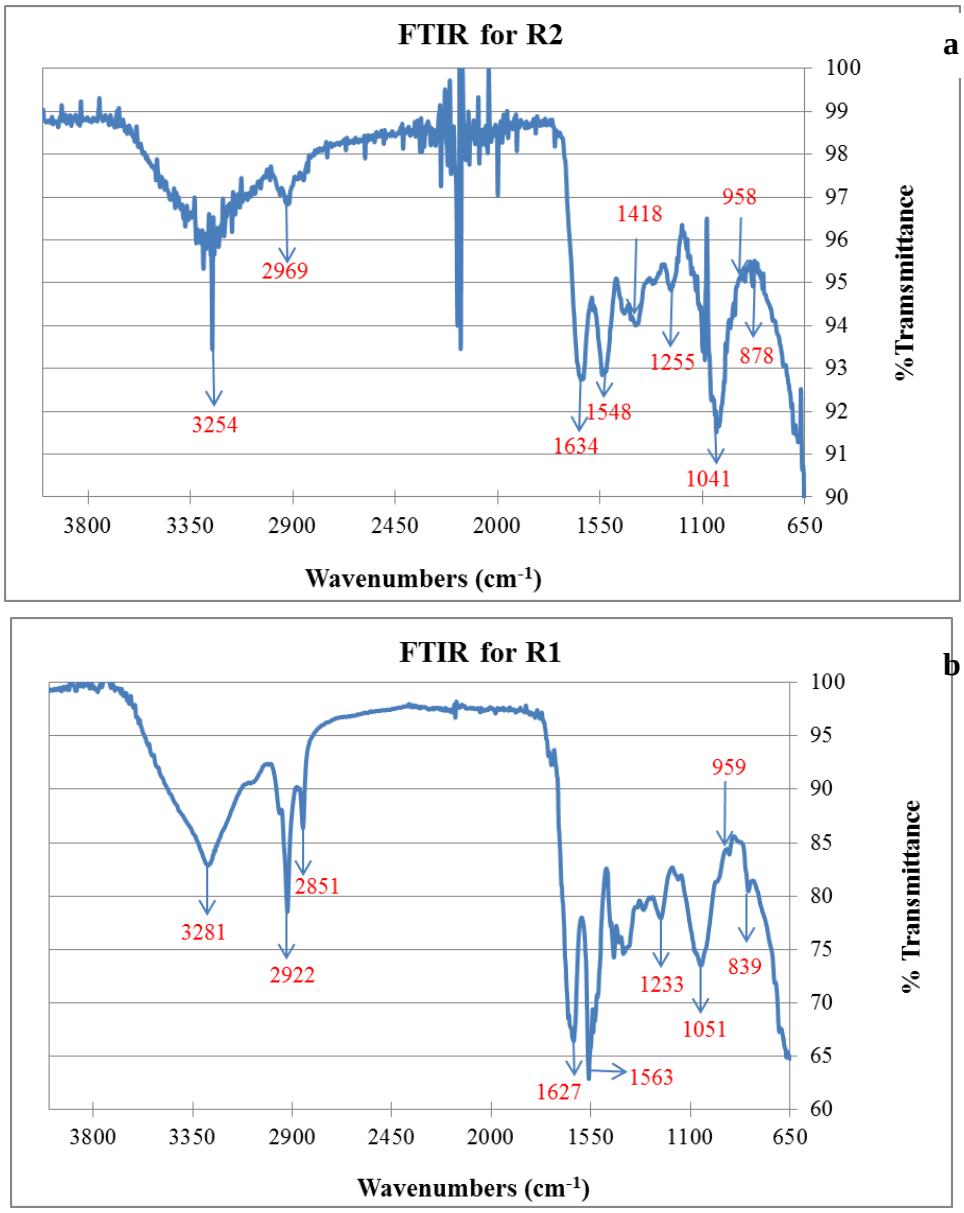

Figure 5.20: FTIR spectra of ExoPS extracted from GAS treating domestic wastewater (a) GAS from R2 (raw ww), (b) GAS form R1 (settled sewage).

Table 5.5: Summary of the FTIR spectroscopy results obtained for the exoPS extracted from GAS collected from the pilot-scale SBRs in comparison with alginate and with some examples from the literature.

References / **Samples**	**Wavenumbers (cm^{-1}) and assigned functional groups (A to I)***	**A**	**B**	**C**	**D**	**E**	**F**[a]	**H**	**I**
Leal et al. (2008)[a]	**Sea-weed pure culture alginic acid**	>3000 peak at 3428	2927	1616	1415		1125, 1091, 1035	**949**	**888 / 820**
Lin et al. (2010) [b,c]	**GAS-exoPS (municipal ww)**	>3000	2927	1654	1400	1240	1000-1200	**902**[b]	**820**[c]
This study	**Alginate (A2158, Sigma)**	>3000 peak at 3258	2914	1597	1407	1294	1027	**948**	**882**
This study	**GAS-exoPS (R2, raw domestic ww)**	>3000 peak at 3254	2969	1634	1418	1255	1041	**958**	**878**
This study	**GAS-exoPS (R1, settled domestic ww)**	>3000 peak at 3281	2922	1627	1403	1233	1051	**959**	**839**

***Assigned functional groups (A to I)**	A: O-H stretching vibrations, B: C-H stretching vibrations, C: O-C-O asymmetric stretching, D: C-OH deformation vibrations and/or O-C-O symmetric stretching vibrations, E: O-acetyl ester, F: C-O-C and/or C-O vibrations, **H:** C-O stretching vibrations of **guluronic** acid residues, **I:** C1-H deformation vibrations of **mannuronic** acid residues / Characteristic peak for mannuronic acid

[a] Values reported by Leal et al. (2008) for C-O stretching of pyranose rings, C-O and C-C stretching vibrations of pyranose rings, and C-O stretching vibration of uronic acid, respectively. [b, c] Second derivative values reported by Lin et al. (2010) for anomeric C-H deformation vibrations of L-guluronic acid residues and for C1-H deformation vibrations of mannuronic acid residues, respectively.

5.8 Block fractionation

Alginate as a sugar polymer consist of repeating guluronic and mannuronic acid residues forming homo-monomeric [-GG-, MM-] and/or hetero-monomeric [-MG-] blocks. The length, proportion and distribution of these blocks are considered to dictate the physical and chemical characteristics of alginate (Lin et al 2010). Furthermore, the structure of these polymers upholds a high gelation capacity when introduced to a $CaCl_2$ solution. Ca^{2+} serves as a cross-linker, bridging the individual polymer fragments and thus enhancing aggregation.

Block distribution of alginate/exoPS is necessary to establish the relationship between the structure and function of these polymers. The distribution of the different block of the polymer dictates properties such as the gel forming capacity (which is attributed the guluronic acids residues in homo-polymeric blocks) and also the flexibility of its gels. According to Leal et al (2008), the block fractions of

alginates can be characterised by the FTIR spectrum, especially in the fingerprint region. Bands approximately 960 cm^{-1} signify C-O stretching vibrations and contribution from C-C stretching and C-C-O deformation vibrations of uronic acid residues in a heteropolymeric block whereas peaks at 815 cm^{-1} were described as representing deformation vibrations of D-mannopyranuronic acid residues. The precise wavenumbers of these uronic acid residues distinguishes the 3 blocks MM, MG and GG.

FTIR spectra of the individual blocks obtained for the GAS-ExoPS (from R2) after block fractionation application are given in Figure 5.21. From the results, the MG blocks which are obtained through partial acid hydrolysis showed similarities with alginate within 3500-1000 cm^{-1} region. In the fingerprint region, contrary to the results obtained by Leal et al (2008), where characteristic bands of mannuronic and guluronic acid residues were observed, no signal was observed in this fraction as seen in figure 5.21(a).

The MM fraction which is soluble at pH 2.85 also displayed a spectrum similar to alginate within 3500-1000 wavelength region. Contrary to the MG blocks, the MM fraction revealed a signal at 843cm^{-1} which can be assigned to deformation vibrations of b-D-mannuronic acid residues according to Leal et al, (2008). As can be seen clearly in figure 5.21(b), signature peaks of guluronic acid residues were not seen in the MM spectrum as was observed by Leal et al (2008) in the characterisation of the block fractions of alginate.

The spectrum of GG blocks presented in figure 5.21(c) is similar to that of MM blocks. The fingerprint region reveals a signal at 852cm^{-1} which is slightly shifted higher in relation to that of the MM block. This signal can be attributed to mannuronic acid residues which are component of the MM fraction that did not solubilise at pH 2.85 and as such indicate the presence of mannuronic acid residues in the homopolyguluronic fraction.

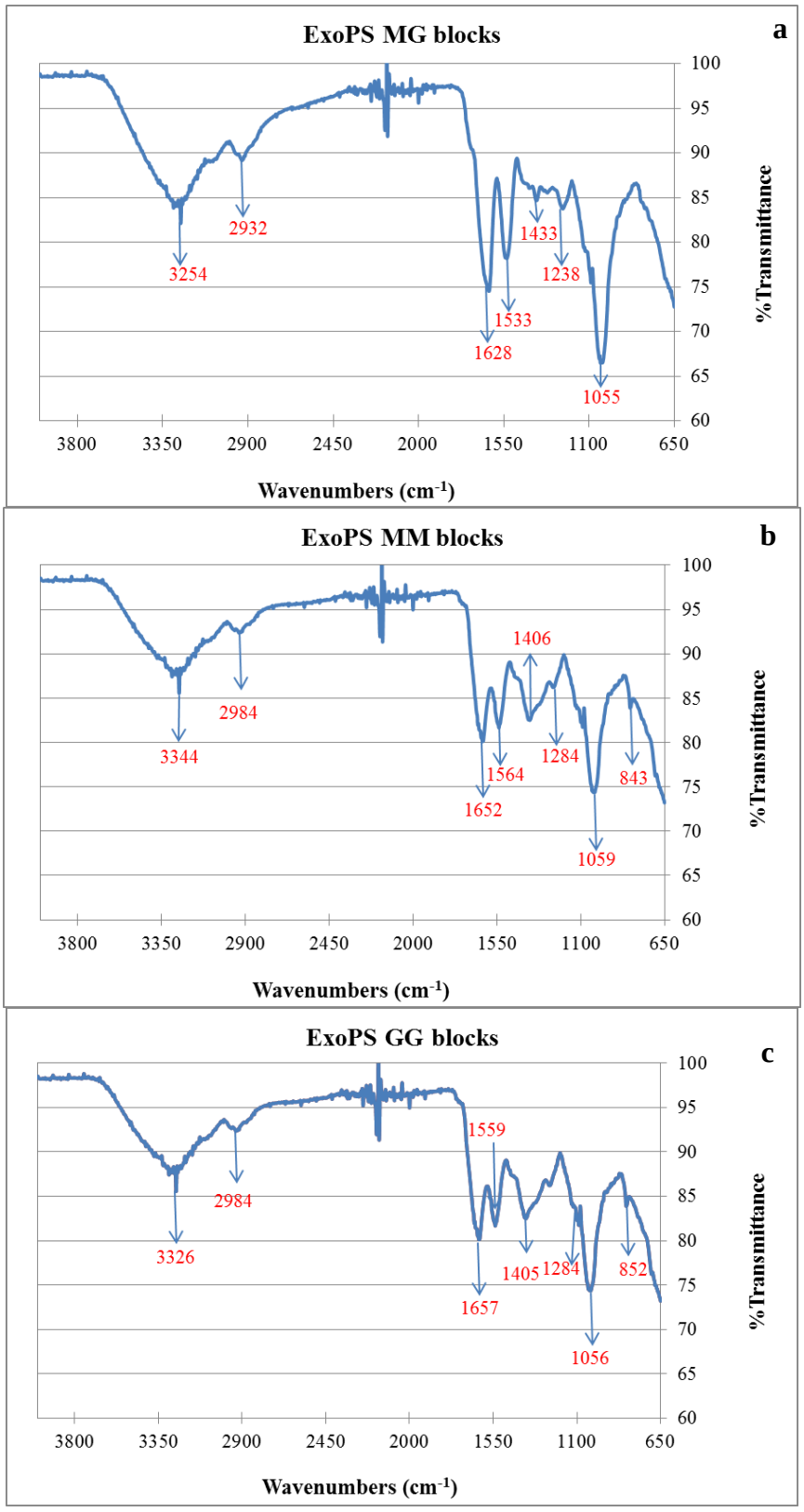

Figure 5.21: FTIR spectra of the individual blocks obtained for R2 GAS ExoPS after block fractionation: (a) MG blocks (b) MM blocks (c) GG blocks.

According to Leal et al (2008), the block fractions of alginate is characterised by the bands in the fingerprint print region of their FTIR spectra. The table below indicates the bands that were recorded for alginate block fractions

Table 5.6: Peaks of the block fractions of alginic acid from pure cultures (Leal et al., 2008).

F1 MG block	F2 MM block	F3 GG block
960.7	948.8	947.3
911.5	-	-
890.3	888.8	903.6
815.1	820.3	812.5

As shown in figure 5.21, the peaks of the 3 fractions display different values. The values of the peaks obtained in this study for R2 GAS ExoPS were different from that reported by Leal et al (2008) and this might be due to differences in the purity of the samples analysed. To make a much better comparison, it will be necessary to repeat fractionation of the alginate.

ExoPS were found to contain about 16.5% of GG blocks and 18.3% of MG blocks (Table 5.7) which is contrary to the results obtained by (Lin et al 2010). The amount of GG blocks in this case was slightly lower than that of MG blocks. This is due to the fact that the presence and amount of the different fractions is dependent primarily on the microbial flora and probably the time of harvest. The biosynthesis of G residues is a post epimerization step (Lin et al, 2010), which may or may not occur depending on the bacteria involved.

Hence the percentages of GG and MG blocks are likely to differ with different wastewater sources having different microbial populations. Although GG block fraction was less than GM fraction, the formation of good hydrogel in $CaCL_2$ by the extracted ExoPS clearly indicates that gelation may occur even at lesser GG block concentration. Such gels may however be more flexible with less mechanical strength than those with higher GG block fractions. The high amount of MG blocks corresponds to an excellent flexibility which increases the elasticity of the gel produced. Improvement in the elasticity of the gel translates into a more stable activated sludge that can withstand strong shear stress in the reactor (Lin et al 2010).

Apparently, the formation of strong gels and a more stable activated sludge due to the composition of GG and MG blocks results in increasing the settling abilities of the sludge. A stable sludge with increased density cannot be washed out of the reactor.

Following are some further details on extraction efficiency and yield of the Na_2CO_3 extraction method obtained for the GAS-ExoPS isolated from the pilot-scale SBR (R2), together with % recovery of the block fractionation step and that of the whole procedure (Table 5.7):

- Extraction efficiency was determined as 8.9% (g exoPS/g VSS).
- Extraction yield was calculated as the ratio of dry mass of the exoPS extract after lyophilisation (1520 mg exoPS) to the dry weight of the biomass in the sample volume centrifuged for extraction-lyophilisation(17.8 g VSS). Resulting yield value was 85.4 mg exoPS/g VSS.
- % distribution of the individual blocks, % recovery of block fractionation and extraction + block fractionation are listed below in Table 5.7 in comparison to relevant values reported by Lin et al. (2010).

Table 5.7: Summary* of the block fractionation and FTIR spectroscopy results obtained for the GAS-exoPS isolated from the pilot-scale SBR (R2, raw domestic wastewater) in comparison to the values reported by Lin et al. (2010).

A	**B**	**C**		**D**	**E**
Blocks	**Mass of fractions (mg)**	**% distribution of 3 blocks**		**% recovery block fractionation**	**Overall % recovery**
	Measured	C= B_{each}/B_{total}	Lin et al. (2010)	D= B_{each}/A (250mg)	
F1 MG	45.6	**50.72%**	14.57% (± 2.25)	**18.24%**	1.6% mg/mg 15.6 mg/g
F2 MM	3.1	**3.45%**	2.10% (± 1.43)	**1.24%**	0.11% mg/mg 1.06 mg/g
F3 GG	41.2	**45.83%**	69.07% (± 8.95)	**16.48%**	1.4% mg/mg 14.1 mg/g
Total	89.9 calculated as sum of the above three (B_{total})	100%	*85.39% (± 7.22)*	*35.96% mg fract. exoPS/ mg total ini. exoPS*	*3.1% mg/mg 30.7 mg sum of 3 blocks/g VSS*

*A: The signature polymeric blocks found in alginate and alginate-like-exoPS. B: dry weight values obtained after each separation and lyophilisation. C: % mg individual block/mg sum of 3 blocks (all lyophilized). D: % mg individual block/mg total initial exoPS (all dry weight). E: % recovery of extraction and block fractionation steps together, in units of % mg individual block/mg VSS (all dry weight).

As seen from the values obtained in this study and given in Table 5.7, % contribution of the MG, MM, and GG blocks of the exoPS extracted from GAS from the pilot-scale SBR treating raw domestic wastewater (R2) were not exactly the same as those reported by Lin et al. (2010) for those of GAS from another pilot-scale SBR treating municipal wastewater mixed with slaughterhouse effluents (2010). Apparently, those two exopolymers are somewhat different in terms of their chemical composition, although both were produced by GAS and exhibited similarities with alginate. The difference was considered to be originating mainly of two reasons: (i) different influents to the systems, (ii) probably different microbial populations grown inside the systems.

6. CONCLUSION

- In accordance with the extraction method described by Lin et al, (2010) for the extraction of exopolysaccharides from municipal wastewater, exopolysaccharides were also extracted both from floccular and aerobic granular sludge fed with acetate and/or treating brewery wastewater. Although the extraction efficiency was lower than that reported by Lin et al, (2010), the Na_2CO_3 extraction method was employed successfully for isolation of the exoPS from AS. Gelation reactions in $CaCl_2$, investigation of formed hydrogels, and FTIR spectroscopy all showed that the AS-associated exoPS resembled alginate in terms of their physical and chemical properties, and thus can be referred to as Alginate-Like-Exopolysaccharides (ALE), as proposed by Lin et al (2010).
- The method of extraction influences the state of the end-products of the extractions (aqueous or lyophilised) and hence the characteristics of the extract. The DOWEX procedure involves extraction of extracellular polymeric substances including proteins, nucleic acids and some humic substances in an aqueous state while extraction with Na_2CO_3 is specific for isolation of exopolysaccharides coming out in a solid lyophilised form. In terms of most of the parameters considered, the best results were achieved with lyophilised exoPS extracted with the Na_2CO_3 method. This observation suggests the importance of the extraction method for EPS studies.
- Though less informative, the UV-VIS spectra of the AS-ExoPS and alginate demonstrate similar significant bands of carbohydrates within 190-210nm wavelength range and no peaks at 260nm and 280nm imply the absence of DNA and proteins respectively.
- Estimation of the total carbohydrate content of biological polysaccharides by digestion followed by spectroscopy can be affected by the type of the reference carbohydrate selected for quantification. Using glucose as the

reference carbohydrate results in underestimation of the actual carbohydrate content of both alginate and AS-exoPS. Hence, the choice of reference material is crucial in accurate estimation of total carbohydrates content of activated sludge-associated samples.

- Best gelation result are obtained with 2% alginate solution; maintaining a 1:1 ratio with $CaCL_2$. On the other hand, even when keeping a 1:1 ratio, the gelation capacity decrease with decreasing alginate concentration and gelation was undetectable at alginate concentrations below 0.01%.
- Gelation experiments as well as SEM examination of the formed hydrogels provide evidence for the gel forming capacity of the AS-ExoPS and the similarities with alginate.
- The % moisture content values indicate that the gels formed by the exoPS extracts are in fact hydrogels, like those formed by sodium alginate
- The chemical composition of the AS-exoPS revealed by FTIR clearly shows that the AS-associated exoPS resembles alginate. Typical polysaccharide bands as well as the significant peaks of mannuronic and guluronic acid residues are present in both alginate and the AS-exoPS.
- The amounts of the blocks fractions indicate that MG blocks are higher (18.24%) than GG (16.48%), an observation different from that reported by Lin et al, (2010). However, good hydrogel beads were formed by the extracted exoPS, which indicates that gelation can occur even at lower GG block % contribution.
- Contrary to other works, this study showed that exoPS produced by floccular sludge fed with acetate can form self-standing hydrogels in $CaCl_2$ just like alginate and the exoPS extracted from aerobic granular sludge. Thus, exoPS originating from both floccular and granular sludge share similarities with alginate.
- Similarities of AS-exoPS with alginate signify the role of extracellular polymers in granular and floccular activated sludge. The gel forming capacity of alginates due to its interaction with divalent cations is a property that has been utilised for the immobilization of cells and enzymes. Likewise, ALE secreted by activated sludge biomass seemingly contributes to the structural

strength of both floccular and granular activated sludge. The gel forming properties of the exoPS extracts, as well as the presence of the GG and MM blocks- the former known as the sites of crosslinking through divalent cations promoting aggregation and gelation of alginates, and the latter described as providing flexibility- are properties providing strength, compactness, and elasticity and thus contributes to the structural strength and coherence of activated sludge, especially to the compact and durable structure of the "excellently settling aerobic granular biomass.

- Future work for further characterization and comparison of the AS-exoPS and alginate should focus on resolving the structure of the exoPS with i.e., NMR. This will aid in the identification of the base sugar units of the polymer, the monomeric sequence and linkage sites and also to determine the location and nature of any substitutions. This will give more information on whether AS-exoPS are complex hetero-exopolysaccharides.

7. REFERENCES

Adav, S.S. and Lee, D.J. (2008). Extraction of extracellular polymeric substances from aerobic granule with compact interior structure. *Journal of Hazardous Materials* **154** (1-3), 1120-1126.

Beun, J.J., Hendriks, A., van Loosdrecht, M.C.M., Morgenroth, E., Wilderer, P.A., Heijnen, J.J. (1999). Aerobic granulation in a sequencing batch reactor. *Water Res,* **33,** 2283 – 90.

Beun, J.J., van Loosdrecht, M.C.M., Heijnen, J.J. (2000). Aerobic granulation. *Water Science and Technology,* **41,** (4-5), 41-48.

Bindhu, B.K. and Madhu, G., (2013). Aerobic granulation - an economically viable option for the treatment of wastewater. *International Journal of Innovative Research in Science, Engineering and Technology*, Volume 2, Special Issue 1.

Bossier, P., and Verstraete, W. (1996). Triggers for microbial aggregation in activated sludge? *Appl Microbiol Biotechnol*., **45**, 1 – 6.

Bruus, J.H., Nielsen, P.H. and Keiding, K. (1992). On the stability of activated sludge flocs with implication to dewatering. *Water Research* **26,** 1597-1604.

Calleja, G.B. Microbial aggregation. Florida: CRC Press; 1984 Boca Raton.

de Kreuk, M.K., van Loosdrecht, M.C.M. (2004). Selection of slow growing organisms as a means for improving aerobic granular sludge stability. *Water Sci Technol*., **49**, 9–17.

de Kreuk, M.K., McSwain, B.S., Bathe, S., Tay, S.T.L., Schwarzenbeck, N. and Wilderer, P.A. (2005). Discussion Outcomes, in *Aerobic Granular Sludge*, pp. 155-169, Eds. Bathe, S., de Kreuk, M. K., McSwain B. S. and Schwarzenbeck, N., Water and Environmental Management Series, IWA Publishing, London, UK.

Draget, K. I. and Taylor, C, (2011). Chemical, physical and biological properties of alginates and their biomedical implications. *Food Hydrocolloids*, **25**, 251–256.

Driessen, W. and Vereijken, T. (2003). Recent developments in biological treatment of brewery effluent. The Institute and Guild of Brewing Convention, Livingstone, Zambia, March 2-7.

Dulekgurgen, E., Ovez, S., Artan, N., Orhon, D. (2003). "Enhanced Biological Phosphate Removal by Granular Sludge in a Sequencing Batch Reactor", *Biotechnology Letters*, **25**(9): 687-693.

Dulekgurgen, E. (2006). "Hydraulic- and/or Metabolic Selection-Pressures Influenced Aerobic Granulation and its Application in Enhanced Biological Phosphorus Removal (EBPR)", *PhD dissertation*, ITU FBE, 2006.

Dulekgurgen, E., Yilmaz M., Wilderer, P.A. (2008a). Shape and surface topology of anaerobic/aerobic granules influenced by shearing conditions.4th IWA Specialized Conference on Sequencing Batch Reactor Technology, Roma, Italy, 7–10 April, 311–320.

Dulekgurgen, E., Artan, N., Orhon, D., Wilderer, P.A. (2008b). How does shear affect aggregation in granular sludge sequencing batch reactors? Relations between shear, hydrophobicity, and extracellular polymeric substances, *Water Science and Technology*, **58** (2): 267-276.

Dülekgürgen, E., **Karahan-Özgün, Ö.** (2012), **"***A New Approach in Industrial Wastewater Treatment: Aerobic Granular Activated Sludge Technology*", TUBITAK Project No: 108Y313, Final Report (*in Turkish*).

Fillaudeau, L., Blanpain-Avet, P., Daufin G., Water, wastewater and waste management in brewing industries, *Journal of Cleaner Production*, **14,** (2006) 463–471.

Gao, D., Liu, L., Liang, H. and Wei-Min, W. (2011). Aerobic granular sludge: characterization, mechanism of granulation and application to wastewater treatment. *Critical Reviews in Biotechnology*; **31** (2): 137–152.

Gebara F., (1999). Activated sludge biofilm wastewater treatment system. *Water Research*, Vol. **33**, No. 1, pp. 230-238.

Gerhardt, P., Murray, R.G.E., Wood, W.A., Krieg, N.R., 1994. Methods for General and Molecular Bacteriology, ASM, Washington D.C., ISBN 1-55581-048-9, p 518.

Goldammer, T., The Brewers' Handbook, 2nd edition, Apex Publishers, Clifton, 2008.

Jia, S., Yu, Y., Lin, H., Dai, Y. (2007). Characterization of extracellular polysaccharides from *Nostoc flagelliforme* cells in liquid suspension culture. *Biotechnology and Bioprocess Engineering*, **12**, pp. 271–275.

Jiang, H.L, Tay, J.H., Liu., Y. (2003). Ca^{2+} augmentation for enhancement of aerobically grown microbial granules in sludge blanket reactor. *Biotechnol Lett*., **25**, 95–103.

Lattner, D., Flemming, H.C., and Mayer, C. (2003). 13C-NMR study of the interaction of bacterial alginate with bivalent cations. *Biological Macromolecules* **33**, 81-88.

Leal, D., Matsuhiro, B., Rossi, M., Caruso, F. (2008). FT-IR spectra of alginic acid block fractions in three species of brown seaweeds, *Carbohydrate Res*, **343**, 308-316.

Lettinga, G., van Velsen, A.F.M., Hosma, S.W., de Zeeuw, W., Klapwijk, A. (1980). Use of the upflow sludge blanket (USB) reactor

concept for biological wastewater treatment, especially for anaerobic treatment. *Biotechnol Bioeng*, **22**, 699–734.

Lin, Y., de Kreuk M, van Loosdrecht, M.C.M., Adin, A. (2010).Characterization of alginate-like exopolysaccharides isolated from aerobic granular sludge in pilot-plant. *Water research*, **44**, 3355-3364.

Lin, Y.M., Wang, L., Chi, Z.M., Liu, X.Y. (2008). Bacterial alginate role in aerobic granular bio-particles formation and settleability improvement. *Separation Science and Technology* **43** (7), 1642-1652.

Lin, Y.M., Van Loosdrecht, M.C.M., Sharma P.K. (2013). The chemical and mechanical differences between alginate-like exopolysaccharides isolated from aerobic flocculent sludge and aerobic granular sludge. *Water research*, **47**, 57-65.

Liu, Y. and Tay, J.H. (2004). State of the art of biogranulation technology for wastewater treatment. *Biotechnol Adv*., **22**, 533–563.

Liu, Y. and Tay, J.H. (2002). The essential role of hydrodynamic shear force in the formation of biofilm and granular sludge. *Water Res*; **36**:1653 – 65 (24).

Liu, Q.S., Tay, J.H., Liu, Y. (2003). Substrate concentration-independent aerobic granulation in sequential aerobic sludge blanket reactor. *Environ Technol*., **24**, 1235–43.

Mahoney, E.M., Varangu, L.K., Cairns, W.L., Kosaric, N., Murray, R.G.E. (1987). The effect of calcium onmicrobial aggregation during UASB reactor start-up. *Water Sci Technol*., **19**, 249–6.

McSwain, B.S., Irvine, R.L., Hausner, M., Wilderer, P.A. (2005). Composition and distribution of extracellular polymeric substances in aerobic flocs and granular sludge. *Applied and Environmental Microbiology*, **71** (2), 1051-1057.

McSwain, B.S., Irvine, R.L, Wilderer, P.A. (2004). The influence of settling time on the formation of aerobic granules. *Water Sci Technol*., **50**, 195−102.

Moy, B.Y.P., Tay, J.H., Toh, S.K., Liu, Y., Tay, S.T.L. (2002). High organic loading influences the physical characteristics of aerobic sludge granules. *Lett Appl Microbiol*, **34**, 407–412.

Ohashi, A. and Harada, H. (1994). Adhesion strength of biofilm developed in an attached-growth reactor. *Water Sci Technol*., **29**:10.

Qin, L., Liu, Y., Tay, J.H. (2004). Effect of settling time on aerobic granulation in sequencing batch reactor. *Biochem Eng J*., **21**, 47–52.

Saarai, A., Kasparkova, V, Sedlacek, T., Saha, P. (2013). On the development and characterisation of crosslinked sodium alginate/gelatine hydrogels. *Journal of the mechanical behaviour of biomedical materials*, **18**, 152–166.

Seviour, T., Pijuan, M., Nicholson, T., Keller, J., Yuan, Z., (2009). Understanding the properties of aerobic sludge granules as hydrogels. *Biotechnology and Bioengineering* **102** (5), 1483-1493.

Seviour, T., Yuan Z., van Loosdrecht M.C.M., and Lin Y. (2012). Aerobic sludge granulation: A tale of two polysaccharides? *Water research*, **46**, 4803-4813.

Simate, G.S., Cluett, J., Iyuke, S.E., Musapatika, E.T., Ndlovu, S., Walubita, L.F., Alvarez, A.E. (2011). The treatment of brewery wastewater for reuse: State of the art. *Desalination*, **273**, 235-347.

Sun, J.Y., Zhao, X., Illeperuma, W.R.K., Chaudhuri, O., Oh, K.H., Mooney, D.J., Vlassak, J.J., Suo, Z. (2012)**.** Highly stretchable and tough hydrogels. *Nature*, **489** (7414): 133-136, doi: 10.1038/nature11409.

Surtherland, I.W. (1999) Biofilm exopolysaccharides. In: Wingender, J., Neu, T.R., and Flemming, H.-C. Microbial extracellular polymeric substances: Characterization, structure, and function. Berlin: *Springer*. 73-92.

Tay, J.H., Liu, Q.S., Liu, Y. (2002). Characteristics of aerobic granules grown on glucose and acetate in sequential aerobic sludge blanket reactors. *Environ Technol*., **23**, 931–936.

Tay, J.H., Liu, Q.S., Liu, Y. (2001). Microscopic observation of aerobic granulation in sequential aerobic sludge blanket reactor. *J Appl Microbiol*, **91**:168–75.

Tay, J.H., Liu, Q.S., Liu, Y. (2002) Aerobic granulation in sequential sludge blanket reactor. *Water Sci Technol*., **46**:13– 8.

Toh, S.K., Tay, J.H., Moy, B.Y.P., Ivanov, V., Tay, S.T.L. (2003) Size-effect on the physical characteristics of the aerobic granule in a SBR. *Appl Microbiol Biotechnol*, **60**:687 – 95.

Wang, Z.W., Li, Y., Zhou, J.Q., Liu, Y. (2006). The influence of short term starvation on aerobic granules. *Process Biochemistry*, **41**, 2373-2378.

Wong, T.W., Chan, L.W., Kho, S.B., Heng, P.W.S. (2002). Design of controlled-release solid dosage forms of alginate and chitosan using microwave. *Journal of Controlled Release*, **84**, 99–114.

Yılmaz, G., Övez, S., Dulekgurgen, E. (2014). "On-Site Treatment of Domestic Wastewater Using Aerobic Granular Sludge Technology", TUBITAK Project No: 111Y036, Interim Report No5, (*in Turkish*).

Yun, U.J. and Park H.D., (2003). Physical properties of an extracellular polysaccharide produced by *Bacillus* sp. CP912. *Letters in Applied Microbiology*, **36**, pp. 282–287.

Zheng, Y.M., Yu, H.Q., Sheng, G.P. (2005) Physical and chemical characteristics of granular activated sludge from a sequencing batch airlift reactor. *Process Biochem*, **40**: 645–50.

Zheng, Y.M., Yu, H.Q., Liu, S.J., Liu, X.Z. (2006). Formation and instability of aerobic granules under high organic loading conditions. *Chemosphere* **63**, 1791–1800.

Zheng, Y.M., Yu, H.Q. (2007). Determination of the pore size distribution and porosity of aerobic granules using size-exclusion chromatography. *Water Res.*, **41**, 39–46.

CURRICULUM VITAE

Name Surname: STANLEY BORTSE SAM

Place and Date of Birth: PRESTEA 25/04/1984

Address: Abdi Ipekçi Öğrenci Yurdu, Vişnezade Mah, Silahhane Sk. No1

Maçka Beşiktaş- Istanbul

E-Mail: borsam@itu.edu.tr , nkbsam@yahoo.com

B.Sc.: Biochemistry and Biotechnology

PUBLICATIONS/PRESENTATIONS ON THE THESIS

- **Sam, S.B. and Dulekgurgen, E., 2014:** Characterization of EPS/ExoPS of floccular and aerobic granular sludge and similarities with alginate - *International Water Association: The perfect Slime Conference*, September 10-12, 2014 Essen, Germany.

- **Sam, S.B. and Dulekgurgen, E., 2014:** Characterization of Exopolysaccharides from Floccular and Aerobic Granular Activated Sludge as Alginate-Like-ExoPS – *International conference on recycling and reuse*, June 04-06 2014 Istanbul Turkey.