EVALUATION OF DIFFERENT WASTE STREAMS AS FERMENTATION SUBSTRATES FOR MICROBIAL BIOPOLYMER PRODUCTION

by

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So close no matter how far, Couldn't be much more from the heart

To Immortal Beloved

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ABSTRACT

The growing reliance on synthetic polymers has raised a number of environmental and human health concerns. Most plastic materials are derived from nonrenewable resources and not biodegradable. Their distinguishing properties like durability and strength also ensure their persistence in the environment and complicate their disposal. Replacing synthetic polymers with biopolymers offers advantages in terms of biodegradability and biocompatibility however still suffers from high production costs. Therefore most research has shifted towards development of cost-effective ways of producing these biopolymers. Levan is a homopolymer of fructose with many outstanding properties like high solubility in oil and water, strong adhesivity, good biocompatibility and film-forming ability. However its industrial use has long been hampered by costly production processes which rely on mesophilic bacteria and plants. *Halomonas* sp. AAD6 halophilic bacteria were found to be the only extremophilic species producing levan at high titers and hence considerable research effort has been directed to combine the advantages of osmoadaptation and halophilicity to favor high yield levan production. As part of this on-going research, in this thesis work, the potential uses of different waste streams like sugar beet and starch molasses, cheese whey powder, sugar beet pulp, olive mill wastewater, and orange bagasses were investigated and based on EPS yields, pretreated sugar beet molasses was found to be superior to others. FT-IR analysis showed that EPSs produced under different conditions were all levan-type polymers and rheological studies revealed that these EPSs have shown the characteristic of low viscosity and appeared to exhibit pseudoplastic behaviours. Flocculating activity of these EPSs were dynamically monitored with a photometric dispersion analyser and one sample was found to exhibit flocculation performance and particles removal efficiency comparable with commercial synthetic polyelectrolytes. Finally, a rough economic analysis revealed that replacing pure sucrose with molasses not only increased the production yields but also resulted in five-fold lower costs of fermentation medium per gram of levan produced.

ÖZET

Sentetik polimerlere gittikçe artan güven, pek çok çevresel ve insan sağlığına yönelik endişelere sebep olmaktadır. Plastik maddelerin çoğu biyoçözünür olmamanın yanında yenilenemeyen kaynaklardan üretilmektedir. Kalıcılık ve dayanıklılık gibi özellikler bu maddeleri çok kullanılışlı yapmakla beraber doğada bozulmamalarını sağlayarak imha edilmelerini güçleştirmektedir. Polimerlerin yerine biopolimerlerin kullanılması biyoçözünürlük ve biyouyumluluk adına avantaj sağlasa da biyopolimerlerin yüksek üretim maliyetleri hala sorun teşkil etmektedir. Bu yüzden birçok araştırma biyopolimerlerin üretiminde maliyet düşürücü yöntemlerin geliştirilmesine kaymıştır. Levan, yağ ve suda yüksek çözünürlük, kuvvetli yapışkanlık, biyouyumluluk ve film oluşturma gibi üstün özelliklere sahip, bir fruktoz homopolimeridir. Bununla birlikte, endüstriyel kullanımı mezofilik bakterilere ve bitkilere dayanan yüksek maliyetli üretim süreçleri yüzünden uzun süreden beri engellenmektedir. Halofilik *Halomonas* sp. AAD6 bakterileri yüksek miktarda levan üreten tek ekstremofil tür olarak belirlenmiştir ve kayda değer sayıda araştırma levan üretiminde yüksek verimliliği desteklemek için halofilikliğin ve ozmoadaptasyonun avantajlarını birleştirmeye yönelmiştir. Devam eden bir projenin parçası olarak bu tez çalışmasında, şeker pancarı ve nişasta melası, peynir suyu altı tozu, şeker pancarı küspesi, zeytin kara suyu, ve portakal posası gibi değişik gıda atıklarının ve yan ürünlerinin biyopolimer üretimindeki potansiyel kullanımları araştırıldı. EPS verimleri temel alındığında, önişlemden geçmiş şeker pancarı melasının diğerlerine üstün olduğu bulundu. FT-IR analizleri değişik şartlarda üretilmiş EPSlerin hepsinin levan türü biopolimerler olduğunu gösterdi. Reolojik çalışmalar bu EPSlerin düşük akışkanlığa sahip olduklarını ve psödoplastik davranış gösterdiklerini açığa çıkardı. Bu EPSlerin flokulasyon aktiviteleri fotometrik dispersiyon analizör cihazıyla dinamik olarak takip edildi ve bir tanesinin ticari sentetik polielektrolitlerle parçacık giderim veriminde ve flokulasyon performansında kıyaslanabileceği görüldü. Son olarak, yaklaşık bir ekonomi analizi sonucunda melasın sükroz yerine kullanımının sadece üretim verimini arttırmakla kalmayıp aynı zamanda levanın gram üretimine karşı gelen fermentasyon ortam masrafını beş kat düşürdüğü belirlendi.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Unit
$\dot{\gamma}$	Shear rate	(s^{-1})
σ	Shear stres	(Pa)
a_w	Water activity	
AC	Activated carbon	
BM	Beet molasses	
CL	Clarification	
CpH	Clarification and pH adjustment	
CWP	Cheese whey powder	
DCW	Dry cell weight	
EPS	Exopolysaccharide	
K	Consistency coefficient	($Pa.s^n$)
n	Flow behaviour index	
OMW	Olive mill wastewater	
SM	Starch molasses	
TCP	Tricalcium phophate	

1. INTRODUCTION

During the last century, global economic activities have increased 19 fold (I.M.F., 2000). This extraordinary growth has raised serious concerns about current models of production and consumption (Huang, 1995; Scott, 1999; Ljunberg, 2007; Barnes et al., 2009). As society has increased its understanding of the environmental implications of its industrial practices, greater attention has been given to the concept of sustainable economic systems that rely on renewable sources of energy and materials. The use of biologically derived polymers, biopolymers, could emerge as an important component of this new paradigm of economic development (I.B.A.W., 2005; S.T.E., 2008).

By transforming agricultural or marine feedstocks, or harnessing the enzymes found in nature, a new class of renewable, biodegradable, and biocompatible materials is being introduced. Emerging applications of biopolymers range from packaging (Markarian, 2008) to industrial chemicals (Hermann et al., 2007; Hamed et al., 2009) to medical implant devices (Mani et al., 2007), and construction engineering (Plank, 2003). Hence, biopolymers is an important and exciting new field in biotechnology which promises to help save the environment as well as slow the depletion of non-renewable resources (Sanchez-Garcia et al., 2008; Ntaikou et al., 2009; Pillai et al., 2009). In addition to producing “green” materials with unique physical and functional properties, the processes used to create biopolymers could lead to new manufacturing approaches that minimize energy consumption and waste generation (U.S. Congress Report, 1993). Yet, biopolymer technology is still at an early stage of its development and many tests and experiments need to be done to see if biopolymers are actually feasible as a replacement for the incredibly versatile petroleum based polymers.

The possibility of using proteins, carbohydrates, and other biopolymers to meet the materials requirements of an expanding economy is likely to receive increasing attention. However, like every emerging technology, difficult

engineering and economic hurdles stand in the way of biopolymer commercialization efforts (Schwark, 2009).

Of many biopolymers produced by diverse microorganisms present in the environment, only few can maintain their integrity in the presence of extreme environmental conditions such as pH, temperature, pressure, radiation and salinity. Extremophiles, microorganisms specifically adapted themselves to these particular conditions, are of significant biotechnological importance as their enzymes and biopolymers possess unique properties that offer insights into their biology and evolution (Shaw et al., 2003; Dickinson, 2004; Hoyoux et al., 2004; Nichols et al., 2005; Paul et al., 2008).

Considering the numerous applications of microbial polysaccharides in cosmetics, food production, petroleum and chemical industries due to their rheological and viscosity-enhancing properties, halophilic bacteria and archaea isolated from Çamaltı Saltern Area in İzmir, Turkey were screened for their polysaccharide-producing abilities with the aim of finding EPS with novel and valuable properties (Gürleyendağ, 2006). The best producer strain, *Halomonas* sp AAD6 was further investigated in terms of its carbon and nitrogen source requirements for EPS production and using statistical optimization tools, a chemical medium was designed for maximum EPS yield (Kazak et al., 2007). Chemical and biological characterization studies revealed that this EPS was a linear homopolymer made up of β(2-6)-linked fructose residues, i.e. a levan type of fructan with high biocompatibility and hence *Halomonas* sp. has been described as a levan producer microorganism for the first time (Poli et al., 2009).

Like starch, fructans are another form of reserve carbohydrates in plants. Interacting with phospholipids and hence protecting lipids from undergoing phase transition, fructans are reported to maintain the integrity of membranes during temperature changes, drought and salt stress (Livingston et al., 2009). As a water soluble, strongly adhesive and film-forming biopolymer, levan has many valuable properties like low viscosity, high solubility in oil, compatibility with salts and surfactants, stability to heat, acid and alkali, high holding capacity for water and

chemicals and good biocompatibility. Hence levan has many potential uses as emulsifier, stabilizer and thickener, an encapsulating agent, osmoregulator and cryoprotector in foods, feeds, cosmetics, and the pharmaceutical and chemical industries. Moreover, in medicine levan is used as plasmasubstitute, prolongator of drug activity, radio protector, antitumor and antihyperlipidemic agent (Kang et al., 2009).

These properties distinguishing levan from other polysaccharides have long been the focus of interest however because of its high cost, levan has only been available in small quantities. Therefore, high level levan producing microbial systems have great industrial importance.

At present, levan production by *Halomonas* sp AAD6 is being done under fully controlled bioreactor conditions using chemical fermentation media containing sucrose as fermentation substrate and current research is focused on elucidating the market potential of levan from *Halomonas* sp. AAD6 by exploring its various properties as a bioflucculating agent and bioemulsifyer, its biosorption properties and its role in various pharmaceutical areas. However, no matter how high the market potential of this new polymer may be, it can not find its place on the polymer market unless it can be produced economically.

Within the scope of this thesis, the production process and the applications of EPSs from *Halomonas* sp. were investigated with the ultimate goal of developing a cost-effective and environmentally friendly biopolymer production process by evaluating the potential uses of different waste streams like sugar beet and starch molasses, cheese whey powder, sugar beet pulp, olive mill wastewater, and orange bagasses as fermentation substrates. FT-IR spectra of EPSs were measured for their preliminary identification and also, the rheological characteristics of the biopolymers were investigated under different conditions. Moreover,flocculation performance and particles removal efficiency of the EPSs as well as three synthetic polyelectrolytes (cationic, anionic and nonionic) were monitored dynamically by a photometric dispersion analyser and compared. Finally the economic advantage provided by this work was also discussed.

2. THEORY

2.1. Biopolymers

The last 50 years have been marked by the astounding growth in the use of plastics and polymers to provide packaging for safer foods, lighter weight and longer lasting appliances, safer cars, and an increased general level of convenience in all manner of applications. These advantages have created a global industry in which consumption is increasing continuously, from 60 million tonnes world-wide in 1980 to an estimated 260 million tonnes in the year 2010 valued at more than 400 billion dollars (Lörcks, 2006).

Yet the vast majority of these materials are based upon the extraction and processing of the finite nature of fossil raw materials, typically petroleum and gas, leading ultimately to increases in greenhouse gases in the atmosphere and the accumulation of persistent plastic materials in the environment at the end of useful life in any acceptable way.

Biopolymers are polymers that are either biological -but non-fossil- in origin, or susceptible to digestion by microorganisms or chemical breakdown in the environment (e.g., hydrolysis), or ideally, both (Johnson et al., 2003). In contrast to fossil resources, they are largely CO_2 neutral. The material and energy cycles are closed (Figure 2.1).

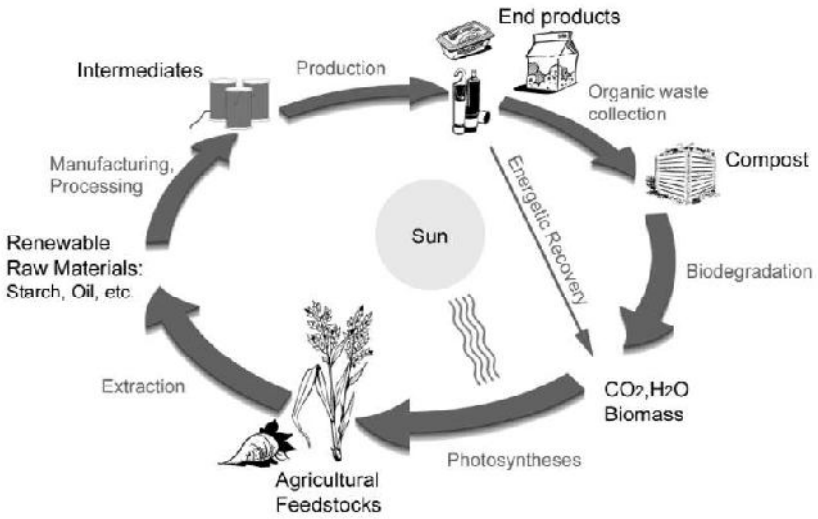


Figure 2.1. Idealized closed loop life cycle (IBAW, 2005)

During recent years, a variety of biopolymers have become available for use in many applications that are not only compatible with human lifestyle but also are friendly to the environment. In nature, biopolymers often play important roles in maintaining cell viability by conserving genetic information, by storing carbon based macromolecules, by producing either energy or reducing power, and by defending an organism against attack from hazardous environmental factors (Lee et al., 2002).

A natural polymer is a macromolecule in a living organism that is formed by linking together several smaller molecules as occurs in protein formation from amino acids or DNA synthesis from nucleotides. The synthesis of the natural polymers involves enzyme catalysed, chain growth polymerisation reactions of activated monomers, which are typically formed within cells by a complex metabolic process. Naturally occurring biopolymers include the carbohydrates, proteins, oils and fats (lipids), and nucleic acids. Also included in the group of biopolymers are chemically derived carbohydrates, and bacteria and fungi derived biodegradable polyesters such as polylactic acid (PLA). The range of materials is shown in Figure 2.2. Biopolymers are superior to petrochemical-derived polymers in several aspects that embrace biocompatibility, biodegradability, and both environmental and human compatibility (Kale et al., 2007; Wang et al., 2007; Huhtala et al., 2008; Zou et al., 2008).

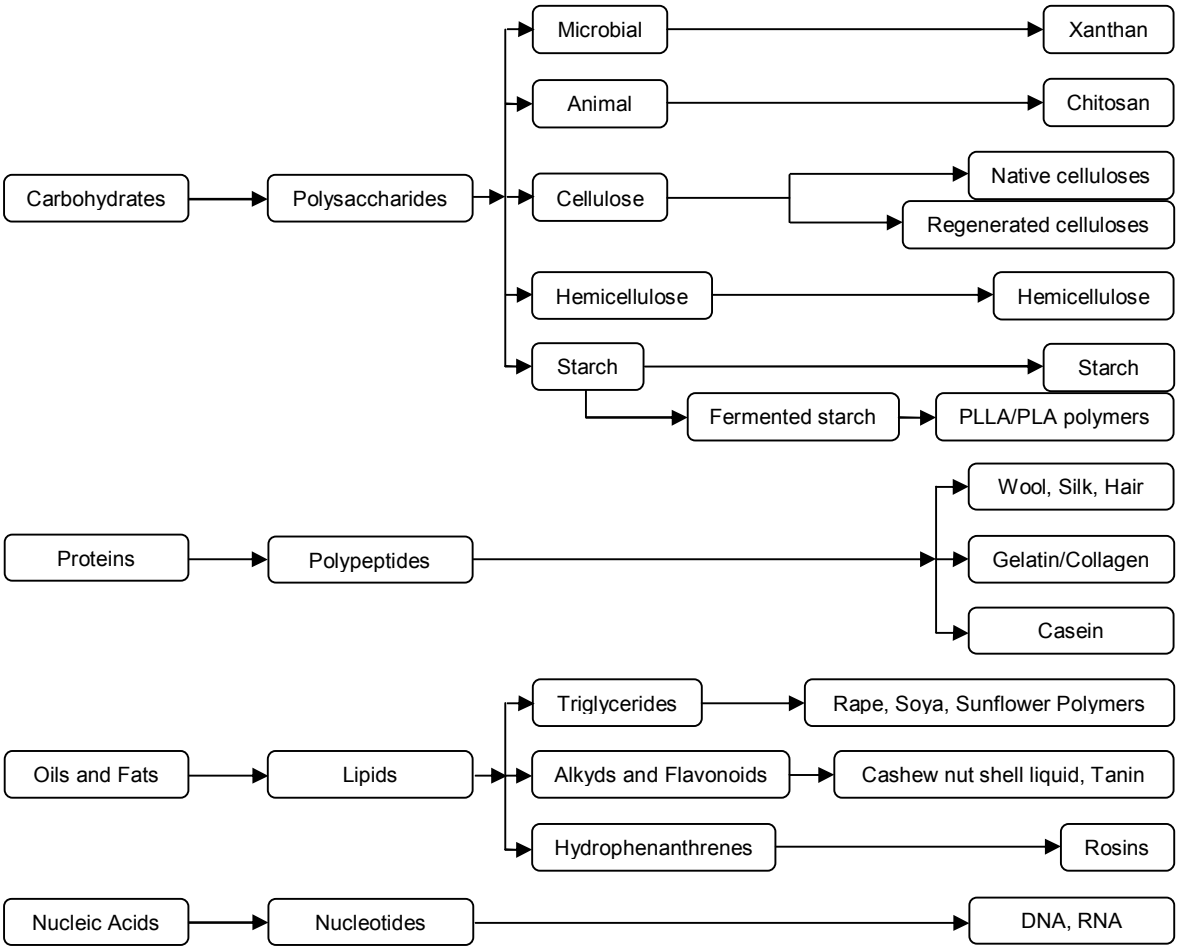


Figure 2.2. Classification of naturally occurring biopolymers (Johnson et al., 2003).

Four major classes of biopolymeric compounds are:

- Lipids
- Nucleic acids
- Proteins
- Carbohydrates

2.1.1. Lipids

By definition, lipids are biological compounds which are soluble in nonpolar solvents such as benzene, chloroform, ether, and are practically insoluble in water. Their relative insolubility leads to their presence predominantly in the nonaqueous biological phases. Lipids also constitute portions of more

complex molecules such as lipoproteins and lipopolysaccharides, which again appear predominantly in biological membranes of cells (Bailey et al., 1986).

Fatty acids are relatively simple lipids. The hydrocarbon chain is constructed from identical two-carbon monomers, so that fatty acids may be regarded as noninformational biopolymers with a terminal carboxylic group (Kavitha et al., 2010).

The long hydrocarbon chain which is hydrophobic (water insoluble) and not very reactive chemically. On the other hand, the carboxylic acid group, which is ionized in solution, extremely hydrophilic (water soluble) and readily reacts with a hydroxyl or an amino group on a second molecule to form esters and amides. Almost all of the fatty acid molecules in a cell are covalently linked to other molecules by their carboxylic acid group.

Fatty acids are a valuable source of food since they can be broken down to produce more than twice as much usuable energy, weight for weight, as glucose. They are stored in cytoplasm of many cells in the form of droplets of triglyceride molecules, which consist of three fatty acid chains, each joined to a glycerol molecule.

The most important function of fatty acids is in the construction of cell membranes. These thin impermeable sheets that enclose all cells and surround their internal organelles are composed largely of phospholipids, which are small molecules that resemble triglycerides in that they are constructed mostly from fatty acids and glycerol. In phospholipids, however, the glycerol is joined to two rather than three fatty acid chains. The remaining site on the glycerol is coupled to a negatively charged phosphate group, which is in turn attached to another small hydrophilic compound. Each phospholipid molecule, therefore, has a hydrophobic tail composed of the two fatty acid chains and a hydrophilic polar head group where the phosphate is located. A small amount of phospholipid will spread over the surface of water to form a monolayer of phospholipid molecules; in this thin film, the hydrophobic tail regions pack together very closely facing the air and the

hydrophilic head groups are in contact with water. Two such films can combine tail to tail in water to make a phospholipid sandwich, or lipid bilayer, an extremely important assembly that is the structural basis of all cell membranes.

2.1.2. Nucleic acids

The term "nucleic acid" is the generic name for a family of biopolymers, named for their role in the cell nucleus. The monomers from which nucleic acids are constructed are called nucleotides. In nucleotides, one of several different nitrogen containing ring compounds, often referred to as bases because they can combine with H^+ in acidic solutions, is linked to a five carbon sugar that carries a phosphate group. There is a strong family resemblance between the different nitrogen containing ring found in nucleotides. Cytosine, thymine, and uracil are called pyrimidine compounds because they are all simple derivatives of a six membered pyrimidine ring. Guanine and adenine are purine compounds with a second five membered ring fused to the six membered ring (Alberts et al., 1983).

Nucleic acid types differ in the structure of the sugar in their nucleotides. DNA contains 2-deoxyribose while RNA contains ribose, where the only difference is the presence of a hydroxyl group. Also, the nitrogenous bases found in the two nucleic acid types are different: adenine, cytosine, and guanine are found in both RNA and DNA, while thymine only occurs in DNA and uracil only occurs in RNA.

In both DNA and RNA, the nucleotides are linked together to form very long polynucleotide chains. The linkage consists of chemical bonds running from the phosphate group of one nucleotide to the deoxyribose (or ribose) group of the adjacent nucleotide. Each deoxyribose (ribose) residue contains several atoms to which phosphate groups might attach (Watson et al., 1983).

Nucleotides can act as carriers of chemical energy. The triphosphate ester of adenine, ATP, participates in the transfer of energy in hundreds of individual cellular reactions. That is the means by which the cell temporarily stores the energy derived from nutrients or sunlight for subsequent use such as

biosynthesis of polymers, transport of materials through membranes, and cell motion.

2.1.3. Proteins

Proteins are the most abundant organic molecules within the cell: 30 to 70 percent, typically around 50 percent, of the cell's dry weight is protein (Gao et al. 2007; Wang et al., 2009). All proteins contain the four most prevalent biological elements: carbon, hydrogen, nitrogen, and oxygen. Average weight percentages of these elements in proteins are C (50%), H (7%), O (%23), and N (16%). In addition, sulfur, up to 3%, contributes to the three dimensional stabilization of almost all proteins by the formation of disulfide (S-S) bonds between sulfur atoms at different location along the polymer chain. The size of these nonrepetitive biopolymers varies considerably, with molecular weights ranging from 6000 to more than 1 million kDa.

All polypeptides and proteins are specific biopolymers with regular arrangements of different types of α-amino acids. All amino acids contain a carboxylic acid group and an amino group, both linked to a single carbon atom called the α-carbon. Proteins and polypeptides are formed by condensation of amino acids. The condensation reaction occurs between the amino group of one amino acid and the carboxyl group of another, forming a peptide bond. This natural synthesis is an extremely complex process involving many different types of enzymes and is not as yet practical as a complete production route for commodity polymers.

2.1.4. Carbohydrates

The carbohydrates are organic compounds with the general formula $(CH_2O)_n$, where $n \geq 3$. These compounds are found in all animal, plant, and microbial cells; the higher molecular weight polymers serve both structural and storage functions. In the biosphere, carbohydrate matter, including starches and cellulose, exceeds the combined amount of all other organic compounds. When

photosynthesis occurs, carbon dioxide is converted to simple sugars C_3 to C_9 in reactions driven by the incident sunlight. These sugars are then polymerized into the forms suitable for structure (cellulose) or sugar storage (starch).

Monosaccharides or simple sugars are the smallest carbohydrates. Containing from three to nine carbon atoms, monosaccharides serve as the monomeric blocks for noninformational biopolymers with molecular weights ranging into the millions. Although glucose is the most common monosaccharide, other simple sugars (galactose, fructose, mannose) are also found in living organisms.

The condensation product of two monosaccharides, by eliminating a water molecule and forming glycosidic bond, is a disaccharide. In addition to sucrose, which is formed from one molecule of glucose and one molecule of fructose, maltose and lactose are other relatively common disaccharides.

Continued polymerization of monosaccharides can occur by formation of new glycosidic bonds resulting in polysaccharides. These structures are often linear, but may contain various degrees of branching. There are two types of polysaccharides (Lindberg, 1990):

- Homopolysaccharides (e.g., cellulose, dextran, levan)
- Heteropolysaccharides (e.g., xanthan, gellan)

A homopolysaccharide is defined to have only one type of monosaccharide repeating in the chain. Monosugars are bound to form linear chains (bacterial cellulose, curdlan or pullulan) or ramified chains (dextran) (Banchathanakij et al., 2009; Purama et al., 2009). The sample forms of branched and unbranched homopolysaccharides are given respectively in Figure 2.3.

Figure 2.3. Unbranched and branched form of homopolysaccharides

On the other hand, a heteropolysaccharide is composed of two or more types of monosaccharides (Xu et al., 2009; Das et al., 2009). Both unbranched and branched forms of heteropolysaccharides, different colors represent different monosaccharides, are shown in Figure 2.4.

Figure 2.4. Unbranched and branched form of heteropolysaccharides

The surface of the microbial cell is a rich source of carbohydrate containing molecules (Alsteens et al., 2008). Some of these are unique types, confined to a limited range of microorganisms. These are the components of the microbial cell walls such as yeast mannans (Kogan et al. 2008), bacterial teichoic and teichuronic acids (Ward, 1981), lipoppolysaccharides and peptidoglycan. However, in addition to these wall components (Lipopolysaccharides, LPS) (Mangoni et al. 2008; Lins et al., 2008), polysaccharides may be found either tightly associated with the microbial cell surface in the form of discrete capsules, capsular polysaccharides (CPS) (Cortes, et al., 2002; Vinogradov, 2005), or as loose slime secreted by the microorganisms but not directly attached to the cell surface (Nicolaus et al., 1999; Sutherland, 2001; Feng et al., 2009). Those which are secreted into extracellular environment are called exopolysaccharides (EPS).

Exopolysaccharide is a term first used by Sutherland (1972) to describe high molecular weight carbohydrate polymers produced by many marine bacteria (Nichols et al., 2005). Extracellular polysaccharides show considerable diversity in their composition and structure. Some of these polymers may bear a strong

chemical similarity to cell wall components, but the majority are distinct chemical structures totally unrelated to cellular constituents (Sutherland, 1990).

The exopolysaccharides are an amazing group of biopolymers that have evolved to serve a wide range of specific functions in the host organism as shown in Table 2.1.

Table 2.1. Biological functions of exopolysaccharides (reconstructed from Wingender et al., 1999)

Function	Relevance	Reference
Adhesion to surfaces	Initial step in colonization of inert and tissue surface, accumulation of bacteria on nutrient-rich surfaces in oligotrophic environments	Irigaray et al., 2008; Harding et al., 2009
Aggregation of bacterial cells, formation of flocs and biofilms	Bridging between cells and inorganic particles trapped from the environment, immobilization of mixed bacterial population, basis for development of high cell densities, generation of a medium for communication processes, cause for biofouling and biocorrosion events	Li et al., 2008; Quintelas et al., 2008
Cell to cell recognition	Symbiotic relationship with plants or animals, initiation of pathogenic processes	Mora et al., 2008; Weijers et al., 2008
Structural elements of biofilms	Mediation of mechanical stability of biofilms (frequently in conjunction with multivalent cations), determination of the shape of EPS structure (capsule, slime, sheath)	Kristensen et al., 2008; Li et al., 2009
Protective barrier	Resistance to nonspecific and specific host defenses (complement-mediated killing, phagocytosis, antibody response, free radical generation), resistance to certain biocides including disinfectants and antibiotics, protection of cyanobacterial nitrogenase from harmful effects of oxygen	Aouidi et al., 2009; Miqueleto et al., 2010
Retention of water	Prevention of desiccation under water deficient conditions	Ayala-Hernandez et al., 2009; Freitas et al., 2009
Sorption of exogeneous organic compounds	Scavenging and accumulation of nutrients from the environment, sorption of xenobiotics (detoxification)	Quintelas et al., 2008; Kang et al., 2010
Enzymatic activities	Digestion of exogeneous macromolecules for nutrient acquisition, release of biofilm cells by degradation of structural EPS of the biofilms	Sutherland, 2001
Interaction of polysaccharides with enzymes	Accumulation/retention and stabilization of secreted enzymes	Ramchandran et al., 2009; Hassan, 2008
Sorption of inorganic ions	Accumulation of toxic metal ion (detoxification), promotion of polysaccharide gel formation, mineral formation	Hammaini et al., 2007

Despite having these astonishing characteristics, many of these functions are not fully understood (Mironescu, 2003). Very often, a subtle change in the structure of the monomer or the type of glycosidic linkage employed has a profound effect on the properties and function of the resultant polysaccharide (Stick, 2001). Main functions of carbohydrates in plants are storage, structural, and defense, which are tabulated in Table 2.2.

Table 2.2. Functions of carbohydrates in plants (reconstructed from Ramesh et al., 2003).

Carbohydrate	Function	Reference
Starch	Food Reserve	Kennedy et al., 2008
Pectin	Cementing, metal ion balancing	Verma et al., 2008
Cellulose and hemicellulose	Structural	Revanappa et al., 2010
Gums	Defense	Pintus et al., 2009
Arabinogalactan	Cell Adhesion	Boudjeko et al., 2009

In the natural environments in which the microorganisms are found, such polymers may be associated with virulence as in the case of plant or animal pathogens (Sik Oh et al., 2005, Koczan et al., 2009), with plant microbial interactions or even it is associated with the protection of the microbial cell against desiccation (Landini, 2009) or other environmental stresses (Chen et al., 2004), and with the attack by bacteriophages and protozoa (Sutherland, 1998).

Exopolysaccharides occur widely, especially among procaryotic species, both those that are free living saprophytes and those that are pathogenic to humans, animals, and plants. Most microalgae yield some type of exopolysaccharide but they are less common among yeasts and fungi. However, some of those isolated from fungi do possess interesting physical and pharmalogical properties (Forabosco et al., 2006; Crognale et al., 2007; Singh et al., 2008).

Definition of exopolysaccharides is more difficult than definition of the carbohydrate containing polymers found in microbial cell walls. The term

exopolysaccharide has been widely used to describe polysaccharides found external to the structural outer surface of the microbial cell and it can be applied to polymers of very diverse composition and of different physical types (Rodriguez-Carvajal et al., 2008). The term glycocalyx fails to differentiate between the different chemical entities found at the microbial surface. It has been used to represent a complex array of micromolecular species including components which are truly extracellular, together with wall polysaccharides and many other non-carbohydrate containing chemical species. Although it may describe structures seen under the microscope or electron microscope, it is inadequate in chemical terms (Sutherland, 1990).

Actually, the exopolysaccharides do not normally take part in microbial structure; the other components of the cell surface, the intracellular functions are unaltered if the exopolysaccharides are absent. They do, however, form structures which can be recognised by either ligth or electron microscopy as in Figure 2.5 below.

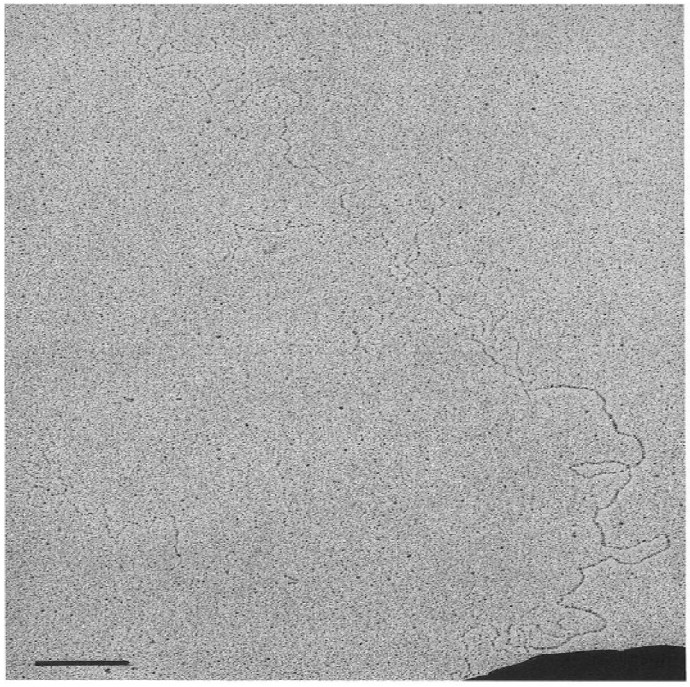


Figure 2.5. Electron micrograph of a xanthan preparation showing the apparently double stranded chain separating into two thinner strands (Sutherland, 1990).

The presence of exopolysaccharides associated with microbial cells grown on solid surfaces under the proper culture conditions is frequently recognisable from the mucoid colony morphology (Malik et al., 2009). In liquid medium, exopolysaccharide producing cultures may become very viscous (Schultheis et al., 2008) or, exceptionally, may solidify as a gel (Kumar et al., 2007; Liu et al., 2009). On solid surfaces exposed to aqueous environments, whether within the human or animal body, in fresh water or in the oceans, bacterial growth is seen as biofilms (Kim et al., 2009; Rinaudi et al., 2009), in which the microbial cells are associated with large amounts of exopolysaccharide. Exopolysaccharides play a major structural role in the formation of biofilms. These biopolymers are believed to assist in the attachment process and protect the underlying cells from fluctuations in the surrounding environment (Allison, 1998). Several of these microbial polysaccharides are now widely accepted industrial products, while others are in various stages of development (Lee et al., 2002). Changes in the growth conditions can drastically alter the composition, physical properties and organisation of the polysaccharides at the bacterial surface (Orr et al., 2009).

Polysaccharides are defined in terms of composition (type and relative abundance of monomers), structure (relative distribution of monomers and type of chemical bounds between them), conformation (arrangement of monomers chains and bounds between them), relative molecular mass and type and arrangement of substitutes (Janelle et al., 1999; Jin et al., 2003; Lo et al., 2007a; Lo et al., 2007b). These informations are used to analyse the functional properties of polysaccharides, like solubility in water, relative viscosity and rheological behaviour (Fissore et al., 2009; Fan et al., 2009), ions binding capacity (Zhu et al., 2009).

The first step in structure analysis is the determination of chemical composition, especially the identification of monomers. Exopolysaccharides are primarily composed of carbohydrates, but in adddition to the various sugars, there may be organic and inorganic substituents (Chi et al., 2007). The carbohydrates found in microbial exopolysaccharides are extremely diverse. Most of the sugars

are those commonly found in animal and plant polosaccharides. D-glucose, D-galactose and D-mannose in the pyranose forms are present in many exopolysaccharides (Maina et al., 2008). The 6-deoxyhexoses, L-fucose and L-rhamnose, are also frequently present. A distinction between eucaryotes and procaryotes can be seen in the presence of pentoses. Eucaryotic polysaccharides may contain pentoses such as D-ribose or D-xylose, but they are of less common occurence in extracellular polymers derived from procaryotes. In addition to the more common monosaccharides, some polysaccharides may contain one or more rare sugars. These may include L-hexose or furanose forms of the hexoses, glucose and galactose. There are also various N-acetylamino sugars, although as yet these have not been found in any of the microbial polysaccharides of industrial importance. As long as more microbial sources of exopolysaccharides are researched, new monosaccharides will be discovered by all means (Sutherland, 1990).

Most of the microbial exopolysaccharides are polyanionic in nature. This results from the presence, in many of them, of uronic acids, with D-glucuronic acid being easily the commonest (Ozturk et al., 2009). Although most polysaccharides contain only a single uronic acid, D-mannuronic acid is usually present in bacterial alginates together with L-guluronic acid (Gimmestad et al., 2009).

Many microbial exopolysaccharides, including several potential industrial importance, are homopolymers. These include a number of glucans, which, because of their different structures, possess significantly different properties even though the sole monosaccharide component is D-glucose. The fungal exopolysaccharide scleroglucan has high viscosity (Augsten et al., 2008) whereas curdlan, a product of several bacterial species, is gel forming (Zhang et al., 2009), and bacterial cellulose is microcrystalline and insoluble (Vuong et al., 2009; Wong et al., 2009). Curdlan and cellulose are composed of a single linkage type but several other homopolysaccharides, including scleroglucan and pullulan, possess two types of glucosyl linkage. Dextran, also a glucan, is more complex and contains three different types of linkage. Although most homopolysaccharides are composed of neutral sugars, a small number are polyanionic.

The majority of microbial polysaccharides are probably heteropolysaccharides. These range from polymers with two sugar components to others with four or five monosaccharides. The possible range of structures and of resultant differences in properties is very great indeed because of the number of possible linkages and configurations. Each hexose can be α- or β- linked; in the pyranose or furanose form; and linked through the 2,3,4 or 6 position. However most of the polysaccharides that have been discovered so far are formed from two or three sugars and various acyl substituents (Maina et al, 2008). It is also probable that most of the polymers with industrial potential will contain certain types of linkage, at least in the main chain, as these will confer the desired physical properties. However, a much wider range of structures can be expected among polysaccharides of medical interest.

The rhelogical behaviour of polysaccharides solutions and the influence of physical or chemical factors on the rheological properties are important because they offer informations on the bioprocess, the biopolymer quality, the relations between microstructure and physical properties (Pelletier et al., 2001; Xu et al. 2008; Harrington et al., 2009), textural analysis (Moreno et al., 2000; Purwandari et al., 2007). Rheological characteristics depend on a large number of factors such as temperature, pH, solvent, ionic strength, molar mass, concentration (Mironescu, 2003).

For instance, temperature influences rheological behaviour of gellan gum. Gellan gum at 1% concentration produces a highly viscous solution, with the help of a sequestrant, which is more pseudoplastic than a high molecular weight sodium alginate solution. Gellan gum solution is sensitive to increases in temperature and undergoes a dramatic drop in viscosity over a very small temperature range at a relatively low temperature. This change in viscosity is completely reversible and most probably reflects a conformational change from some form of relatively ordered, non-aggregated double helix to a random coil (Imeson, 1999).

Because of their versatile and fascinating physico-chemical and rheological properties, the microbial exopolysaccharides act as new biomaterials and find themselves broad areas of applications in many industries like food (Dlamini et al., 2009), medicine (Miranda et al., 2008; Novak et al., 2009) textiles (Fijan et al., 2009), detergents (Friedmann et al., 2009), adhesives (Hoffmann et al., 2009), microbial enhanced oil recovery (MEOR) (Singh et al., 2007), wastewater treatment (Pant et al., 2007; Chen et al., 2008), dredging (Yim et al., 2007), brewing (Alsteens et al., 2008), downstream processing (Maina et al., 2008), cosmetology (Im et al., 2009), pharmacology (Yan et al., 2009), (Kumar et al., 2004).

The industrial use of polysaccharides based until recently on materials extracted from plants (starch, cellulose, pectins, galactomanans, gums) or algae (carragenan, alginates, agar) (Ruas-Madiedo et al, 2005). In the last three decades, biotechnologies for the microbial production of extracellular polysaccharides were developed. Some of them have proved to be useful industrial products which compete with plant and algal polysaccharides as well as synthetic products (Zhao et al., 2009; Singh et al., 2009). While dextran was the first microbial polysaccharide to be commercialized and to receive approval for food use, several such polymers now have a variety of commercial uses. The success is because of their properties and to the diversity of producing microorganisms and synthesised polysaccharides (Sutherland, 2002)

Bacteria are the microorganisms used in the industrial production of exopolysaccharides. Species of *Xanthomonas, Leuconostoc, Pseudomonas, Alcaligenes* which produce xanthan, dextran, gellan, curdlan are the most well known and industrially used ones. Actually, high attention is given to the exopolysaccharides produced by lactic bacteria (Sutherland, 2002; Sugimoto et al., 2008) which are already accepted as GRAS (Generally Recognised As Safe) and the most adequate for the food industry (Girard et al., 2007; Girard et al., 2008).

In spite of these developments, only one microbial polysaccharide, xanthan, finds itself a place among the 10 industrial polysaccharides utilized in the largest amount. However, the enormous range of polysaccharides synthesized by microorganisms has yet to be adequately explored. The list of major food and industrial polysaccharides employed was given in Table 2.3.

Table 2.3. Major food and industrial polysaccharides in order of decreasing consumption (Glazer et al., 2007)

Polysaccharides
Cornstarch and derivatives
Cellulose and derivatives
Guar gum and derivatives
Gum arabic
Xanthan
Alginate
Pectin
Carrageenan
Locust bean gum
Ghatti gum

Xanthan also ranks high among fermentation products when it is considered its market value. Below, Table 2.4 denotes the market value of xanthan between the predominant fermentation products.

Table 2.4. Estimated global market for fermentation products by category, in millions of U.S. dollars (Glazer et al., 2007)

Fermentation Products	
Crude antibiotics	5000
Amino acids	3435
Organic acids	2321
Enzymes	2006
Vitamins	1013
Xanthan	335

Xanthan is the benchmark product, being a major commercial biopolymer for many ways. It received food approval many years ago. It is relatively

inexpensive product because of the high conversion of substrate to polymer (60-70%) (Sutherland, 1998).

Solutions of xanthan, which are highly pseudoplastic, remain stable over a wide range of pH and temperature, rapidly regain viscosity on removal of shear stress and show very good suspending properties; they show high viscosity at low shear rates. The polysaccharide is incorporated into foods to alter the rheological properties of the water present, and has found applications that take advantage of many of its physical properties. Principle applications of xanthan are shown in Table 2.5.

The fungal polysaccharides are limited, pullulan produced by *Aureobasidium pullulans* (Manitchotpisit et al., 2009) and scleroglucan synthesized by *Sclerotium glucanicum* (Marchetti et al., 2009) being the most known and already obtained at technical scale. The main bacterial and fungal polysaccharides produced at industrial and technical level as presented in Table 2.6.

Table 2.5. Major industrial applications of xanthan gum (reconstructed from Garcia-Ochoa et al., 2000)

Application	Concentration (%w/w)	Function	Reference
Foods and beverages			
Salad dressing	0.1–0.5	Emulsion stabilizer	Silva et al., 2010
Dry mixes	0.05–0.2	Facilitates dispersion in hot or cold water	Nakai, 2006
Syrups, relishes, sauces, toppings	0.05–0.2	Thickener; provides heat stability and uniform viscosity	Kambourova et al., 2009
Beverages	0.05–0.2	Stabilizer	Mirhosseini et al., 2008
Frozen foods	0.05–0.2	Improves freeze–thaw stability	Fernandez et al., 2008
Pharmaceuticals and cosmetics			
Creams and suspensions	0.1–1.0	Emulsion stabilizer	Papagianni et al., 2009
Controlled-release tablets		Regulates disintegration rate	Baumgartner et al., 2008
Shampoo, liquid soaps, toothpaste	0.2–1.0	Improves flow properties and lather stability	Colquitt, 2002
Agriculture			
Additive in animal feeds and pesticide formulations	0.03–0.4	Suspension stabilizer; increases spray cling and permanence	Fitzsimons et al., 2008; ElShafei et al., 2010
Oil production			
Oil drilling aid	0.1–0.4	Thickener in drilling fluids that flush rock fragments away from drill bit	Hamed et al., 2009
Enhanced oil recovery	0.05–0.2	Facilitates oil displacement by increasing water viscosity	Maia et al., 2009
Other			
Textile printing and dyeing	0.2–0.5	Forms temperature-stable foams for printing and finishing; acts as flow modifier for dyeing heavy fabrics	Abdel-Halim et al., 2008
Ceramic glazes	0.3–0.5	Suspending agent	Raschip et al., 2007

Table 2.6. Principal bacterial and fungal polysaccharides (reconstructed from Glazer et al., 2007)

Polysaccharide	Organism	Composition	Reference
Bacteria			
Xanthan	*Xanthomonas campestris*	A pentasaccharide repeating unit containing glucose, mannose, glucuronic acid, acetyl, and pyruvate substituents	Vorhölter et al., 2008
Dextran	*Acetobacter* sp. *Leuconostoc mesenteroides* *Streptococcus mutans*	Polyglucose linked by *α*-1,6-glycosidic bonds; some 1,2-, 1,3-, or 1,4-bonds are also present in some dextrans	Guezennec et al., 1998 Sarwat et al., 2008 Saburi et al., 2007
Alginate	*Pseudomonas aeruginosa* *Azotobacter vinelandii*	Blocks of *β*-1,4-linked D-mannuronic residues, blocks of *α*-1,4-linked L-guluronic acid residues, and blocks with these uronic acids in either random or alternating order	Hay et al., 2009 Gaona et al., 2004
Curdlan	*Alcaligenes faecalis*	*β*-1,3-glucan (polyglucose)	Matsushita, 1990
Gellan	*Pseudomonas elodea*	Partially *O*-acetylated polymer of glucose, rhamnose, and glucuronic acid	Jansson et al., 1983
Cellulose	*Gluconacetobacter xylinu*	*β*-1,4-linked D-glucopyranose polymer	Nguyen et al., 2008
Fungi			
Scleroglucan	*Sclerotium glutanicum*	Glucose units primarily *β*-1,3-linked with occasional *β*-1,6-glycosidic bonds	Sutherland, 1998
Pullulan	*Aureobasidium pullulans*	Glucose units primarily *α*-1,4-linked with occasional *α*-1,6-glycosidic bonds	Gniewosz et al., 2008

2.2. Extremophiles

The reasonable environments are important for a sustainable human life. Reasonable environments mean a pH near neutral, temperature between 20 and 40°C, air pressure 1 atm and adequate levels of available water, nutrients and salts. Any ecological system beyond these limitations such as acidic, hot springs, salt and soda lakes, deserts, ocean beds is considered severe for the continuity of a normal life. Any environmental condition that can be perceived as beyond the acceptable range is an extreme condition. However, there are various microorganism survive in such environments (Satyarana et al., 2005)

Extremophiles are microorganisms that can thrive in environments that would kill most other organisms like radiation, metals, low levels of nutrient, light or water. Not only do they survive in these devastating environments, but also they grow, reproduce, and do better in extreme environments than in any other place (Cavalier-Smith, T., 2002)

Extreme conditions can be physical (temperature, radiation, pressure) or geochemical (desiccation, salinity, pH, oxygen species, redox potential, metals and gases) (Javaux, 2006).

Extremophiles keep up with these extreme environments in two general ways. They either exclude the environmental stress from their cytoplasm by the unusual properties of the cell membrane or all intracellular components have to be functional at the extreme conditions (Kazak et al., 2009)

Life in extreme environments has been studied great deal giving attention to the diversity of organisms and molecular and regulatory mechanisms involved. The products obtainable from extremophiles such as polysaccharides, proteins, enzymes are of great interest to biotechnology.

Notably the thermophiles have yielded a range of enzymes used in biotechnology (Hough and Danson, 1999). The properties of acidophilic metal

leaching microorganisms were used to recover copper and other metals from metal ores long before the nature of the involvement of the bacteria was understood. A famous example of extremophile based biotechnology is the *Taq* polymerase used for the amplification of DNA in the polymerase chain reaction. This enzyme, obtained from the thermophile *Thermus aquaticus*, has found applications in biotechnology and industry (Gibbs et al., 2009). Other examples are the exploitation of exoenzymes of alkaliphilic bacteria in washing powders and other detergents, the present and predicted future use of bacteriorhodopsin, the light driven proton pump of halobacteria, in a range of applications from holography to computer memories and processors. A recent success story is the industrial production of ectoine (1,4,5,6-tetrahydro-4-pyrimidine carboxylic acid), an organic compatible solute accumulated by many halophilic members of the domain Bacteria to withstand the osmotic pressure of their highly saline environment. This compatible solute is being used both as a stabilizer of enzyme preparations and as an additive to skin treatment cosmetics (Buenger and Driller, 2004). Superbugs, the name given to extremophiles for their unusual properties, signifies both the abilities of these organisms to live in unusual environments and the possibilities for their economic exploitation (Horikoshi, 1991).

The range of environmental extremes tolerated by microbes is much broader than other life forms (Table 2.7). Many of these extremophiles cannot live under the more moderate conditions preferred by most living organisms. Examples of such specialists with a low level of adaptability to changing environmental conditions are the acidophile *Picrophilus*, which dies after even a short exposure to a pH higher than 4, and the halophile *Halobacterium salinarum*, which lyses as soon as the salt concentration in its surroundings decreases below 100-150 g/L (Rainey and Oren, 2006).

Table 2.7. Examples of extremophiles and extreme environments (reconstructed from Cavicchioli, 2002)

Class of Extreme	Environment	Organism	Defining Growth Condition	Reference
High temperature growth (hyperthermophile)	Submarine vent, terrestrial hot spring	*P. fumarii* (A)	T_{max} 113°C	Goncalves et al., 2008
High temperature survival	Soil, growth media contaminant	*Moorella thermoaceticum* (spore) (B)	2 h, 121°C, 15 psi	Byrer et al., 2000; Li et al., 2009
Cold temperature (psychrophile)	Snow, lakewater, sediment, ice	Numerous [e.g., *Vibrio, Arthrobacter, Pseudomonas* (B), and *Methanogenium* (A) spp.]	-17°C	Murray et al., 2007 Hildebrandt et al., 2009; Singh et al., 2009
High acid (acidophile)	Dry solfataric soil	*Picrophilus oshimae/torridus* (A)	pH_{opt} 0.7 (1.2 *M* H_2SO_4)	Hess, 2008
High salt (halophile)	Saline lakes, evaporation ponds, salted foods	Mainly archaeal halophiles (e.g., *Halobacterium* spp. and *Halorubrum* spp.)	Saturated salt (up to 5.2 *M*)	Kish et al., 2009; Camacho et al., 2010
High alkaline (alkaliphile)	Soda lakes	*Bacillus* spp., *Clostridium paradoxum* (B), *Halorubrum* species (A	pHopt >10	Hu et al., 2008; Wilks et al., 2009
Radiation (radiation tolerant)	Soil, nuclear reactor water core, submarine vent	*D. radiodurans, Rubrobacter* spp., *Kineococcus* sp. (B), *Pyrococcus furiosus* (A)	High, γ, UV, x-ray radiation (e.g., >5,000 Gy γ radiation and .400 J m^2 UV)	Bentchikou et al., 2010; Pasternak et al., 2010
Toxicity (toxitolerant)	Toxic waste sites, industrial sites, organic solutions and heavy metals	Numerous [e.g., *Rhodococcus* sp. (B)]	Substance-specific (e.g., benzene-saturated water)	Urai et al., 2006; Kolomytseva et al., 2009
High pressure (barophile or piezophile)	Deep sea	Various [e.g., *Photobacterium* sp. (B), *Pyrococcus* sp. (A)]	Deep open ocean or submarine vent (e.g., pressure in Mariana Trench is 1.1 tons cm^{-2})	Eloe et al., 2008; El-Hajj et al., 2009; Xiang et al., 2009
Low nutrients (oligotroph)	Pelagic and deep ocean, alpine, and Antarctic lakes, various soils	*S. alaskensis, Caulobacter* spp. (B)	Growth with low concentrations of nutrients (e.g.,<1 mg/L dissolved organic carbon) and inhibited by high concentrations	Williams et al., 2009; England et al., 2010
Low water activity (xerophile)	Rock surfaces (poikilohydrous), hypersaline, organic fluids (e.g., oils)	Particularly fungi (e.g., *Xeromyces bisporus*) and Archaea (e.g., *Halobacterium* sp.)	Water activity (*a*w) <0.96 (e.g., *X. bisporus* 0.6 and *Halobacterium* 0.75)	Saum et al., 2008; Williams et al., 2009
Rock dwelling (endolith)	Upper subsurface to deep subterranean	Various [e.g., *Methanobacterium subterranean* (A), *Pseudomonas* sp. (B)]	Resident in rock	Chazottes et al., 2009; Vogel et al., 2009

A: Algaea; B: Bacteria

There are many kinds of microorganisms, considered extremophilic according to the criteria used above, that are exposed simultaneously to more than one environmental extreme, and many of these are optimally adapted to life under multiple stres factors (Mesbah et al., 2008). Such polyextremophilic organisms, as they were termed by Rothschild and Mancinelli (2001), are quite common, as combinations of different forms of stress are often found in nature. Frequently found combinations are hot+acidic (volcanic areas, hot springs), saline+alkaline (soda lakes), and cold+high pressure (the deep sea). Other combinations are hot+high pressure (deep sea hydrothermal vents) and hot+high pH (certain hot springs). Table 2.8 presents a few examples of such polyextremophiles.

Extremophiles are useful organisms for multibillion dollar industries, including agricultural, chemical synthesis, laundry detergents and pharmaceuticals. Enzymes are sought that are stable and functional in economically preferable environments, such as high or unstable temperatures (Gomes et al., 2004). Enzymes from extremophiles, extremozymes, have potential in multiple areas, either by using the enzymes themselves, or by using them as sources of ideas to modify mesophile derived enzymes. A well known example of extremophile derived enzymes in biotechnology is Taq polymerase, the enzyme widely used polymerase chain reaction (PCR).

Other extremophiles have industrial applications. For example, some Antarctic bacteria produce polyunsaturated fatty acids, an essential dietary ingredient for many aquaculture species such as Atlantic salmon. Antarctic bacteria have potential in bioremediation of waters following oil spills, which is a concern in cold waters. *D. salina* is widely used for the commercial production of b-carotenes, which it produces in response to solar radiation, and glycerol, which it produces to counterbalance external osmotic pressure. Direct uses include marketing of dried *Dunaliella* as a nutritional supplement, primarily as an antioxidant. Antifreeze proteins show potential as cryoprotectants of frozen organs Some examples of extremophiles and their enzymes which are useful for industry are shown in Table 2.9 (Rothschild et al., 2001).

Table 2.8. Some examples of ‘‘polyextremophilic’’ microorganisms, adapted to life at a combination of several environmental extremes (Reconstructed from Seckbach et al., 2004).

Environmental factors	Organism	Habitat	Phylogenetic affiliation	Tolerance to stress	Reference
High temperature+Low pH	*Thermoplasma acidophilum*	Solfatara fields, Coal refuse piles	Archaea - Euryarchaeota	Maximum temperature 63 ^{o}C; Minimum pH 0.5	Shimada et al., 2008
	Picrophilus oshimae	Acidic hot springs	Archaea - Euryarchaeota	Maximum temperature 65 ^{o}C; Minimum pH 0.06	Park et al., 2003
	Metallosphaera spp.	Solfataras, Uranium mines	Archaea - Euryarchaeota	Maximum temperature 80 ^{o}C; Minimum pH 1	Etzel et al., 2008
	Sulfurisphaera	Acidic hot springs	Archaea - Crenarchaeota	Maximum temperature 92 ^{o}C;Minimum pH 1	Aditiawati et al., 2009
	Cyanidium caldarium	Acidic hot springs	Eukarya	Maximum temperature 57 ^{o}C;Minimum pH 0.2	Huss et al., 2002
High pH+High salt concentration	*Natronobacterium magadii*	Soda lakes	Archaea - Euryarchaeota	Maximum pH 12;NaCl saturation	Katoh et al., 2003
High temperature+High pH	*Thermococcus alcaliphilus*	Shallow marine hydrothermal springs	Archaea – Euryarchaeota	Maximum temperature 90 ^{o}C;Maximum pH 10.5	Keller et al., 1995
High temperature+High pressure	*Thermococcus barophilus*	Thermal vent, Mid-Atlantic Ridge	Archaea – Euryarchaeota	Maximum temperature 100 ^{o}C;Requires 15-17.5 Pa pressure at the highest temperatures	Marteinsson et al., 1999
High temperature+High radiation	*Deinococcus geothermalis*	Thermal springs	Bacteria	Maximum temperature 50 ^{o}C. Resistant to at least 10 kGy gamma radiation	Ferreira et al., 1997; Makarova et al., 2007
Low temperature+High pressure	All deep-sea bacteria	Deep sea environments	Bacteria	Live at 2-4 ^{o}C and pressures of 50-110 Mpa	Allen et al., 1999; Simonato et al., 2006; Lauro et al., 2008; Wang et al., 2008

Table 2.9. Examples of extremophiles in industry and biotechnology (Reconstructed from Rothschild et al., 2001)

Industrial process	Biomolecule	Advantages	Source organism	Reference
Hydrolysis of starch to produce soluble dextrins, maltodextrins and corn syrups	α-Amylase	High stability, aciduric, bacterial amylase	*Bacillus stearothermophilus*	Ben Ali et al., 2006
Paper bleaching	Xylanases	Decreases amount of bleach needed	Thermophiles	Oakley et al., 2003
Prevent stalling in range of baked products	α-Amylase	Gives boost to yeast fermentation		Ben Ammar et al., 2002
Food processing, baking, brewing, detergents	Proteases	Stable at high temperatures	Thermophiles	Abusham et al., 2009
PCR reaction	DNA polymerase	No need to add additional enzyme during each cycle	Thermophiles	Harve et al., 2010
Cheese maturation, dairy production	Neutral proteases	Stable at low temperatures	Psychrophiles	Aguilar, 1996
Degradation of polymers in detergents	Proteases, amylases, lipases	Improved performance of detergent	Psychrophiles	Joseph et al., 2008
Degradation of polymers in detergents	Cellulases, proteases, amylases, lipases	Stable at high pH	Alkaliphiles	Ito et al., 1998; Horikoshi, 1999
Mariculture	Polyunsaturated fatty acids	Produced in cold temperatures	Psychrophiles	Sushchik et al., 2010
Bioremediation	Reduction of oil spils	Works efficiently in cold waters	Psychrophiles	Margesin, 2000; Ghaly et al., 2001
Pharmaceuticals	Polyunsaturated fatty acids		Psychrophiles	Nichols et al., 2002
Biosensors	Dehydrogenases		Psychrophiles	Alqueres et al., 2007
Desulphurication of coal	Sulphur oxidation		Acidophiles	Rohwerder et al., 2007
Antibiotic production	Antibiotics		Alkaliphiles	Vasavada et al., 2006
Food colouring	Carotene	Inexpensive to produce	Halophiles/ *Dunaliella*	Fujiwara, 2002; DasSarma et al., 2001; Harun et al., 2010
Pharmaceuticals	Glycerol, compatible solutes	Inexpensive to produce	Halophiles	Roberts, 2005
Surfactants for pharmaceuticals	Membranes		Halophiles	Blamey, 2009

Extremophiles are found in all three domains of life: Archaea, Bacteria and Eukarya. They are classified, according to the conditions in which they exist, such as :

- Acidophile: An organism that grows best at acidic (low) pH values.
- Alkaliphile: An organism that grows best at high pH values.
- Anaerobe: An organism that can grow in the absence of oxygen.
    - Facultative Anaerobe: An organism that grows in the presence or in the absence of oxygen.
    - Obligate Anaerobe: An organism that cannot grow in the presence of oxygen; the presence of oxygen either inhibits growth or kills the organism.
- Endolith: An organism that lives inside rock or in the pores between mineral grains.
- Halophile: An organism requiring high concentrations of salt for growth.
- Methanogen: An organism that produces methane from the reaction of hydrogen and carbon dioxide, member of the *Archaea*.
- Oligotroph: An organism with optimal growth in nutrient limited conditions.
- Piezophile (Barophile): An organism that lives optimally at high hydrostatic pressure.
- Psychrophile: An organism with optimal growth at temperature 15 °C or lower.
- Thermophile: An organism with optimal growth at temperature 40 °C or higher.
    - Hyperthermophile: An organism with optimal growth at temperature 80 °C or higher.
- Toxitolerant: An organism able to withstand high levels of damaging elements (e.g., pools of benzene, nuclear waste).
- Xerophile: An organism capable of growth at very low water activity.

2.2.1. Acidophile

Acidophiles are organisms that can withstand and even thrive in acidic environments where the pH values less than 3 (Austin et al., 2007). Acidophiles include certain types of eukaryotes, bacteria and archaea that are found in a variety of acidic environments, including sulfuric pools and geysers, areas polluted by acid mine drainage, and even our own stomachs.

Types of acidophiles include the red alga *Cyanidium caldarium* and the green alga *Dunaliella acidophila*, which can live below pH 1, as well as the fungi, *Acontium cylatium*, *Cephalosporium* sp., and *Trichosporon cerebriae*, which grow near pH 0. The archean *Picrophilaceae* are the most acidophilic organisms known and are able to grow at negative pH values (Fütterer et al., 2004).

Normally, high acid levels destroy cells. However, acidophiles have evolved a variety of specialized mechanisms to maintain their internal cellular pH at a constant level, usually 7.2. These mechanisms include passive regulation, not requiring the cell to expend energy, and active regulation which requires energy. Passive pH regulation defenses primarily rely on enforcing the cell membrane against an unfavorable environment. Some microorganisms secrete a biofilm to slow down the diffusion of molecules into the cell, while others are able to change their cell membrane to incorporate substances such as fatty acids that protect the cell. Another important way that microbes passively regulate their pH is by secreting buffer molecules that help to raise pH. Some have also evolved active pH regulation, which gives them the ability to pump hydrogen ions out of their cells at a constantly high rate. By doing this they are able to keep their internal pH at around 6.6-7.1 (Messerli et al., 2005).

Acidophiles have been the focus of substantial research in recent years, particularly regarding their role in acid mine drainage. One of the most well known acidophiles with respect to this important environmental problem is *Ferroplasma*, which was found growing at pH 0 in acid mine drainage in Iron Mountain in California (Golyshina et al., 2009).

2.2.2. Alkaliphile

There are no precise definitions of what characterizes an alkaliphilic or alkalitolerant organism. Several microorganisms exhibit more than one pH optimum for growth depending on the growth conditions, particularly nutrients, metal ions, and temperature. Therefore, the term "alkaliphile" is used for microorganisms that grow optimally or very well at pH values above 9, often between 10 and 12, but cannot grow or grow only slowly at the near neutral pH value of 6.5 (Horikoshi, 1999).

Cells face many challenges in an alkaline environment. Of greatest significance is the ability to maintain internal pH. If cells are to survive in an alkaline environment, they must make their cytoplasm more acidic to buffer the alkalinity. Cell walls containing acidic polymers function as a negatively charged matrix and may reduce the pH value at the cell surface. In addition, enzymes, both excreted and surface located, must be resistant to the effects of extreme pH. Finally, the pH gradient must be reversed to carry out ATP synthesis (Horikoshi, 1999).

While alkaliphilic taxa have been identified in each of the three domains of life (Eukarya, Archaea, and Eubacteria), the bioenergetics of alkaliphilic bacteria are perhaps the best understood. Internal pH maintenance is achieved by both active and passive regulation mechanisms in alkaliphilic bacteria. Cytoplasmic pools of polyamines and low membrane permeability are two modes of passive regulation, whereas sodium ion channels drive the active regulation. In the first instance of passive pH regulation, some cells have been found to contain cytoplasmic pools of polyamines. Particularly rich in amino acids with positively charged side groups (lysine, arginine, and histidine), these cells are able to buffer their cytoplasm in alkaline environments. Low membrane permeability is another mode of passive regulation as it ensures that fewer protons move in and out of the cell. Typically, protons enter and leave a cell via diffusion at about the same rate, therefore not having much impact on homeostasis in an extreme pH environment. Some alkaliphilic bacteria, however, have developed sodium ion channels that

actively drive the entry of protons across the membrane, thus decreasing the overall pH of the cytoplasm (Padan et al., 2005).

Enzymes from microorganisms that can survive under extreme pH can be particularly useful for applications under highly alkaline reaction conditions, for example in the production of detergents (van den Burg, 2003).

2.2.3. Anaerobe

Environments without oxygen can be found in the Earth from places as unglamorous as a waste management plant to deep hydrothermal vents on the ocean floor. The commonality between these extremes is that they lack oxygen. Oxygen the gas that humans rely upon as one of the essential components for metabolism many microbes find toxic, these places are where life without oxygen can be found.

Environments without oxygen can be found in a variety of places. These environments are ones where oxygen is absent from the chemicals species in the system. The lack of oxygen in these systems can be because of physical or chemical conditions, and the durations of anoxia can last from extremely long periods to short diurnal periods (De Meester et al., 1997; Debolskaya et al., 2008). Zones of anoxia exists as phenomena know as a dead zones. Dead zones are areas where the bottom water (the water at the sea floor) is anoxic, meaning that it has very low (or completely zero) concentrations of dissolved oxygen. These dead zones are occurring in many areas along the coasts of major continents, and they are spreading over larger areas of the seafloor (Weston et al., 2008).

The cause of anoxic bottom waters is fairly simple: the organic matter produced by phytoplankton at the surface of the ocean sinks to the bottom, where it is subject to breakdown by the bacteria. The problem is, while phytoplankton use carbon dioxide and produce oxygen during photosynthesis, bacteria use the oxygen dissolved in the water and give off carbon dioxide during respiration.

2.2.4. Endoliths

In some terrestrial locations which are devoid of sustainable life due to extremes of temperature, the existence of endolithic organisms is recognized. Endoliths are organisms that live inside rocks or in pores between mineral grains. These creatures provides an example of terrestrial terminal extremophilic behaviour before life finally becomes untenable. They are thought to have been found in a large range of environments, from rocks on the Earth's surface to miles beneath the subsurface, from the shallow surface to the deep terrestrial and ocean crust. In the Antarctic Dry Valleys and in cold desert sites such Mars Oasis, Antarctica, endoliths provide the sole example of life forms for which an ambient temperature of -32 ^{o}C and a humidity of 0% is a normal occurrence. Surface dwelling organisms cannot survive the strong erosion of the polar winds and UV-radiation insolation at the terrestrial surface which is worsened by atmospheric ozone depletion at higher latitudes. A typical Antarctic endolith consists of a stratified colony in which cyanobacterial layers are situated up to 1 cm. below a sandstone surface. The presence of an $Fe_2O_3/Fe(OH)_3$ coating at the rock surface to absorb low wavelength UV-radiation and the porosity of the quartz crystals in the sandstone rock are perceived to be essential for the survival strategy of the endolithic colony (Villar et al., 2005)

There are thousands of known species of endoliths, including members from bacteria, archaea, and fungi (Pointing et al., 2009). Many endoliths are autotrophs, meaning they are able to make their own organic compounds by utilizing gas or dissolved nutrients from water moving through fractured rock. Others may incorporate inorganic compounds found in their rock substrate by excreting acids to dissolve the rock (Bungartz et al., 2004).

2.2.5. Halophile

Halophilic microorganisms dwell hypersaline environments with salt concentrations up to NaCl saturation. They are donated with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects

of salts. Halophiles might be classified as slightly, moderately or extremely halophilic, depending on their requirement for NaCl. Slight halophiles grow optimally at 0.2-0.85 mol/L (2-5%) NaCl; moderate halophiles grow optimally at 0.85-3.4 mol/L (5-20%) NaCl; and extreme halophiles grow optimally above 3.4-5.1 mol/L (20-30%) NaCl (Ozusaglam et al., 2008). Halophiles are found in all three domains of life: Archaea, bacteria, and eucarya. The extremely halophilic archaea, in particular, are well adapted to saturating NaCl concentrations and have a number of novel molecular characteristics, such as enzymes that function in saturated salts, purple membrane that allows phototrophic growth, sensory rhodopsins that mediate the phototactic response, and gas vesicles that promote cell flotation.

Hypersaline environments are characterized by a low water content or water activity (a_w) level because of the high salt concentration. Water activity is defined as the ratio of the vapor pressure of the air in equilibrium with a substance or solution to the vapor pressure of pure water at the same temperature. Values for a_w range from 0 to 1 and are unitless. Pure water has an a_w of 1.00, whereas seawater has an a_w of 0.98, and saturated NaCl has an a_w of 0.72. Typically, growth does not occur at a_w below 0.72 with the halophilic archaea approaching this lower limit : As the a_w decreases, the water needed to dissolve nutrients, to maintain general metabolic processes, and hydrating proteins and nucleic acids decreases a well (Grant, 2004).

The halophilic microbes are colored with carotenoid compounds in their cell membrane, painting the waters with a pink-orange hue (Asker et al., 2002). Some species also have a purple membrane, regions where bacteriorhodopsin (BR) or other rhodopsin like chromo proteins reside.

In general, nonhalophilic organisms tolerate NaCl levels up to 0.2 mol/L. Halophiles, however, thrive at NaCl levels of 3.5 mol/L. To prevent the loss of cellular water to the environment, halophiles accumulate solutes within the cytoplasm. These solutes may be either inorganic ions or organic molecules, and their function is to balance the internal osmotic pressure with the external osmotic

pressure to maintain cellular integrity. Sodium ions, along with many inorganic ions, will passively diffuse across the membrane. This creates a problem for microbes lacking an adaptive mechanism for dealing with the increased salt concentrations. Nonhalophilic cells when exposed to >10-12% salts will lyse because of the excessive osmotic pressure generated by the high external Na^+ concentrations compared to the internal Na^+ concentrations. On the other hand, halophilic archaea, by use of a Na^+ pump, push Na^+ ions out of the cell, while concentrating K^+ ions within the cell in order to balance osmotic pressure. This balance consists of an internal concentration of K^+ at around 5M and an outside concentration of Na^+ at around 4M (Litchfield, 1998).

Although the halophilic archaea survive in the hypersaline environments through the control of inorganic ion concentrations, the halotolerant bacteria and algae have developed an alternative osmotic pressure balancing mechanism by synthesizing specific organic molecules as their compatible solutes. The halotolerant bacteria, for example, adjust their osmotic pressure by taking up the compatible solute from the environment: *Halornonas elongata* concentrates glycine betaine from the environment, but it can also synthesize ectoine. The halotolerant alga, *Dunaliella,* synthesizes glycerol as its compatible solute. Thus, the halophilic bacteria and *Dunaliella* spp. have multiple mechanisms for osmoregulation, whereas the halophilic Archaea rely totally on inorganic salts (Litchfield, 1998).

Microbes that inhabit hypersaline lakes experience intense ultraviolet (UV). In order to survive in this type of environment, halophiles have efficient DNA repair, but they also have mechanisms to prevent damage. For example, halophilic archaea have a low number of UV "targets," thymines, in their genomes. The colorful carotenoids may also be a strategy for photoprotection as mutant colorless halophiles are UV sensitive. These are a class of very important antioxidants that may provide protection from UV damage. Exposure to UV light is necessary for the activation of Bacteriorhodopsin, a purple chromoprotein located within the cell membrane, which acts as a proton pump and drives ATP synthesis. Halobacterium species produce gas vesicles which enable them to float to the

surface of the water column where light and oxygen are readily available (DasSarma and Arora, 2001)

The halophilic microorganisms have found a number of interesting applications in biotechnology. They may be less fashionable in biotechnology research than the thermophilic extremophiles, but the numbers of applications that are either already being exploited or are under development are impressive.

The applications that the halophiles have found in biotechnology can be divided into a number of categories. Firstly, the halotolerance of many of their enzymes can be exploited wherever enzymatic transformations are required to function at low water activities, such as found in the presence of high salt concentrations. Secondly, some organic osmotic stabilizers produced by halophiles have found interesting applications. Thirdly, a number of halophilic microorganisms produce valuable compounds. Some of these are unique and not found elsewhere in the living world. Halophiles may also present distinct advantages for the development of biotechnological production processes of certain products that can also be found in nonhalophiles (Oren, 2002).

Table 2.10 lists the most important applications of the halophilic archaea, bacteria, and eucarya. These applications are already commercially available or are in different stages of development.

Generally, the halophilic microorganisms have some distinct advantages over their nonhalophilic counterparts. Possible contamination with undesired microorganisms is much less of a problem at high salt concentrations than in low salinity media.

Table 2.10. Some presently and potential biotechnological uses of halophilic microorganism (Reconstructed from Oren, 2002)

Product	Producing organism	Uses and present status of technology	Reference
Archaea			
Bacteriorhodopsin	*Halobacterium salinarum*	Holographic storage material, computer memories and processing units, photoelectric converters, and others, all in the experimental stage	Gonzales et al., 2009
Different carotenoid pigments	Different members of the *Halobacteriaceae*	Light absorption, increasing evaporation in saltern crystallizer ponds	Niemi et al., 1997; Manikandan et al., 2009; Oren et al., 2009
Poly-β-hydroxyalkanoate	*Haloferax mediterranei*	The organism has a high potential for PHA production ; not yet industrially exploited	Lu et al., 2008
Extracellular polysaccharides	*Haloferax mediterranei*	The organism has high potential for the production of valuable polysaccharides to be used, e.g., in the recovery of oil from oil wells, no large scale production	Parolis et al., 1996; Zhenming et al., 2005
Salt tolerant enzymes	Different halophilic *Archaea*	Not yet industrially exploited	Tapingkae et al., 2010
Soy sauce, fish sauce	Different halophilic and halotolerant microorganisms, *Bacteria* as well as *Archaea*	Halophilic bacteria are involved in the production; no pure cultures are used; the microbiological aspects are poorly controlled	Taira et al., 2007; Ko et al., 2009
Bacteria			
Ectoine and hydroxyectoine	*Halomonas elongata, Marinococcus* M52	Produced as enzyme stabilizer (molecular chaperone), moisturizer in cosmetics; industrial scale production has recently started	Canovas et al., 1999
Extracellular polysaccharide	*Halomonas* sp AAD6	Potential applications as a industrial gum, sweetener, emulsifier, stabilizer, encapsulating agent, carrier for flavor and fragrance	Poli et al., 2009
Salt tolerant enzymes	Different halophilic *Bacteria*	Not yet exploited on a large scale	Rao et al., 2009
Soy sauce, fish sauce	Different halophilic and halotolerant microorganisms, *Bacteria* as well as *Archaea*	Halophilic bacteria are involved in the production; no pure cultures are used; the microbiological aspects are poorly controlled	Masuda et al., 2008; Satomi et al., 2008
Eucarya			
β-Carotene	*Dunaliella* species	Produced as antioxidant and food coloring agent	Oren, 2005
Halophilic cell biomass for cosmetics	*Dunaliella* species	*Dunaliella* is being used as an additive in cosmetic antiwrinkle preparations.	Lamers et al., 2008; Michel et al., 2009

2.2.6. Oligotrophs

Bacteria can grow rapidly, yet there are some that grow slowly under apparent optimal conditions. These organisms are usually present in environments with low levels of nutrients, and are not found in conditions of more plentiful nutrients. They are known as oligotrophs. Oligotrophs are characterized both by their inability to grow and prosper in environments with high levels of nutrients, and by an ability to use low concentrations of substrates (Koch, 2001).

Natural locations with extremely low nutrient levels (snow, rain water pools, springs, free ocean water, Antarctic rocks and soils) do not contain more than 1-5 mg/L of organic carbon. Oligotrophs found here are especially adapted to constant famine: they frequently live attached to surfaces, form polymers and storage products even while starving, and often aggregate. Many of these oligotrophs alter their morphology with changing nutrient concentrations (Hirsch, 1986).

Characteristics that are considered to be important for oligotrophic microbes include a substrate uptake system that is able to acquire nutrients from its surroundings. Thus, oligotrophs would ideally have large surface area to volume ratio, high affinity uptake systems with broad substrate specificities and an inherent resistance to environmental stresses, e.g., heat, hydrogen peroxide and ethanol (Lauro et al., 2009).

2.2.7. Piezophile (Barophile)

Pressure has a substantial effect on the physiology and biochemistry of living cells. Microorganisms inhabiting the deep sea and subsurface of Earth have the ability to survive at hydro and litho static pressures greater than 1 atmosphere (atm). Piezophiles are microorganisms that possess optimal growth rates at pressures above atmospheric pressure. They are strictly dependent on high pressures. Piezotolerant microorganisms are capable of growth at high pressure, as well as at atmospheric pressure, but can be distinguished from piezophiles

because they do not have optimal growth rates at pressures above one atmosphere (Abe et al., 2001).

Increased pressure changes the volume and compresses packaging of lipids resulting in decreased membrane fluidity. Barotolerant bacteria under high hydrostatic pressure regulate the fluidity of membrane phospholipids to compensate for pressure gradients between the inside of the cell and the environment.

Barophilic bacteria are defined as those displaying optimal growth at pressures more then 400 atmosphere, whereas barotolerant bacteria display optimal growth at pressure less then 400 atmosphere and can grow well at a pressure of one atmosphere. There is also an obligately barophilic species that can grow at 70 to 80 atmosphere, but not below 50 atmosphere (Pikuta et al., 2007).

New findings from deep sea ecosystems have important applications in medicine and biotechnology. Mesophilic *Vibrio diabolicus* isolated from a vent sample, secretes an innovative exopolysaccharide of potential medical interest for its chemical resemblance to heparin, which delays the onset of blood clotting (Raguenes et al. 1997). Another new mesophilic vent strain of *Pseudomonas aeruginosa* is remarkable for its tolerance to high concentrations of cadmium (up to 5 mM). It not only tolerates but completely (>99%) removes the cadmium from solution by precipitation onto the cell wall, making it a prime candidate for applications in heavy metal recovery and environmental remediation (Wang et al. 1997).

2.2.8. Psychrophile

Psychrophiles are defined as organisms having an optimal temperature for growth at about 15ºC or lower, a maximal temperature for growth at about 20º C, and a minimal temperature for growth at 0ºC or below. Psychrophiles are found mostly in the Arctic and Antarctic oceans which remains frozen most of the year.

The food that is needed by psychrophiles is inside the frozen glaciers and seawater, but flows in tiny streams in between cracks and layers of ice.

Cold-adapted microorganisms are generally understood to achieve their physiological and ecological successes in cold environments as a result of their unique features.

Psychrophilic bacteria have the unique ability to survive and grow at low temperatures because of their cold active enzymes which have reduced activation energy leading to high catalytic effieciency which may possibly be attributed to an enhanced local or overall flexibility of the structure of the proteins. Thus, they could serve as excellent model systems to understand the molecular basis of low temperature adaptation. Their adaptation to low temperature is dependent on a number of survival strategies such as the ability to modulate membrane fluidity which helps to transport nutrients under very cold, otherwise rigidifying conditions, ability to carry out biochemical reactions at low temperatures, ability to regulate gene expression at low temperatures, and capability to sense temperature. Unlike mesophilic bacteria, psychrophiles have increased levels of unsaturated fatty acids that further increase with reduction in temperature, so as to modulate membrane fluidity, an important strategy for cold adaption. Carotenoids are also shown to regulate membrane fluidity due to their temperature dependent synthesis. The presence of cold active enzymes and the ability to support transcription and translation in psychrophiles were demonstrated at low temperatures. Studies have also revealed the presence of certain genes which were active at low temperature (Deming, 2002).

2.2.9. Thermophile

For most known species of eucarya, the temperatures around 100 ^{o}C usually denature proteins and nucleic acids, increase the fluidity of membranes to lethal levels, and degrade chlorophyll, above 75 ^{o}C, making photosynthesis impossible. Some members of two other domains bacteria and archaea grow at much higher temperatures. Traditionally all thermophilic microorganisms are

divided into three groups: moderately thermophilic, growth optimum at 50-60 ^{o}C, thermophilic, optimum higher than 70 ^{o}C, and hyperthermophilic, optimum higher than 80 ^{o}C. They are isolated from a number of marine and terrestrial geothermally heated habitats including shallow terrestrial hot springs, hydrothermal vent systems, sediment from volcanic islands, and deep sea hydrothermal vents (Pikuta et al., 2007).

All thermophiles require a hot water environment, but some thrive in more than one extreme, such as those with high levels of sulfur or calcium carbonate, acidic water, or alkaline springs. Regardless of varying environmental conditions, the ability of thermophiles to thrive in extremely hot environments lies in extremozymes, enzymes constructed to work in extremely high temperatures. The amino acids of these extremozymes have special abilities to retain their twisted and folded 3D structures in high heat, where other enzymes would unfold and no longer work (Hough et al., 1999).

Most hyperthermophiles are anaerobic and many are chemolithotrophs. There are no particular carbon use or energy generation pathways that are exclusively linked to growth at high temperatures. For any microbe, lipids, nucleic acids and proteins are generally susceptible to heat and therefore, there is no single factor that enables all thermophiles to grow at elevated temperatures. The membrane lipids of thermophiles, having ether bonds rather than the much more unstable ester bonds, contain more saturated and straight chain fatty acids than mesophiles. This allows thermophiles to grow at higher temperatures by providing the right degree of fluidity needed for membrane function. Many archaeal species contain a paracrystalline surface layer (S-layer) with protein or glycoprotein and this is likely to function as an external protective barrier (Claus et al., 2002).

2.2.10. Xerophile

Environments without water are ones where desiccation is common and water is a scarce resource. These environments range in temperature from very

hot to very cold. One thing they have in common is the lack of water. Organisms capable of growing under conditions of low water activity (a_w) are called xerophilic.

The apperant features of desiccation tolerance are few: a complete arrest of cellular metabolism, followed by time spent in a state of suspended animation and then subsequent recovery of metabolic functions (Potts, 2001).

Desiccation results in extreme osmotic stress and low water activity values. Below the (a_w) value of 0.70 the life cease. Some microbes are very resistant to drying, e.g., bacteria in the family *Deinococcaceae*. This is believed to stem from their thick cell walls, which help in protecting membrane integrity.. All xerophiles produce or accumulate low-molecular-mass organic compounds that have osmotic potential. These osmolytes, also known as compatible solutes because of the need for them to be compatible with cell machinery must also protect against inactivation, inhibition and denaturation of both enzymes and macromolecular structures under conditions of low water activity. The most important compatible solutes of microorganisms are polyols, sugars or sugar derivatives such as trehalose, sucrose, betaines, amino acids, ectoines (Grant et al., 2004).

2.3. Food Wastes and Byproducts

Over the past few decades, an increasing trend toward efficient utilization of natural resources has been observed around the world. The direct disposal of agro-industrial residues as a waste on the environment represents an important loss of biomass, which could be bioconverted into different metabolites, with a higher commercial value (Vendruscolo et al., 2008).

Food waste results from processing agricultural raw materials to foodstuffs, which arise as liquid, e.g., wastewater, and solid waste, is typically effected by the extraction or separation of the nutritionally valuable portion of the raw materials. The unused remain primarily consist of organic material but further

utilizing it as a food source is limited, because it possesses little nutritional value or contains inedible components (Russ et al., 2007)

The wastewater results from the cleaning processes or in the form of excessive or polluted process water. Its dry material content is typically less than 5% by mass. It possibly also contains organic or inorganic cleaning agents or disinfectants. Solid food wastes with an organic origin have remarkably high water content ,mostly about 80% by mass. They are usually characterized by a constant quality and purity because of the forgone processes.

The fact that these substances are removed from the production process as undesirable ingredients makes them, by definition of most European legislations, wastes. The term ‘‘by-product,’’ which is common in industry, points up that these are mostly ulterior usable substances, often with a market value.

Waste disposal is one of the major problems facing most food processing plants. Agriculture as the traditional way of waste utilization, a consequential outcome because most raw materials are also from agricultural origin, is no longer available because of major changes in law and technology. Furthermore, new kinds of process engineering and resultant new products and markets make the utilization of waste increasingly interesting (Russ et al., 2007).

The interest in polysaccharides has increased considerably in recent years, as they are candidates for many commercial applications in different industrial sectors like food, petroleum, pharmaceuticals. The polysaccharides have several advantages over chemical equivalents including biocompatibility and biodegradability. In spite of the advantages, fermentation must be cost competitive with chemical synthesis, and many of the potential applications that have been considered for exopolysaccharides depend on whether they can be produced economically. Fermentation medium can represent almost 50% of the cost for a microbial fermentation (Van Hoek et al., 2003). Employing complex media for growth are not economically attractive because of their high amount of expensive nutrients such as yeast extract, peptone and salts. Nevertheless, much effort in

fermentation process optimization has been done to produce the biopolymers economically from several inexpensive waste substrates, thereby decreasing their production cost (Table 2.11).

Media used for polysaccharide production in the laboratory or industry, are based on high ratios of carbon substrate:limiting nutrient, where nitrogen is usually the favoured component to induce growth limitation and stimulate EPS formation. Such media have been shown to favour polymer production for *Xanthomonas campestris, Pseudomonas* spp. and many other bacteria and also in EPS producing fungal species. It has to be remembered that in the large scale industrial process, the carbon substrate will almost certainly be a complex product, most probably obtained as a byproduct from the processing of agricultural or other plant material (Sutherland, 1996).

Under conditions employed for industrial production of microbial polysaccharides, the same principle of high carbon:nitrogen ratios is used, but the substrates utilized are the cheapest available form. Thus, corn-steep liquor, distillers solubles, acid or enzymic hydrolysates of starch or other substrates have been used to form the bases for large scale culture media. Some of these materials also provide sources of growth factors and amino acids required by the microorganisms.

The substitution of agricultural wastes for other substrates depends initially on the ability of the microbial species to utilize them for polymer production. Several of the EPS producing microorganisms are relatively limited in their nutritional capabilities and will only grow satisfactorily on relatively complex media such as yeast hydrolysates, corn-steep liquor and appropriate carbohydrate carbon sources such as sucrose, starch hydrolysates or molasses. The nature of the waste material to be used as substrate may also present problems if it contains growth inhibiting compounds or is highly coloured as is the case with alpechin. The use of starch hydrolysates and glucose has found wide acceptance, because the substrates are available in most parts of the world, are pure and

relatively cheap. This is also true of sucrose, although if molasses is employed some decolourization process may be required.

The future development of microbial polymers will depend on a number of factors, but the key question is whether they can they be produced economically or not. This problem can be solved by using agricultural wastes and byproducts which some of them are mentioned below briefly.

Table 2.11. Some examples of inexpensive waste substrates in the production of biopolymers

Microorganism	Biopolymer	Fermentation Substrate	Reference
Sphingomonas paucimobilis ATCC 31461	Gellan	Cheese whey	Fialho et al., 1999
Xanthomonas campestris NRRL B-1459 S4LII	Xanthan	Olive mill wastewater	Lopez et al., 2001
Azotobacter chroococcum	Alginate	Whey	Khanafari et al., 2007
Aureobasidium pullulans.	Pullulan	Olive mill wastewater	Ramos-Cormenzana et al., 1995
Halomonas eurihalina	V2-7	Various hydrocarbons	Martinez-Checa et al., 2007
Aureobasidium pullulans	Pullulan	Beet molasses	Lazaridou et al., 2002
Xanthomonas campestris ATCC 1395	Xanthan	Beet molasses	Kalogiannis et al., 2003
Leuconostoc mesenteroides	Dextran	Beet molasses	Vedyashkina et al., 2005
Leuconostoc mesenteroides NRRL B512(f)	Dextran	Carob pod, Whey	Santos et al., 2005
Agrobacterium	Curdlan	Sugar cane molasses	Lee et al., 1997
Sphingomonas paucimobilis ATCC-31461	Gellan	Sugar cane molasses	Banik et al., 2007
Mixed culture	PHA	Sugar cane molasses	Albuquerque et al., 2007
Acetobacter xylinum IFO 13772	Bacterial cellulose	Sugar cane molasses	Keshk et al., 2006
Lactobacillus rhamnosus RW-9595M	-	Whey permeate	Macedo et al., 2002
Rhizobium hedysari	Succinoglycan	Spent malt grains	Stredansky et al., 1999
Agrobacterium tumefaciens	Succinoglycan	Grated carrots	Stredansky et al., 1999
Paenibacillus jamilae	-	Olive mill wastewater	Morillo et al., 2006
Xanthomonas campestris	Xanthan	Citrus waste	Bilanovic et al., 1994
Xanthomonas campestris	Xanthan	Apple pomace	Stredansky et al., 1999
Xanthomonas campestris	Xanthan	Grape pomace	Stredansky et al., 1999
Lactobacillus delbrueckii ssp. *bulgaricus* RR	-	Whey	Briczinski et al., 2002
Zoogloea sp.	-	Sugar cane molasses	Paterson-Beedle et al., 2000
Acetobacter xylinus	-	Whey	Battad-Bernardo al., 2004
Pseudomonas elodea	-	Whey	Dlamini et al., 1997
Botryosphaeria sp.	Botryosphaeran	Sugar cane molasses	Steluti et al., 2004
Streptococcus thermophilus 1275	-	Whey protein concentrate	Zisu et al., 2003u
Botryosphaeria rhodina DABAC-P82	β-Glucan	Olive mill wastewater	Crognale et al., 2006
Xanthomonas campestris	Xanthan	Olive mill wastewater	Lopez et al., 2001
Lactobacillus delbrueckii subsp.bulgaricus	-	Whey	Shene et al., 2007
Beijerinckia indica	PS-7	Soybean pomace	Jin et al., 2006
Beijerinckia indica	PS-7	Apple pomace, Sikhye	Jin et al., 2002
Xanthomonas campestris	Xanthan	Spent malt grains	Stredansky et al., 1999
Xanthomonas campestris	Xanthan	Citrus peels	Stredansky et al., 1999
Lactic acid bacteria	Lactic acid	Whey, molasses	Hofvendahl et al., 2000
Escherichia coli	P3HB	Cheese Whey	Fonseca et al., 2008
Enterobacter agglomerans	-	Maple sap	Morin et al., 1995

2.3.1. Molasses

Initially the term molasses referred specifically to the final effluent obtained in the preparation of sucrose by repeated evaporation, crystallization and centrifugation of juices from sugar cane and from sugar beets. Today, several types of molasses are recognized and in general, any liquid feed ingredient that contains in excess of 43% sugars is termed molasses (Curtin, 1983).

- Cane Molasses is a byproduct of the manufacture or refining of sucrose from sugar cane. It must not contain less than 46% total sugars.
- Beet Molasses is a byproduct of the manufacture of sucrose from sugar beets. It must contain not less than 48% total sugars.
- Citrus Molasses is the partially dehydrated juices obtained from the manufacture of dried citrus pulp. It must contain not less than 45% total sugars.
- Hemicellulose Extract is a byproduct of the manufacture of pressed wood. It is the concentrated soluble material obtained from the treatment of wood at elevated temperature and pressure without use of acids, alkalis, or salts. It contains pentose and hexose sugars, and has a total carbohydrate content of not less than 55%.
- Starch Molasses is a byproduct of dextrose manufacture from starch derived from corn or grain sorghums where the starch is hydrolyzed by enzymes and/or acid. It must contain not less than 43% reducing sugars expressed as dextrose and not less than 50% total sugars expressed as dextrose.

2.3.1.1. Compostion of Molasses. The average composition and selected nutrient content of the various types of molasses is presented in Tables 2.12. As is often found with many industrial by-products, the chemical composition of molasses shows wide variation. Its composition is influenced by factors such as soil type, ambient temperature, moisture, season of production, variety, production practices at a particular processing plant, and by storage variables. consequently,

considerable variation may be found in nutrient content, flavor, color, viscosity and total sugar content.

Table 2.12. Composition and nutrient content of molasses products (Curtin, 1983)

Item	Cane	Beet	Citrus	Extract	Starch
Total Solids (%)	75	77	65	65	73
Specific Gravity	1.41	1.41	1.36	1.32	1.40
Total Sugars (%)	46	48	45	55	50
Crude Protein (%)	3	6	4	0.5	0.4
Nitrogen Free Extract (%)	63	62	55	55	50
Total Fat (%)	0	0	0.2	0.5	0
Total Fiber (%)	0	0	0	0.5	0
Ash (%)	8.1	8.7	6	5	6
Calcium (%)	0.8	0.2	1.3	0.8	0.1
Phosphorus (%)	0.08	0.03	0.15	0.05	0.2
Potassium (%)	2.4	4.7	0.1	0.04	0.02
Sodium (%)	0.2	1	0.3	-	2.5
Chlorine (%)	1.4	0.9	0.07	-	3
Sulfur (%)	0.5	0.5	0.17	-	0.05

2.3.1.2. Sugars. All types of molasses contain relatively large amounts of total sugars or carbohydrates and these compounds constitute the majority of the feeding value of molasses. Sugar mills can control the amount of sucrose extracted and because of this, the sugar content of molasses produced in different countries will vary according to the production technology employed.

2.3.1.3. Protein. As is presented in Table 2.12, none of the molasses types contain significant levels of crude protein. Also, the nitrogenous materials which are present consist mainly of non-protein nitrogen compounds which include amides, albuminoids, amino acids and other simple nitrogenous compounds.

2.3.1.4. Trace Elements and Vitamins. Molasses also contains many elements in very low concentrations. A considerable portion of the valuable substances withdrawn from the soil are minerals, trace elements, combined with vitamins can be found in the composition of the molasses (Table 2.12.; Table 2.13).

Table 2.13. Trace minerals in molasses

Mineral, mg/kg	Cane	Beet	Citrus
Copper	36	13	30
Iron	249	117	400
Manganese	35	10	20
Zinc	13	40	-

Table 2.14. Vitamins in molasses

Vitamin, mg/kg	Cane	Beet	Citrus
Biotin	0.36	0.46	-
Choline	745	716	-
Panthothenic acid	21	7	10
Riboflavin	1.8	1.4	11
Thiamine	0.9	-	-1

2.3.2. Olive Mill Wastewater

The manufacture of olive oil produces large amounts of a dark coloured juice. This olive mill wastewater (OMW) consists of a mixture of water from the olive, machinery cooling waters, fruit washings and remainder of the fruit. The composition of OMW is rather variable (Table 2.15) depending on crop, variety of fruit and in particular on the technological system used for oil extraction (press, centrifugation or filtration). This waste causes large-scale environmental problems in the Mediterranean area. Toxic effects are derived from the nature of some of their constituents: its extremely high organic load and the presence of recalcitrant organic compounds such as polyphenols with strong antimicrobial properties. OMW comprises about 15% organic material that is composed of carbohydrates,

proteins and lipids as well as a number of other organic compounds including monoaromatic and polyaromatic molecules (Aguilera et al., 2008)

A wide range of technological treatments are available nowadays. Most of them are focused on both bioremediation, as a means of reducing the polluting effect of OMW and biotransformation into valuable products, together with modification of the technology used in oil extraction. The possibility of using this waste as an alternative substrate for biopolymer production is reported in many papers (Israelides et al., 1995; Aguilera et al., 2001; Lopez et al., 2001) because of its high C/N ratio which could limit cellular growth but favour or stimulate EPS production. (Alburquerque et al., 2004; Baddi et al., 2004).

Because (OMW) is a pure vegetative byproduct containing polyphenolic compounds, the process should be optimized depending on the type and quality of the effluent employed (Lopez et al., 2001). The difficulty lies in the antimicrobial character of OMW which limits the concentration of the used waste as culture medium, usually not exceeding 50% (Aguilera et al., 2008).

Table 2.15. Olive mill wastewater composition (Ramos-Cormenzana et al., 1996)

	Concentration, (%)		
Component	**Maximum**	**Minimum**	**Medium**
Water	94.15	82.40	83.40
Organic matter	16.55	3.96	14.80
Fats	2.30	0.03	0.02-1
Organic nitrogen	2.40	0.06	1.2-2.4
Total sugars	8.00	0.10	2-8
Organic acids	1.00	0.20	0.5-1.5
Polyalcohols	1.80	0.30	1-1.5
Pectins, mucilages, tannins	1.30	0.20	0.5-1.5
Polyphenols	2.40	0.13	0.5-1
Polymers	1.50	0.50	-
Mineral matter (P, K, Ca, Fe, Mg, Mn, Na, Zn, Co, Cu, Si, Cl,)	7.20	0.40	1.8

2.3.3. Cheese Whey Powder

Whey is the major byproduct obtained during the preparation of dairy products such as cheese. The nutrient composition of whey is based on the nutrient composition of milk from which it is derived, which in turn is affected by many factors including how the milk was processed. Whey contains a pool of nutrients and growth factors that have the potential stimulate the growth of microorganisms.

Lactose, the major component of whey, is probably the least valuable component and most difficult to utilise. Lactose comprises about 70% of the total solids of whey. Whey proteins have a biological value (BV) of 100, which is a measure of the proportion of absorbed protein from a food which becomes incorporated into the proteins of the organism's body. This value is higher than those of casein, soy protein, beef, or wheat gluten. Whey proteins also have a high content of sulfur containing amino acids such as cysteine and methionine. Whey is a good source of electrolytes including sodium and potassium. Minerals such as calcium, magnesium, and phosphorus are present in solution and also partly bound to proteins. Zinc is present in trace amounts. Whey is also a rich source in terms of vitamins: B12, B6, pantothenic acid, riboflavin, biotin, thiamine, nicotinic acid, folic acid, and ascorbic acid are vitamins present in whey (Goyal et al., 2008).

The composition of cheese whey powder from a conventional cheese production is approximately as follows:

Table 2.16. Cheese whey powder composition (GEA Niro Company)

Composition	Approx. % of dry matter
Lactose	75
Protein	13
Ash	10
Fat	2

Whey and its derivatives could be a promising fermentation substrate for EPS production as reported by researches (Fialho et al., 1999; Macedo et al., 2002; Briczinski et al., 2002; Dlamini et al., 2007; Shene et al., 2007).

2.3.4. Grape Pomace

Only few researchers have published work on grape pomace, which today is a very significant waste product in agriculture industries. Grape pomace is the residue left after juice extraction by pressing grapes in the wine industry. In Spain alone, over 250 million kg of this byproduct, constituted by seeds, skin and stem, are used every year either as animal feed (with low nutritional value) or for ethanol production by fermentation and distillation (low level benefit). This material is underexploited and most of it is generally disposed in open areas, leading to serious environmental problems (Botella et al., 2005).

During vinification, lactic acid bacteria perform malolactic fermentation; they transform malic acid into lactic acid with a consequent decrease of wine acidity and taste improvement. Generally, this second fermentation is due to *0enococcus oeni*, a bacterial species particularly well adapted to the yeast fermented musts. Nevertheless, because of the abundance of microorganisms gathered with grapes, other species or even undesirable strains can grow during or after malolactic fermentation and release compounds which spoil wine quality. One of these microbial alteration is ‘‘ropiness’’, characterized by a viscous and thick texture of the beverage, because of the excretion of small amounts of a β-glucan by the bacteria (Walling et al., 2005)

The potential utility of this waste for value added products is promising. Considering the high carbohydrate content in the seeds (8%), and in the skin (13%), grape pomace can be considered as a rich source of substrates for EPS production.

2.3.5. Orange Bagasse

The processing of orange juice generates huge amounts of orange waste, approximately 50% of the fruit weight, which offers potential applications in biotechnology. The solid material arising from processing oranges for juice consists of peel, seed, and pulp, collectively referred to as orange bagasse, and primarily constitutes a waste product.

Orange bagasse contains large amounts of soluble carbohydrates, particularly fructose, glucose, sucrose, and pectins, as well as insoluble cellulose , and has been used as a fermentation feedstock for the production of fermentation products (Giese et al., 2008).

Orange bagasse is already a good and cheap source of producing enzymes. Pectinases are produced from biodegradation of orange peels and pulps (El-Sheekh et al., 2009).

Orange waste can also be used in producing biopolymers by taking into consideration of its the carbohydrate content.

2.3.6. Sugar Beet Pulp

Sugar beet pulp, a byproduct of the sugar beet industry, is produced annually in large quantities It is fibrous material left over after the sugar is extracted from sugar beets. It consists mainly of cellulose, hemicellulose and pectin. Its composition is suitable for biological degradation. Beet pulp is used in countries with an intensive cattle raising industry, as livestock feed. In other countries, it is dumped in landfills. On the other hand, however, sugar beet pulp can be an important renewable resource and its bioconversion appears to be of great biotechnological importance. The processing of 1 ton of beet produces about 250 kg of exhausted pressed pulp, with a water content of approximately 75–80%. This amount can also be converted into 70 kg of exhausted dried pulp, with about 10% water content (Hutnan et al., 2000)

Sugar beet pulp can be used for the production of pure chemicals (Micard et al., 1996), antioxidants (Sakac et al., 2004), biogas production by an anaerobic treatment (Hutnan et al., 2000).

Taking into consideration that beet pulp is carbohydrate-rich with a high carbon to nitrogen ratio (C/N, 35-40), that sugar beet farming is a widespread and already mature industry, and that beet pulp is abundant and cheap, this coproduct has potential for use as a renewable biomass feedstock for microbial fermentations to biopolymer (Foster et al., 2001).

The components of beet pulp given in Table 2.17 is a good indicator of the fermentability of this potential fermentation substrate.

Table 2.17. Sugar beet pulp composition (South Minnesota Beet Sugar Cooperative)

Constituents	
TDN(Total digestible nutrients), %	74.78
Crude protein, %	9.21
Total sugars, %	9.56
Fat, %	0.70
Ash, %	6.22
Calcium, %	1.72
Phosphorus, %	0.08
Magnesium, %	0.33
Potassium, %	0.36
Sulfur, %	0.38
Boron, ppm	45.00
Manganese, ppm	86.00
Zinc, ppm	21.00
Copper, ppm	16.00
Iron, ppm	308.00
Aluminum, ppm	259.00
Sodium, ppm	911.00
C/N	35-40

2.3.7. Apple Pomace

The industrial processing of apples is performed mainly for the production of juice, jelly, and pulp. Fruits that are not suitable for consumption are processed, generating large amounts of residues. Apple pomace, the solid residue from juice production, represents around 30% of the original fruit and is generated during fruit pressing (Vendruscolo et al., 2008).

Large amounts of apple pomace are produced worldwide, and being highly biodegradable, its disposal represents a serious environmental problem. It is mostly used as animal feed. This utilization is, however, limited because of a low protein and vitamin content, which means a low nutritional value.

Many researchers looking for value-added products, have proposed the use of apple pomace for the production of natural antioxidants (Wijngaard et al., 2010), organic acids (Gullon et al., 2008), ethanol (Mohanty et al., 2009), exopolysaccharide (Jin et al., 2002), natural dye (Guyot et al., 2007), flavours (Laufenberg et al., 2003), enzyme (Favela-Torres et al., 2006).

Apple pomace is a very good cantidate for exopolysaccharide production with its monosaccharides which are found in the seed and skin of apple (Arnous et al., 2008)

2.4. Bioflocculation

Flocculants have been popularly used in wastewater treatment, drinking-water treatment, and industrial downstream processing (Qiu et al., 2009; Wei et al., 2009; Menkhaus et al., 2010). The flocculants used in water treatment can be classified into three groups: synthetic inorganic flocculants such as alum, ferrite flocculants or polyaluminum chloride; synthetic organic flocculants such as polyacrylamide derivatives or polyethyleneimine; and naturally occurring flocculants, shortly bioflocculants, such as sodium alginate or microbial flocculants. Among these flocculants, the use of alum usually leads to the problem of residual

aluminum. Recent epidemiological, neuropathological and biochemical studies suggest a possible link between the neurotoxicity of aluminum and the pathogenesis of Alzheimer's disease (Banks et al., 2006). Ferrite flocculants can be costly and the resultant excess iron may cause unpleasant metallic taste, odor, color, corrosion, foaming or staining. Although the synthetic organic flocculants are most frequently used because of their cost-effectiveness, they are not readily biodegradable and some of their degraded monomers such as acrylamide are neurotoxic and even show strong human carcinogenic potential (Ho et al., 2010). Because of the limitations of these flocculants, biopolymers produced by microorganisms through the synthesis of extracellular polymers by living cells are of urgent needs and alternatives (Yu et al., 2009).

Microbial produced bioflocculants have received increased scientific and technical attention because they are biodegradable and nontoxic and their degradation intermediates are not secondary pollutants (Li et al., 2009; Liu et al., 2010). Bioflocculants are mainly composed of protein, glycoprotein, polysaccharide, and nucleic acid (Liu et al., 2009). Many microorganisms have been shown to produce bioflocculants, e.g., *Pseudoalteromonas* sp. SM9913 (Li et al., 2008), *Bacillus* sp. F19 (Zheng et al., 2008), *Serratia ficaria* (Gong et al., 2008), *Bacillus licheniformis* X14 (Li et al., 2009), *Rhodococcus erythropolis* (Chang et al., 2009), *Chryseobacterium daeguense* (Liu et al., 2010). The high-cost and low yield of bioflocculant production, however, are the major factors limiting the development of bioflocculants for commercial use in wastewater treatment. To overcome these production limitations, mutational methods to obtain more efficient strains and a search for low cost feedstocks will be active areas of research (Wang et al., 2007).

2.5. Biosorption

A world wide environmental problem has became inevitable over the last few decades because of tremendous increase in the metallic contents in the environment. Heavy metals are the main group of inorganic contaminants, and a considerable large area of land is contaminated with them on account of use of

sludge, pesticides, fertilizers, and emissions from municipal waste incinerators, car exhausts, residues from metalliferous mines, and smelting industries (Jiang et al., 2004). The industrial effluents containing heavy metals are being drained into the river, a source of drinking water for downstream towns. Wastewater treatment facilities in most of the developing countries are not well equipped to remove traces of heavy metals, thus exposing every consumer to unknown quantities of pollutants in the water they consume.

The most common methods of removal of metals include chemical precipitation, solvent extraction, dialysis or electrodialysis, electrolytic extraction, cementation, reverse osmosis, evaporative methods, ion exchange resins, carbon adsorption and dilution. The increasing problem of heavy metal contamination has stimulated a search for new mechanisms to remove these pollutants. New attempts have been made to use the metal binding capacity of microorganisms such as yeast, algae and bacteria for the clean up of industrial effluents (Amini et al., 2009).

Biosorption refers to a specific type of sorption based on the used solid phase (sorbent) that is derived from various types of biomaterials or biomass. Biosorption is a process that utilizes low cost biosorbents to sequester toxic heavy metals (Volesky, 2003).

Biological treatment of wastewater is an innovative technology available for heavy metal remediation. Biosorbents such as algae, fungi and bacteria are examples of biomass tested for biosorption of several metal species with very encouraging results and are known to tolerate and accumulate heavy metals (Pahlavanzadeh et al., 2010; Singh et al., 2010; Tsekova et al., 2010). To overcome the disadvantages toxic effect at elevated toxicant concentrations on living biomass; non-viable or dead biomass is preferred (Velasquez et al., 2009).

The advantages of biosorption over the conventional methods are:

- low operating cost
- selectivity for specific metal
- short operational time
- no chemical sludge.

In the natural environment, bacterial exopolymers play an importanl: role in bacterial attachment to surfaces. Biopolymers, which are biodegradable and the degradation intermediates are harmless to human being and the environment, are used as biosorbents in wastewater treatment and performed well at common conditions. Most of the biopolymers are the exopolysaccharides (EPS) produced by microorganisms. It has also been demonstrated that they can be involved in binding metal ions (Kiran et al., 2008). Owing to their chemical composition, many extracellular polysaccharides derived from microorganisms and plants are known to bind metals strongly (Lamelas et al., 2006; Sharma et al., 2008; Lakshmanraj et al., 2009).

The adsorption of heavy metals by EPS is considered non-metabolic, energy independent and can be caused by interaction between metal cations and negative charge of acidic functional groups of EPS (Zhou et al., 2009).

All these features make exopolysaccharides promising material for wastewater treatment, especially for heavy metal removal.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1. Microorganism

Halophilic bacterial strain *Halomonas* sp. AAD6 (GenBank accession number DQ131909) used in this study was isolated from Çamaltı Saltern Area in Turkey (Latitude: 38° 25' N, Longitude: 27° 08' E).

3.1.2. Chemicals

All chemicals and solutions used in this study were supplied by MERCK (Germany), SIGMA (USA), DIFCO (USA) and FLUKA (Switzerland).

3.1.3. Molasses, Sugar Beet Pulp, Cheese Whey Powder, Olive Mill Wastewater, Orange Bagasse

Sugar beet molasses with average composition and properties given in Table 3.1. and sugar beet pulp (104 g/kg crude protein, 9 g/kg crude fat (ether extract), 18–30% dry matter, 40–90 g/kg total ash) were supplied by Kütahya Sugar Factory (Kütahya, Turkey). Starch molasses was supplied by Akmaya Yeast Factory (Lüleburgaz, Turkey). Olive mill wastewater was supplied from Verde Olive Oil Factory (İzmir, Turkey). Orange bagasse was provided from Starbucks coffee shops (Istanbul, Turkey). Cheese whey powder was provided from a wholesaler in Eminönü (İstanbul, Turkey).

Table 3.1. Average composition of sugar beet molasses (Kütahya Sugar Factory, Turkey)

Component	
Moisture, %	15-18
Sucrose, %	48-51
Chloride, %	0.90
Potassium, %	3.61
Sodium, %	0.45
Calcium, %	0.53
Magnesium, %	0.01
Sulphate, %	0.27
Nitrate, %	0.09
Betaine, %	8.00
Total Carbohydrate,%	3.00
Other organic compounds, %	7.50
Ash, %	11-13
pH	8.76

3.1.4. Sea Water

Sea water for the bioflocculation experiments was obtained from Seraglio Point (Sarayburnu) region of İstanbul (Latitude: 41° 1' 1" N, Longitude: 28° 59' 11" E).

3.1.5. Culture Medium

Culture medium used for the growth of microorganisms and for the production of EPS is described below (Kazak, 2007).

Optimum Medium

K_2HPO_4	7 g
KH_2PO_4	2 g
$MgSO_4 \cdot 7H_2O$	0.1 g
$(NH_4)_2SO_4$	1 g

Pepton	0.5 g
NaCl	137.2 g
Carbon Source	Appropriate conc.

pH = 7.0

per liter of distilled water.

The pH was adjusted to 7.0 by using 1 M NaOH or with 1 M HCl. While preparing medium, NaCl and the rest of the medium components, excluding carbon source, were dissolved in 500 mL and 400 mL, respectively and then sterilized separately by autoclaving at 1.06 bar and 121ºC for 15 minutes. Carbon sources were sterilized separately applying the same procedure but for 3 minutes to avoid their deterioration by caramelization. Sterile salt and carbon source solutions were combined to one liter final volume.

3.1.6. Buffers and Standard Solutions

3.1.6.1. Solution Used in Phenol/Sulphuric Acid Method

Phenol Solution (80% by weight)

Phenol	80 g
dH_2O	20 g

3.1.6.2. Solutions Used in Preparing Stock Culture

Sea Water Stock Solution (30%)

NaCl	240 g
$MgSO_4 \cdot 7H_2O$	35 g
$MgCl_2 \cdot 6H_2O$	30 g
KCl	7 g

pH = 7.0

After the contents were dissolved in 1000 mL distilled water, 5 mL of sterile $CaCl_2 \cdot 2H_2O$ (1 M) was added to the solution.

80% Glycerol, 6% Sea Water Solution

Glycerol	80 ml
Sea Water Stock Solution (30%)	20 mL

After the solution was sterilized at 121°C 1.06 bar for 15 minutes, 0.2 mL of sterile 0.5 M $CaCl_2$ was added to the solution aseptically.

3.1.8. Activated Carbon

3.1.8.1. Characteristics of Activated Carbon (Norit SA4 PAC)

Powdered activated carbon was provided from Norit Inc., USA.

Table 3.2. Characteristics of activated carbon used in the experiments

Composition	
Apparent density (g/L)	190
Moisture	%5
Ash content	%6
Phenol adsorption	%4
Molasses number	525
Methylene blue adsorption (g/100g)	10
Iodine number (mg/g)	750
Internal surface area (m^2/g)	650
Particle size	
>10 micron	%80
>44 micron	%37
>79 micron	%20
>150 micron	%5

3.1.7. Laboratory Equipments

Table 3.3. Laboratory equipments used in the experiments

Equipment	Model
Analytic Balances	Mettler Toledo AB204-S, PG403-S, PG40002-S (Sweden)
Atomic Absorption. Spectrophotometer	Perkin Elmer Aanalyst 300 (USA)
Autoclaves	Systec 3870 ELV (Germany) OT 032 Nüve (Turkey)
Automatic Pipettor Sets	4500080, 4500110 (Finipipette, Finland)
Centrifuges	Sigma 3K30, 8K10 (Germany)
Deepfreezers	-80°C, Heto Holten (Denmark) -20°C, Arçelik (Turkey) -20°C, Heto Holten (Denmark) Liebherr Fridge Freezer (England)
Freeze-Dryer	Lyovac GT 2/GT 2-E (SRK-System technik GmbH, Germany)
FT-IR	Nicolet 6700 (Thermo Scientific, USA)
Laminar Flow Cabinet	Class II Safety Cabinet, Safe 2010 0.9 (Heto Holten, Denmark)
Orbital Shakers	New Bruinswick Excella E24 (USA) Certomat BS1 (B. Braun, Germany)
Ovens	Binder BD115, Ed115(Germany)
pH meter	Mettler Toledo MP-220 (USA)
Photometric Dispersion Analyzer	PDA 2000 (Rank Brothers Ltd., UK)
Refrigerators	+ 4°C, Arçelik (Turkey)
Rheometer	AR 1500 EX (TA Instruments, USA)
Spectrophotometer	Perkin Elmer Lambda 35 UV/VIS (USA)
Turbidimeter	Turb 550 (WTW, Italy)
Viscometer	Brookfield R/S (USA)
Vortex	Heidolph REAX top (Germany)
Water Purification System	ELGA LabWater (UK)
Zeta Potantiometer	Nano-ZS Zen 3600 (Malvern Ltd., UK)

3.2. Methods

3.2.1. Pretreatments of Molasses

Starch and sugar beet molasses were subjected to different pretreatment methods like clarification, pH adjustment, sulfuric acid, tricalcium phosphate and activated carbon treatment as well as their different combinations. Hence eight pretreated molasses sample was prepared for each source making a total of 16 different molasses samples. Before and after the pretreatments, both sugar beet and starch molasses were analyzed for their total carbohydrate concentration by the phenol/sulfuric acid method (Dubois et al., 1956) and then diluted with distilled water to an appropriate final carbohydrate concentration.

3.2.1.1. Clarification (CL). Both molasses were centrifuged at 5000g for 15 minutes and the clear supernatants were directly used in the experiments without any pH adjustment.

3.2.1.2. Clarification and pH Pretreatment (CpH). Both molasses were centrifuged at 5000g for 15 minutes, the pH of the clear supernatants were adjusted to 7.0 and then used for fermentation.

3.2.1.3. Sulphuric Acid Pretreatment (H). First, pH of both molasses was adjusted to 3.0 with concentrated H_2SO_4 and then they were allowed to mix for 24 hours. After centrifugation at 5000g for 15 minutes, the pH of the clear, acid treated supernatants were adjusted to 7.0 and then used as the carbon source for the experiments.

3.2.1.4. Activated Carbon Pretreatment (AC). 3% (w/v) activated carbon was added to the molasses and after one hour of mixing, insolubles were removed by centrifugation at 5000g for 15 minutes. The supernatants were subjected to the same procedure for the second time and then their pH values were adjusted to 7.0 and used for fermentation.

3.2.1.5. Tricalcium Phosphate Pretreatment (TCP). Both molasses were treated with 2% (w/v) tricalcium phosphate (TCP) followed by autoclaving at 105 ^{o}C for 5 minutes, cooling down to room temperature and then clarification by centrifugation at 5000g for 15 minutes. The supernatants were used for in the experiments after their pH was adjusted to 7.0.

3.2.1.6. Tricalcium Phosphate and Sulphuric Acid Pretreatment (TCPH). TCP pretreated supernatants were acidified with concentrated H_2SO_4 by mixing for 24 hours at pH 3.0. Afterward, they were centrifuged at 5000g for 15 minutes, their pH were adjusted to 7.0 and then used as carbon source for fermentation.

3.2.1.7. Sulphuric Acid and Activated Carbon Pretreatment (HAC). Acid treated liquors obtained as explained in Methods 3.2.1.3. above, were subjected to 3% (w/v) activated carbon pretreatment as explained in Methods 3.2.1.4.

3.2.1.8. Tricalcium Phosphate, Sulphuric Acid and Activated Carbon Pretreatment (TCPHAC). Molasses solutions pretreated with tricalcium phosphate followed by acidification with sulfuric acid (Methods 3.2.1.6.) were subjected to 3% (w/v) activated carbon pretreatment as explained in Methods 3.2.1.4.

3.2.2 Sterilization

Throughout this study, sterilization was carried out by killing the microorganisms using steam. The culture media used in the experiments were sterilized in an autoclave. Preparation of media, inoculation and all other experimental work that require sterile environment were carried out under sterile laminar flow.

3.2.2.1. Sterilization of Media. All media were prepared in Erlenmeyer flasks. The Erlenmeyer flasks were sealed with cotton stoppers and wrapped with aluminum foils. The chemical and optimum media were sterilized by autoclaving at 1.06 bar and 121ºC for 15 minutes. Carbon sources were separately sterilized in the same manner but for 3 minutes to avoid their degradation and left to cool before using.

All the solutions of media were allowed to cool after sterilization and then they were combined aseptically.

3.2.2.2. Sterilization of Equipments. Micropipette tips, eppendorfs and glass equipments were sterilized by autoclaving at 1.06 bar and 121ºC for 15 minutes.

3.2.3. Cultivation Conditions

Microorganisms were cultured in liquid media. Shake flask cultures were grown in Erlenmeyer flasks with medium volumes up to 1/3 of the flask volume. For optimal aeration, the Erlenmeyer flask cultures were incubated in an orbital shaker at 180 rpm.

3.2.4. Preparation of Preculture

100 mL of sterile liquid medium in 250 mL Erlenmeyer flask was inoculated with 1% (v/v) frozen glycerol culture and incubated at 37°C at 180 rpm for 24 hours until its growth reached the early exponential phase in an orbital shaker. 1% (v/v) of this preculture was used as an inoculum.

3.2.5. Batch Experiments

The batch shake flask experiments were carried out in 250 mL Erlenmeyer flasks. Certomat BS1 and New Brunswick Scientific Excella E 24 orbital shaker incubators were used with the set agitation rate of 180 rpm and the set temperature of 37°C.

3.2.6. Measurement of Bacterial Growth

The rate of growth of bacterial cells is usually monitored by measuring the increase in cell number. The optical density (OD) is a measure of the amount of light passing through a cell suspension determined with a spectrophotometer. The growth of the cells was monitored by measuring offline the optical densities at

660 nm using Lambda35 UV/Vis spectrophotometer. At the desired time intervals an aliquot of the cell suspension was taken and OD_{660} was measured using cell-free medium as the blank. When necessary, samples were diluted to keep the spectroscopic reading within reliable limits of 0.2-0.8.

3.2.7. Isolation and Purification of EPS

For the purification of EPS, cells at their early stationary phase of growth were harvested by centrifugation at 10733g for 20 min and the supernatant phases were treated with an equal volume ethanol, held at -18ºC overnight, and then centrifuged at 15455g and 4°C for 30 min using a refrigerated centrifuge. The pellets were dissolved in hot distilled water, dialyzed against several runs of distilled water for 3 days, lyophilized and then weighed. The EPS samples were analyzed for total carbohydrate, protein, nucleic acid contents.

3.2.7.1. Determination of Total Carbohydrate Concentration. To determine the total carbohydrate concentration, EPS samples were dissolved in ultra pure distilled water (1% w/v). Carbohydrate content was determined using phenol/sulfuric acid method using glucose as standard. According to the method; 200 µL of sample was pipetted into an eppendorf, and 0.5 µL of 80% phenol and 1 mL of concentrated sulfuric acid were added, respectively. They were shaken in order to obtain good mixing and after waiting for 30 minutes at room temperature, optical densities were measured at 490nm. Blanks were prepared by substituting distilled water for the sugar solution (Dubois et al., 1956).

In order to convert absorbencies to grams per liter, a calibration chart was prepared with known amounts of dried EPS. EPS solutions within a suitable concentration range (0.25-2.0 g/L) were subjected to phenol/sulfuric acid method as described above. Optical densities measured at 490nm were plotted against EPS concentration and a line was fitted through the data points using least square analysis. The calibration curve demonstrates a linear relationship between absorbance and EPS concentration, with R^2 values greater than 0.98. This

calibration curve given in Appendix A was used to convert the results of phenol/sulfuric assay to carbohydrate concentration in g/L.

3.2.7.2. Determination of Protein Concentration. Protein concentration was determined by the Bradford test using Bovine Serum Albumin (BSA) as standard (Bradford, 1976). To determine the protein concentration of samples, 0.3 mL of dye stock (BioRad) was added to 1.2 mL of dilutions of sample to be assayed and vortexed. After incubation at room temperature for at least 5 minutes, absorbance was measured at 595nm. A standard curve of absorbance versus protein concentration was prepared by using known amounts of Bovine Serum Albumin (BSA). The calibration curve shows a linear correlation between absorbance and BSA concentration. This calibration curve given in Appendix B was used to convert the absorbancy results of Bradford assay to protein concentration in g/L.

3.2.7.3. Determination of DNA Concentration. Spectrophotometric measurement was used to determine the quantity of DNA in the EPS samples. For that, samples were diluted with distilled water and their UV absorbancies were read at 260nm using quartz cuvettes.

3.2.7.4. FT-IR Analysis

Fourier transform-infrared (FT-IR) spectroscopy spectra of EPS samples were obtained with Nicolet 6700 FT-IR Spectrometer between 400 and 4000 wave numbers (cm^{-1}).

3.2.8. Bioflocculation Studies

The bioflocculant properties of EPS samples were analyzed in kaolin/distilled water suspension media. Flocculation dynamics of different concentrations of EPS samples were investigated in normal tap water (100 mg $CaCO_3$/L hardness and alkalinity, pH 7.3), synthetic sea water (2.40 g/L NaCl, 0.35 g/L $MgSO_4 \cdot 7H_2O$, 0.30 g/L $MgCl_2 \cdot 6H_2O$, 0.07 g/L KCl, $CaCl_2 \cdot 2H_2O$, pH 7.3), and real sea water (filtered to remove particles, pH 7.3) online through photometric

dispersion analyzer (PDA). Into each water, kaolin was added as model particulate matter to give a concentration of 50 mg/L and turbidity of 100 nephelometric turbidity unit (NTU). The formation, size, stability, and reformation of flocks produced by EPS samples were compared with commercial cationic (Magnafloc LT 22), nonionic (Magnafloc LT 20), and anionic (Magnafloc LT 25) polyelectrolytes.

The capability of floc formation of EPS samples was based on the decrease of turbidity of 50 mg/L kaolin-distilled water suspension. EPS-kaolin suspension was mixed during 10 seconds at 400 rpm, then the mixing speed was reduced to 50 rpm and floc formation was observed using PDA at 850 nm absorbance value by circulating the suspension with peristaltic pump. When floc formation was completed, turbidity of the supernatant was mesured with turbidimeter. Flocs formed were disrupted by increasing rpm to 400 for 10 seconds, then mixing speed was reduced again down to 50 rpm to let the particles reform flocs. After the flocs reformation were completed, the flocs were let to settle down for 20 minutes. Then, the turbidity of samples taken from the supernatant phase were measured as nephelometric turbidity unit (NTU).

3.2.9. Rheological Studies

Lyophilized EPS samples and standard polymers including Xanthan (Sigma no: G1253), Sodium Alginate (Sigma no: A2033) and Pullulan (Sigma no: P4516) were dissolved in either distilled water or 10% NaCl solution at varying concentrations and the shear viscosity measurements of the aqueous polymer solutions were recorded using AR 1500 EX (TA Instruments, USA) Rheometer at 20^{o}C and 30ºC. The shear stress used was in the range of 0.1-1.0 Pa for low, 1.0-10.0 Pa for medium and 5.0-15.0 Pa for high stress conditions, respectively.

3.2.10. Heavy Metal Biosorption Experiments

To study the heavy metal removal efficiency of exopolysaccharides, lead (Pb), iron (Fe), zinc (Zn), copper (Cu), cobalt (Co), and nickel (Ni) were selected

as heavy metal cations to be investigated. Batch equilibrium dialysis experiments were carried out in 250 ml flasks containing 100 ml of aqueous solution with 5 mg/L heavy metal concentration. Solutions were prepared using double distilled deionised water.

Dialysis tubing was boiled in 2% $NaHCO_3$ and 1 mM EDTA and finally in deionized water to remove any contaminating metals. It was stored at 4 ^{o}C before use. 5 mL EPS (0.1% w/v solutions) was placed in dialysis tubing and then the sealed tube was immersed in 100 mL metal solution. Flasks were shaken in an orbital shaker set to 100 rpm and 40 ^{o}C until equilibrium sorption was reached The final metal concentration was determined by atomic absorption spectrophotometry. Appropriate blanks were examined throughout the sorption experiments to correct the glassware and dialysis tubing sorption of metals and other potential side effects

4. RESULTS AND DISCUSSION

Biopolymers are alternative materials for petrochemical-derived polymers. Considering their relatively high cost of production because of the ingredients in the fermentation medium, a production process which has been aimed to seek the ways of decreasing the cost by utilizing different organic wastes and byproducts was investigated. Halophilic *Halomonas* sp AAD6 bacteria isolated from Çamaltı Saltern Area was used in fermentation experiments. Different pretreatment methods, fermentation conditions, fermentation media were studied to optimize microbial growth and biopolymer production. Molecular characterization of the biopolymers obtained from fermentation experiments were realized by FT-IR and also the rheological properties of the biopolymers were investigated under different conditions. Moreover, flocculation performance and particles removal efficiency of the EPSs were monitored dynamically by a photometric dispersion analyser and results were compared with commercial polyelectrolytes. Finally, the economical advantages provided by this thesis work were discussed.

4.1. Fermentation Studies

Fermentation is an extremely versatile process technology for producing value added products like biopolymers (Barnabe et al., 2009; Castilho et al., 2009; Keshwani et al., 2009; Levin et al., 2009). Reaching optimized fermentation conditions, particularly associated with physical and chemical parameters, is of primary and great importance for the development of any process, because of their impact upon its economics and practicability (Lynd et al., 2005; Isar et al., 2006; Xiros et al., 2008; Prasertsan et al., 2008).

Fermentation medium can represent 30-50% of the total cost for a microbial fermentation (van Hoek et al., 2003). Complex media commonly employed for growth and production are not economically attractive because of their high amount of expensive nutrients such as yeast extract, peptone and salts.

Hence to achieve high production yields as well as to compete with synthetic petrochemical products in performance and cost, it is a prerequisite to design an optimal cost-effective production medium.

The greatest expense in producing biopolymers has traditionally been the substrate which is used as the fermentation feedstock: The carbon source, e.g., sugar, accounts for over 50% of the fermentation cost (van Hoek et al., 2003). Until the late 1990's, researchers working in the development of microbial systems to produce biopolymers generally focused on using a single type of microorganism, with a minimum component synthetic feedstock to achieve defined culture conditions. However, to maximize the cost effectiveness of the process, recent work has shifted to use multi-component feedstock systems and the synthetic media were replaced by cheaper alternatives such as cheese whey and molasses (Salehizadeh et al., 2004; Treichel et al., 2006; Cha et al., 2007; Shene et al., 2007; Aguilera et al., 2008; Khardenavis et al., 2009; Leung et al.,2009).

In this thesis work, the potential uses of different waste streams like sugar beet and starch molasses, cheese whey powder, sugar beet pulp, olive mill wastewater, and orange bagasses as fermentation substrates were evaluated.

4.1.1. Molasses

Molasses is generally defined as any liquid feed with an excess of 43% sugars content (Curtin, 1983). Besides its valuable constituents like amino acids and vitamins, they also contain undesirable components like heavy metals, impurities, and dark brown pigments (melanoidins) that may have adverse effects in microbial fermentation processes such as growth inhibition or inactivation of enzymes associated with the biosynthesis of the products.

Within the scope of this thesis, two different types of molasses were used for biopolymer production. Sugar beet molasses (BM) which is a byproduct of the manufacture of sucrose from sugar beets and starch molasses (SM) which is a byproduct of dextrose manufacture from starchy substrates like corn. Whereas BM

was supplied by Kütahya Sugar Factory (Kütahya, Turkey), SM was provided by Akmaya Yeast Factory (Avcilar, Turkey).

4.1.1.1. Pretreatment of Molasses. Sugar beet molasses and starch molasses were subjected to five different physical and chemical pretreatment methods, i.e., clarification, pH adjustment, sulfuric acid, tricalcium phosphate and activated carbon treatment. Together with combinations of these methods, eight different pretreatment procedure was applied to both sugar beet and starch molasses making a total of 16 different pretreated molasses samples.

As also explained in more detail in Methods 3.2.1., in clarification, centrifugation was used to recover the clear supernatants which were either used directly in the experiments (CL) or after their pH were adjusted to 7.0 (CpH). For sulfuric acid treatment, concentrated H_2SO_4 was added to molasses and the resulting acidic solution (pH 3.0) was allowed to mix for 24 hours. In activated carbon pretreatment, 3% (w/v) activated carbon was added to the molasses and mixed for one hour. In tricalcium phosphate (TCP) pretreatment, molasses containing 2% (w/v) TCP was autoclaved at 105 ^{o}C for 5 minutes followed by cooling down to room temperature. These methods, as well as their combinations involved a final clarification step before their use as carbon source in the experiments. With the exception of CL, the final pH values of all the pretreated molasses samples were adjusted to 7.0 and then used for microbial fermentation. The initial pH values of the untreated molasses samples were found to vary within the range of 7.35 - 7.65. Before and after the pretreatments, all molasses samples were analyzed for their total carbohydrate concentration by the phenol/sulfuric acid method (Dubois et al., 1956). Total carbohydrate content of untreated sugar beet and starch molasses were found to be around 550 g/L and 500 g/L, respectively. Moreover, negligable differences in carbohydrate contents of the samples before and after the pretreatments suggested the fact that pretreatments have not affected the sugar contents of the molasses as also reported by Kundu et al., (1984). The pretreatments applied in this study are summarized in Figure 4.1

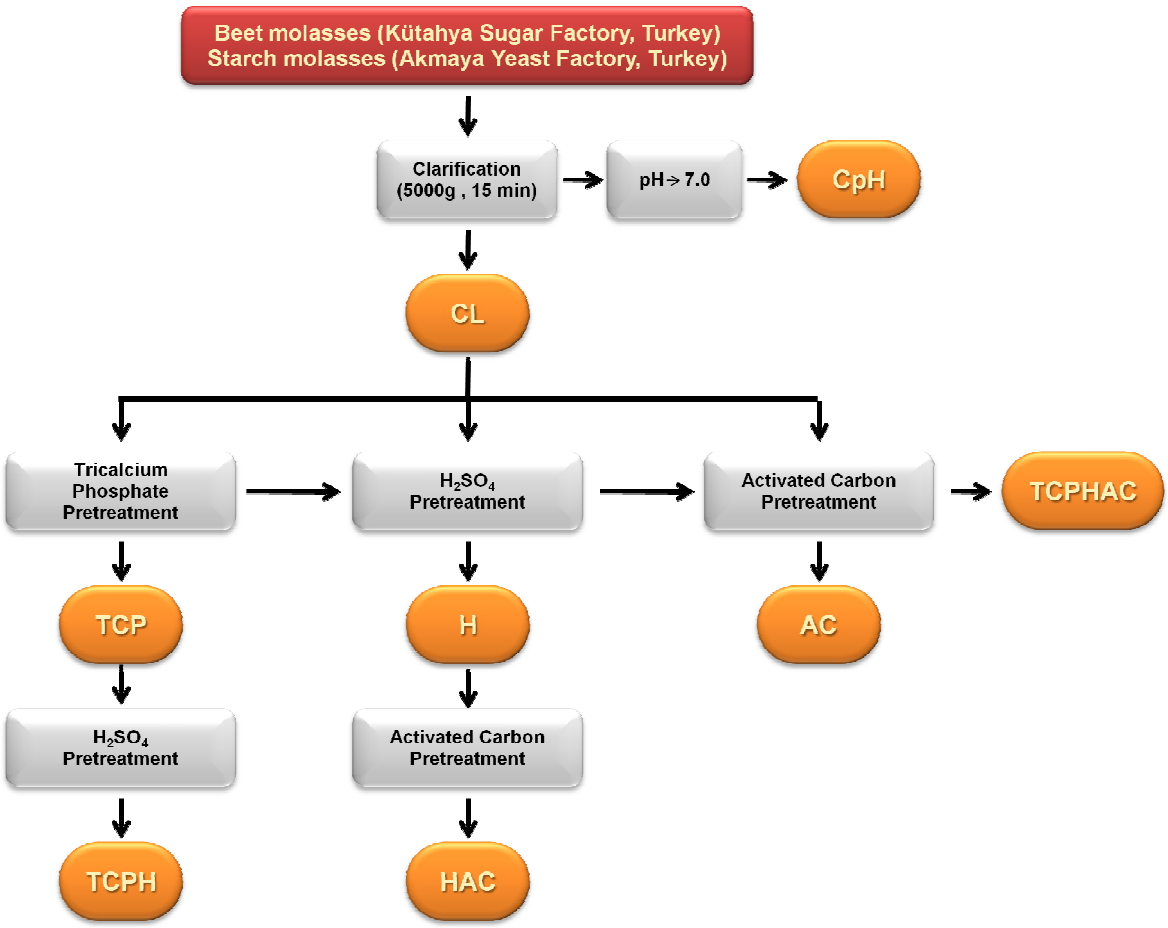


Figure 4.1. Flowchart of pretreatments applied to molasses

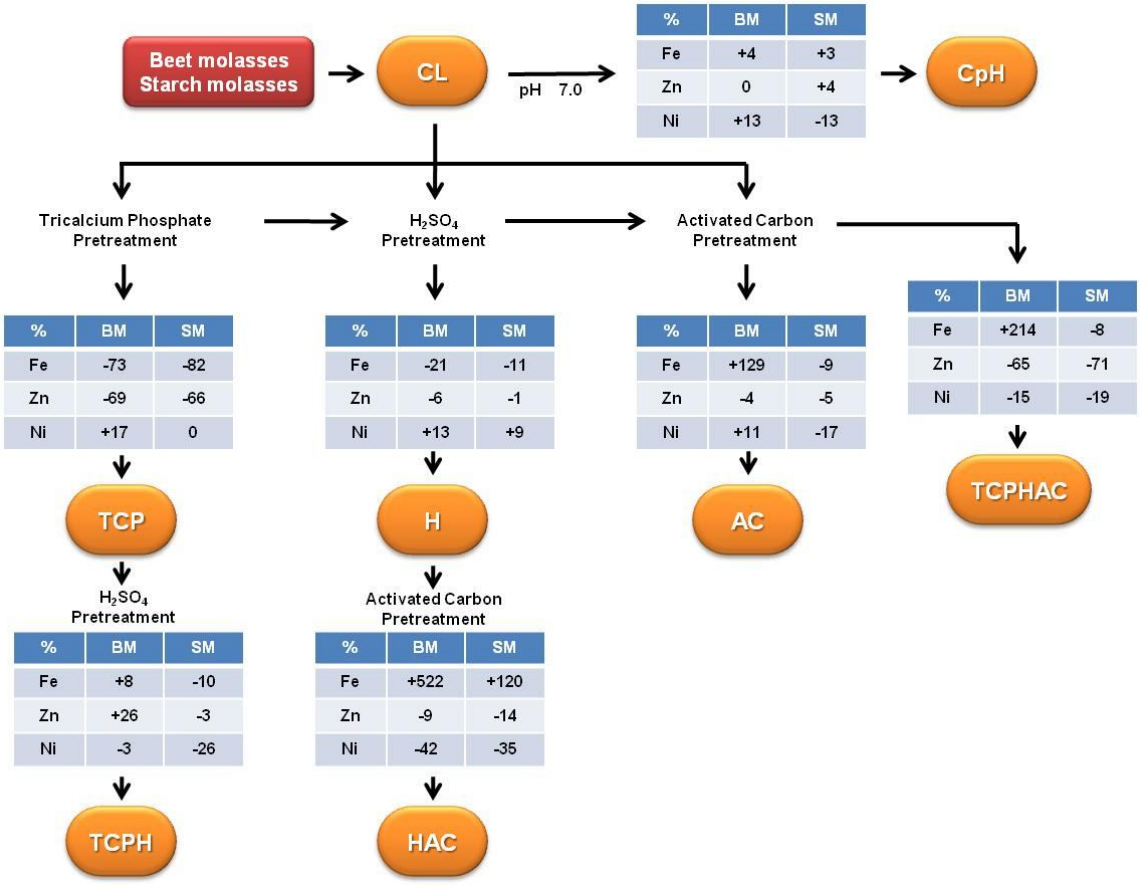
4.1.1.2. Effect of Pretreatments on Heavy Metal Content of Molasses. In order to investigate the effect of pretreatments on the removal of heavy metal from the molasses, the pretreated samples were analyzed by using Perkin Elmer Aanalyst 300 Atomic Absorption Spectrophotometer for their iron, zinc and nickel content and results were tabulated in Table 4.1.

Generally, iron, zinc and nickel concentrations were within 0.5-13 mg/L, 0.3-2.3 mg/L and 0.4-0.6 mg/L range, respectively. The clarified and pH adjusted molasses samples indicated that whereas starch molasses was richer in its iron content, sugar beet molasses was richer in its zinc content. No significant differences between the two types of molasses could be detected about their nickel content.

Table 4.1. Changes in heavy metal concentration of pretreated molasses

Sample	Fe (mg/L)	Zn (mg/L)	Ni (mg/L)
BM CL	1.965	2.270	0.530
BM CpH	2.043	2.270	0.600
BM H	1.555	2.125	0.600
BM AC	4.493	2.170	0.590
BM HAC	9.667	1.940	0.350
BM TCP	0.533	0.710	0.620
BM TCPH	0.575	0.895	0.600
BM TCPHAC	6.188	0.790	0.450
SM CL	6.650	0.890	0.520
SM CpH	6.865	0.930	0.455
SM H	5.905	0.880	0.565
SM AC	6.050	0.840	0.430
SM HAC	12.975	0.755	0.370
SM TCP	1.213	0.300	0.520
SM TCPH	1.093	0.290	0.385
SM TCPHAC	6.110	0.255	0.420

To interpret the metal concentration data in a systematic manner, their % changes in each pretreatment were also calculated and shown in Figure 4.2.



%	BM	SM
Fe	+4	+3
Zn	0	+4
Ni	+13	-13

%	BM	SM
Fe	-73	-82
Zn	-69	-66
Ni	+17	0

%	BM	SM
Fe	-21	-11
Zn	-6	-1
Ni	+13	+9

%	BM	SM
Fe	+129	-9
Zn	-4	-5
Ni	+11	-17

%	BM	SM
Fe	+214	-8
Zn	-65	-71
Ni	-15	-19

%	BM	SM
Fe	+8	-10
Zn	+26	-3
Ni	-3	-26

%	BM	SM
Fe	+522	+120
Zn	-9	-14
Ni	-42	-35

Figure 4.2. Effect of pretreatments on heavy metal concentration

When CL and CpH treatments were compared, pH adjustment was found not to change the heavy metal concentration in both molasses types as expected. Effect of the strong acid pretreatment (H) on both molasses in terms of iron, zinc and nickel removal was also low. About 10% decrease in iron content was observed whether the acid treatment was applied to clarified or TCP treated starch molasses samples. Iron in the clarified beet molasses was reduced 21% by acid treatment, however a slight increase (8%) in iron content was observed in the TCP treated beet molasses. This could most probably because of the fact that TCP treatment already removed more than 70-80% of the iron content of both types of molasses. Not only iron, but also zinc was removed up to 70% from both molasses by the TCP treatment making TCP an effective compound for selective removal of iron and zinc from molasses or other mixtures of comparable composition. Similar results were also reported in literature. For citric acid production using *Aspergillus* niger T55, 81% of iron in the cane molasses was removed by both TCP and TCPH

methods and zinc removal was 84% for TCP and 96% for TCPH pretreatments (Kundu et al., 1984). Again for the citric acid production from a novel *Aspergillus niger* strain, 9.5% iron removal and 56% zinc removal by TCP method was reported (Lofty et al., 2007). Heavy metals like iron, zinc and nickel are known to enter the apatite crystal structure of TCP ($Ca_3(PO_4)_2$) by replacing the Ca atom and causing some distortion in the crystal structure (Yin et al., 2002).

Whereas TCP treatment was not effective for nickel removal, AC adsorbed 20-40% of the nickel from the treated samples. Removal of Zn, on the other hand, remained within 4-14% range. As such, in the TCPHAC treated samples, from the three consecutive steps, TCP step removed Zn and AC adsorbed Ni from the clearified molasses. Activated carbon is especially known for its effectiveness in removing heavy metal pollutants (Zang et al., 1989). However in this study, after the AC treatment, a drastic increase in the dissolved iron (Fe^{+2}) content was observed. This could be because of the reduction of iron from its impregnated Fe^{+3} form to its soluble form since this increase in soluble iron was more profound when acid treated samples were subjected to AC treatment to yield HAC and TCPHAC.

The iron, zinc and nickel content of the pretreated molasses samples were expected to influence the growth and biopolymer production efficiency of the *Halomonas* sp. AAD6 cultures.

4.1.1.3. Effect of Pretreatments of Molasses on Cellular Growth and EPS Production. In previous studies, the ability of halophilic *Halomonas* sp. AAD6 cells to use different carbon sources for growth and EPS production was analyzed by growing cells in chemical medium containing 14 different carbon sources (glucose, lactose, sucrose, arabinose, xylose, maltose, raffinose, fructose, galactose, mannose, acetate, glycerol, trisodiumcitrate, rhamnose) and reported (Poli et al., 2009). In the light of the results shown in Table 4.2, sucrose was chosen as the best carbon source for both biomass and EPS production in chemical medium. In general, maltose, fructose, glucose and galactose favored biomass formation however from these, only cells grown in the presence of maltose were found to

produce EPS. Interestingly, raffinose and xylose reached almost the same EPS concentrations like maltose but the biomass yields were about ten fold lower. Acetate, trisodium citrate, rhamnose and glycerol were most probably not utilized by the microorganisms' metabolism resulting in very poor growth (< 0.1 gDCW/L) and negligible biopolymer production levels.

Table 4.2. Effect of carbon sources on growth and EPS production by *Halomonas* sp. AAD6 (Poli et al., 2009)

Carbon Source	Biomass, (gDCW/L)	EPS, (g/L)
Glucose	1.108	0
Lactose	0.103	0.060
Sucrose	0.863	1.073
Arabinose	0.104	0.027
Xylose	0.096	0.267
Maltose	1.374	0.189
Raffinose	0.135	0.206
Fructose	1.548	0
Galactose	1.199	0
Mannose	0.607	0
Rhamnose	0.085	0
Acetate	0.051	0
Glycerol	0.001	0
Trisodium Citrate	0.003	0

Sugar beet molasses with its high sucrose content is a relatively inexpensive and renewable material that is widely used as a substitute for sucrose in various industrial fermentation media (Han et al., 1992). Hence in this study, sugar beet molasses subjected to different pretreatments, was used instead of sucrose which was reported as the best fermentation substrate for both biomass and EPS production in chemical medium. Starch molasses was also used to ascertain the importance of molasses type.

Fermentation experiments were conducted with shake flask cultures incubated in an orbital shaker at 37^{o}C and 180 rpm rotational speed. The

molasses samples pretreated as explained in 4.1.1.1. were added aseptically to the optimum medium with composition given in 3.1.5. at 10 g/L final carbohydrate concentration. Growth media were inoculated with 1% (v/v) of fresh preculture and samples were taken from *Halomonas* sp. AAD6 shake flask cultures at certain times, analyzed for biomass and EPS production. Growth was followed by measuring the optical densities at a wavelength of 660 nm using Lambda35 UV/Vis spectrophotometer. Time dependent growth profiles of the cultures were given in Figures 4.3 and 4.4.

Growth on sugar beet molasses was apparently not dependent on the treatment for the first 50 hours whereas in starch molasses, two different growth patterns were observed, so that retarded growth of clarified (CL), acid treated (H) and pH adjusted (CpH) cultures were clearly distinguishable from the other fast growing ones. As also mentioned before in 4.1.1.2., CL, CpH and H treatments were found not to change the heavy metal concentration in both molasses types. Hence the obvious improvement in growth may result from TCP or AC treatments that changed the starch molasses composition in favor of the metabolic needs of *Halomonas* sp. AAD6 cells. In sugar beet molasses, TCPH and TCPHAC cultures reached higher turbidities than the others suggesting the importance of TCP treatment for beet molasses.

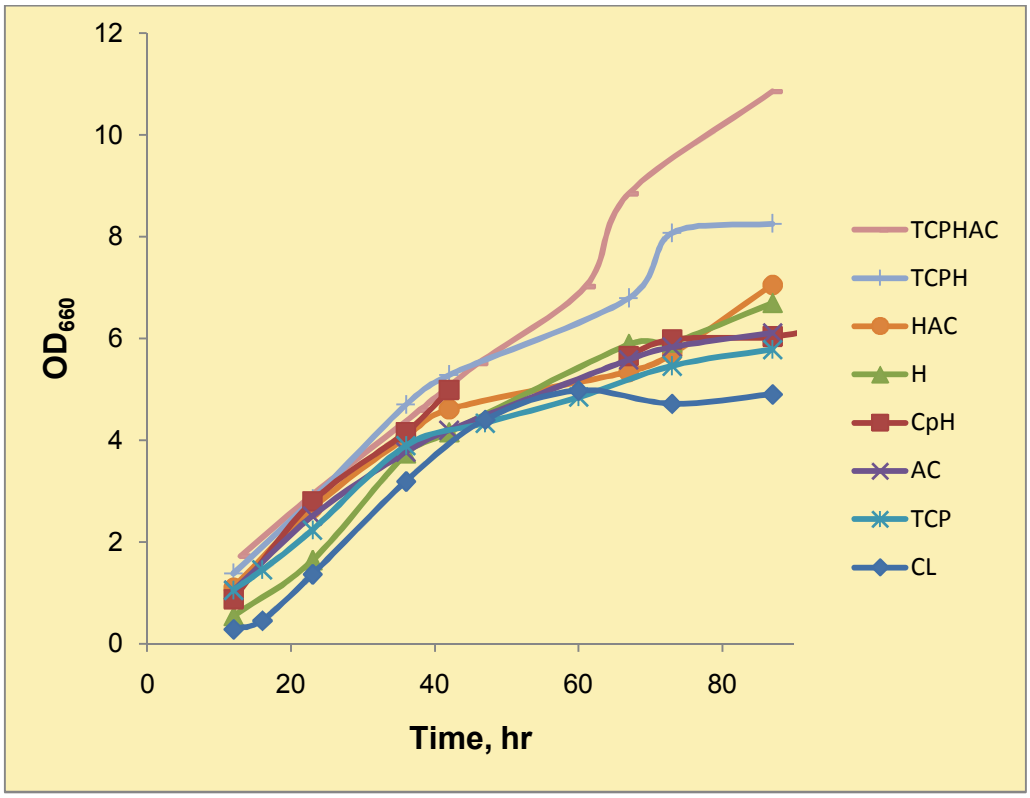


Figure 4.3. Optical densities of cells grown in different pretreated beet molasses, 10 g/L initial sugar concentration

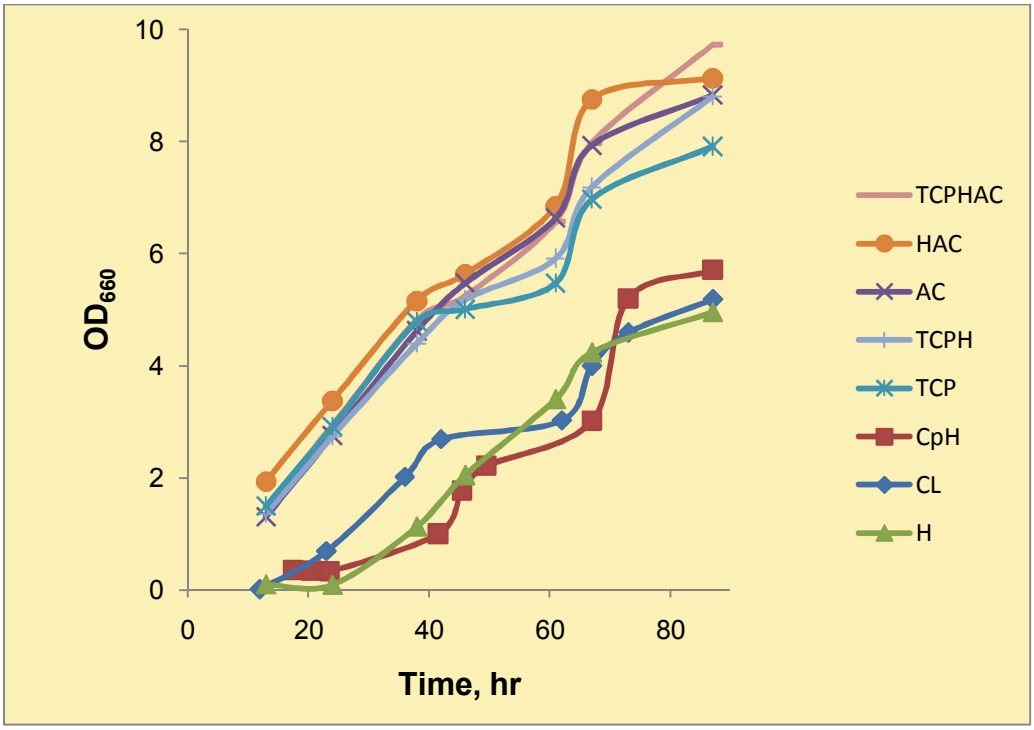


Figure 4.4. Optical densities of cells grown in different pretreated starch molasses, 10 g/L initial sugar concentration

However, these results should be interpreted together with the dry cell mass yields and EPS yields of the cultures. For the biomass yields, stationary phase cultures were centrifuged and the cell pellets were dried in oven until constant weight. The biopolymers in the clear supernatants were recovered as explained in 3.2.7. The carbohydrate, protein and DNA content of the samples were also analyzed to evaluate their composition. Results were summarized in Table 4.3.

Considering the dry cell weight (DCW), microorganisms were grown better in beet molasses as fermentation substrate in comparison to starch molasses in every applied pretreatment (Table 4.3). The low biomass yields of the CL, CpH and H treated starch molasses cultures ranging between 4-6 gDCW/L were in good agreement with their retarded growth profiles observed in Figure 4.4. Sulphuric acid-activated carbon (HAC) pretreatment for beet molasses gave the highest amount of cellular mass whereas activated carbon (AC) pretreatment was the best pretreatment for bacterial growth in starch molasses.

Generally, biopolymer amounts that were produced from pretreated sugar beet molasses were higher than those produced from starch molasses. This could most probably because of their predominant monosaccharides which are sucrose and glucose for sugar beet and starch molasses, respectively. This result is also in accordance with the reported fact that sucrose was the best carbon source for both biomass and EPS production (Poli et al., 2009).

In both molasses types, pretreatments like clarification, pH adjustment were not adequate as also reflected by the low EPS production yields. This is an expected situation because of the retained undesirable constituents (e.g., heavy metals, impurities) which influence the growth of microorganism and associated polysaccharide production (Kundu et al., 1984; Roukas, 1998; Jiang et al., 2009). The growth promoting effects of the AC and TCP pretreatments of starch molasses were also reflected in the EPS production yields which were comparable to sugar beet molasses making starch molasses a potential fermentation substrate besides sugar beet.

Table 4.3. Biomass and EPS production yields of *Halomonas* sp AAD6 cultures growing in pretreated sugar beet and starch molasses and composition of the EPSs

Pretreatment	Cells (gDCW/L)		EPS (g/L)		DNA (%)		Protein (%)		Carbohydrate (%)		Yield (gEPS/gCells)	
	BM	SM	BM	SM	BM	SM	BM	SM	BM	SM	BM	SM
CL	11.141	6.085	1.210	1.030	5.457	4.991	0.569	1.017	100	100	0.109	0.169
CpH	19.260	5.949	2.329	0.341	4.577	9.444	0.374	2.228	100	90	0.121	0.057
H	20.776	4.262	2.856	0.820	5.921	10.281	0.637	0.945	80	80	0.137	0.192
AC	20.617	18.217	2.780	3.290	4.241	4.307	0.409	0.107	100	100	0.135	0.181
HAC	22.335	9.390	3.103	3.379	4.596	5.235	0.417	0.297	100	100	0.139	0.360
TCP	14.977	9.816	2.283	2.722	4.248	4.583	0.419	0.282	100	100	0.152	0.277
TCPH	15.399	11.182	3.688	3.372	4.229	4.676	0.350	0.192	100	100	0.239	0.302
TCPHAC	14.814	11.361	4.194	3.681	5.190	4.593	0.356	0.193	90	100	0.283	0.324

Highest EPS concentrations were reached by cultures grown in tricalcium phosphate-sulphuric acid-activated carbon (TCPHAC) pretreated sugar beet and starch molasses with yields of 4.19 g/L and 3.68 g/L, respectively. This may be because of the effective removal of unwanted constituents including heavy metals, impurities and color.

All exopolysaccharides, both from beet and starch molasses, were composed of only carbohydrate molecules excluding of acid pretreated (H) molasses of both kinds where the carbohydrate content dropped to 80%. This could be because of cell lysis as also verified by the high DNA and protein contents of these samples. Similar observation could also be made for the CpH treated starch molasses with 90% carbohydrate content.

The EPS yields on biomass ranged between 0.109 to 0.283 g EPS/g DCW for beet molasses with the highest yields obtained from the TCPH and TCPHAC cultures where the EPS yields were also higher than the other cultures. For the starch molasses though, besides the TCPH and TCPHAC, HAC cultures were also found to reach high EPS concentrations (3.379 g/L) and highest yield on biomass (0.360 g EPS/g DCW).

Since the growth and EPS yields of both types of molasses were close, they were compared at a higher initial concentration (30 g/L). The biomass yields and EPS concentrations were given in Table 4.4.

Table 4.4. Biomass and EPS production yields of *Halomonas sp.* AAD6 cultures growing in 30 g/L pretreated sugar beet and starch molasses.

	Cells (gDCW/L)		**EPS (g/L)**		**Yield (gEPS/gCells)**	
Pretreatment	**BM**	**SM**	**BM**	**SM**	**BM**	**SM**
CpH	22.64	17.16	3.49	2.52	0.154	0.147
HAC	23.90	30.10	7.37	4.44	0.308	0.148
TCPHAC	31.98	23.86	7.56	4.38	0.236	0.184

When the biomass yields of CpH and HAC were compared, for both initial concentrations (Tables 4.3 and 4.4), no change and an about two-fold increase in biomass yields were detected for beet and starch molasses, respectively. Generally, for all the pretreatments, higher EPS concentrations and yields were reached with the beet molasses. Therefore, sugar beet molasses subjected to TCPHAC treatment was chosen as fermentation substrate for the forthcoming experiments.

4.1.1.4. Effect of Initial Sugar Concentration of Molasses on Cellular Growth and EPS Production. In order to determine the optimum initial substrate concentration, preliminary shake flask experiments were conducted in optimum medium containing TCPHAC treated sugar beet molasses at 50, 100, 150 and 200 g/L concentrations. The growth of the cultures were followed and the turbidity of the stationary phase cultures measured as OD_{660} were found to decrease from 10.8 (for 30 g/L) down to 2.77, 0.136, 0.144 and 0.110 for 50, 100, 150 and 200 g/L initial sugar concentrations, respectively. This result was in agreement with the growth profiles of the *Halomonas* sp. AAD6 shake flask cultures growing in optimum medium containing 10 – 300 g/L sucrose as carbon source. At sucrose concentrations exceeding 50 g/L, considerable retardation of growth and associated low biopolymer yields were observed (results not shown). This could be because of the inhibitory osmotic stress the cells were exposed at high sugar concentrations as also suggested by Liu et al. (2008). Therefore, same experiments were performed with 45 g/L initial sugar concentration and the biomass and EPS yields were compared in Table 4.5.

As shown in Table 4.5, 30 g/L initial concentration of TCPHAC pretreated beet molasses was found to yield highest EPS concentration. At concentrations exceeding this optimum concentration, decreases in cellular growth and associated decreases in EPS yields were observed. Therefore, 30 g/L initial concentration was used for the subsequent optimization studies..

Table 4.5. Effects of initial total carbohydrate concentrations on growth and biopolymer production in TCPHAC pretreated sugar beet molasses

BM TCPHAC (g/L)	Biomass (gDCW/L)	EPS (g/L)	Yield (gEPS/gDCW)
10	14.81	4.19	0.283
30	31.98	7.56	0.236
45	26.60	6.86	0.258

4.1.1.5. Effect of Mineral Salts in the Culture Medium on Cellular Growth and EPS Production. TCPHAC pretreated sugar beet molasses at 30 g/L initial carbohydrate concentration was used in the experiments in order to understand the effect of mineral salts composition of the medium (7 g/L **K**$_2$HPO$_4$, 2 g/L **K**H$_2$PO$_4$, 0.1 g/L **M**gSO$_4$·7H$_2$O, 1 g/L (**N**H$_4$)$_2$SO$_4$, 0.5 g/L **P**epton) **(KKMNP)** on biomass growth and EPS production. Experiments were conducted in orbital shaker operated at 37 ^{o}C, 180 rpm and initial pH of 7.

In salt drop-out experiments, the salts, except K_2HPO_4, were removed one in each experiment by excluding the previous one as well in the next experiment. In other words, media without pepton **(KKMN)**, without pepton and $(NH_4)_2SO_4$ **(KKM)**, without pepton, $(NH_4)_2SO_4$ and $MgSO_4 \cdot 7H_2O$ **(KK)** and finally media containing only K_2HPO_4 **(K)** were used for the first set of experiments.

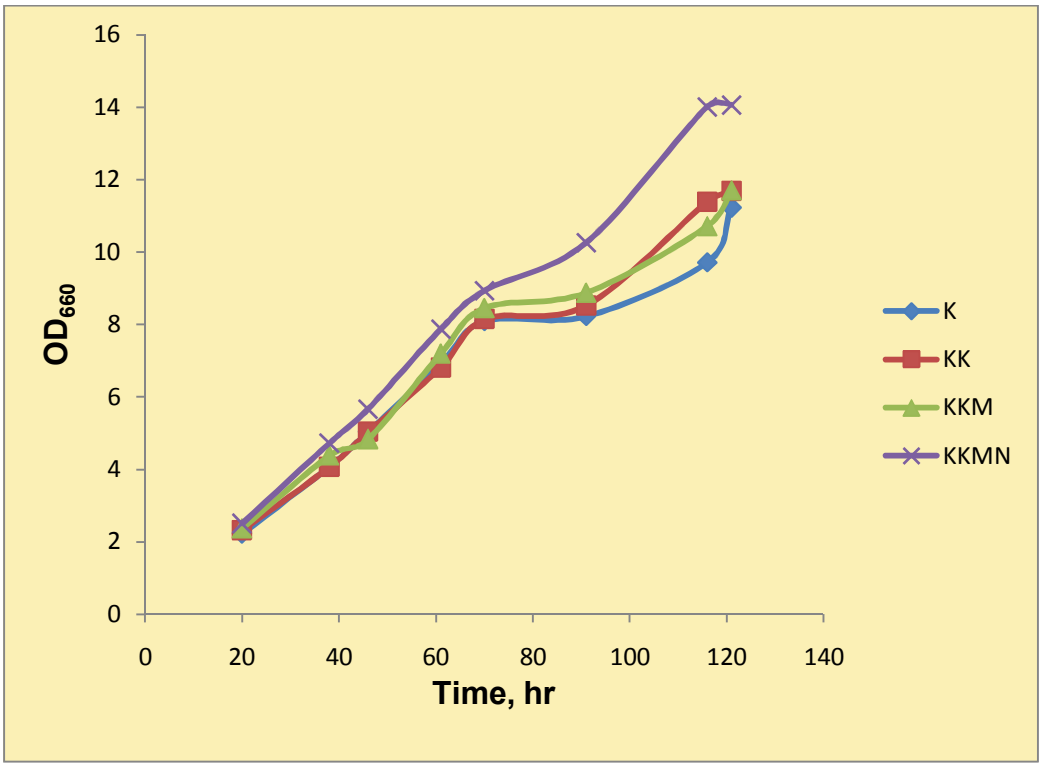


Figure 4.5. Optical densities of cells grown in different mineral salts

As shown in Figure 4.5, growth profiles of the cultures were found to be very similar for the first 70 hours after which the culture containing $(NH_4)_2SO_4$ **(KKMN)** was found to continue growing whereas others entered a stationary phase of growth with no increase in culture turbidity. This result indicated the importance of $(NH_4)_2SO_4$ as a nitrogen source in the medium. At the same time it may also point to the phase where cultures were subjected to N starvation and since exopolysaccharides production was known to occur at high C:N values, this phase could be associated with high EPS production. To elucidate this fact more experiments should be done to obtain the EPS production profiles of each culture

After 100 hours, cultures were found to enter a new phase of growth with probably a different metabolic activity that also calls for more in dept studies. The biomass concentrations of K, KK, KKM and KKMN were found to be 11.88, 11.70, 15.88 and 15.38 gDCW/L, respectively. Experiments were repeated for a longer period and for the biomass yields, stationary phase cultures were centrifuged and the cell pellets were dried in oven until constant weight. The biopolymers in the clear supernatants were recovered as explained in 3.2.7. Results summarized in

Table 4.6 clearly pointed to the fact that by a careful control of the fermentation conditions with respect to the requirements for the EPS production by *Halomonas* sp. AAD6 shake flask cultures, high yields could be attained even by using a medium with a more simple composition and hence more economical also.

Table 4.6. The effects of mineral salts used in complex medium on cellular growth and EPS production

Mineral Salts	Cells (gDCW/L)	EPS (g/L)	Yield (EPS/Cells)
K	11.875	5.164	0.434
KK	11.680	4.686	0.401
KKM	15.878	4.995	0.314
KKMN	15.380	5.241	0.340

By considering the results gathered above, a second set of experiments were performed where K_2HPO_4 which was the last mineral salt that was kept in the culture medium was also removed. The clarified sugar beet molasses (BM CpH) was the carbon source for this set of experiments. Optimum culture medium using BM CpH as carbon source was used as control. Results tabulated in Table 4.7 are averages of multiple runs.

Table 4.7. The effect of dipotassium phosphate salt used in complex medium on cellular growth and EPS production

Mineral Salts	Cells (gDCW/L)	EPS (g/L)	Yield (EPS/Cells)
BM CpH	14.788	4.890	0.331
BM CpH (-K), ($Time_1$)	19.450	4.367	0.225
BM CpH (-K), ($Time_2$)	21.097	5.316	0.252

These results clearly indicated that by a systematic salt drop out strategy, the composition of the production media can be simplified without any losses in EPS yields.

4.1.1.6. Effect of Initial pH of Culture Medium on Cellular Growth and EPS Production. To find out the effect of initial medium pH on cellular growth and polysaccharide production, microorganisms were grown in shake flasks containing clarified sugar beet molasses (BM CpH) at 30 g/L initial total carbohydrate concentration. Before the onset of fermentation, culture media were adjusted to four different initial pH values being 6, 7, 8 and 9. Shake flasks were placed in the orbital shaker working at 180 rpm and 37 ^{o}C and bacterial growth was monitored by measuring the optical densities at 660 nm using Lambda35 UV/Vis spectrophotometer. Time dependent growth profiles of the cultures were given in Figure 4.6. At pH 6, no detectable change in the turbidity of the shake flask cultures suggested that the microorganism was not able to grow at such low pH values. The cultures were found to grow and reach steady state faster when grown at pH 8 and pH 9. At pH 7 on the other hand, growth was slower within the first 30 hours however after that, it increased steadily to reach high optical densities and steady state. Fermentation was terminated at 160 hours of fermentation time for pH 7 culture and at 100 hours for the pH 8 and pH 9 cultures with final pH of the cultures measured to be between 7.6-7.8 (Table 4.8). Gravimetric method based on the weight measurements of the oven-dried cells was used to analyze the biomass yields. The biopolymers in the clear supernatants were recovered as explained in 3.2.7. The carbohydrate, protein and DNA content of the samples were also analyzed to evaluate their composition. Results were summarized in Table 4.8. At pH 9, very high EPS yields were obtained however the carbohydrate content of the samples were low. With pH 8, EPS yields were lower than pH 9 but higher than pH 7 cultures however only very low carbohydrate contents were attained. Generally, EPS yields were found to increase with initial pH of the culture broth. Hence these results highlighted the importance of pH for the production of EPS by *Halomonas* sp. AAD6 shake flask cultures.

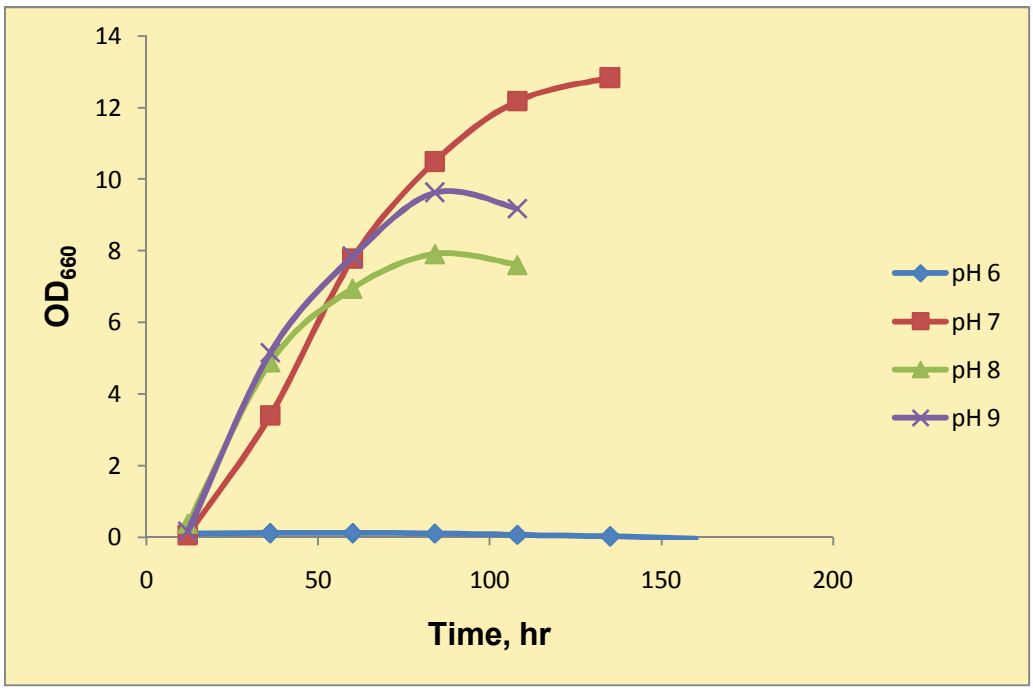


Figure 4.6. Growth profiles of *Halomonas* sp. AAD6 cells grown in culture media at different initial pHs

Table 4.8. Effect of initial pH of the culture medium on biomass and biopolymer production

Initial pH	Cells (g DCW/L)	EPS (g/L)	DNA (%)	Protein (%)	Carbohydrate (%)	Yield (EPS/Cells)	Final pH
6	-	-	-	-	-	-	5.30
7	14.788	4.890	10.34	48.5	48.9	0.331	7.63
8	22.242	5.492	10.47	13.3	38.0	0.247	7.63
9	19.050	7.517	10.67	12.5	48.4	0.395	7.80

4.1.1.7. Effect of Ethanol Volume on EPS Recovery. Exopolysaccharides are generally recovered from the culture broth by the addition of polar organic solvents miscible with water, such as lower alcohols or acetone. The proportion of solvent used is variable, which can be one, two or three volumes of the culture broth, although two volumes are most oftenly used (Han et al., 1990; Kumar et al., 2007). To investigate the effect of ethanol volume on polysaccharide recovery, ethanol has been added to the cell-free fermentation broth at 1:1, 2:1, 3:1 and 4:1 (v/v) and the precipitated EPSs were recovered by centrifugation, dialyzed and lyophilized. As shown in Table 4.9, at least two volumes of ethanol was found to be necessary to achieve low water activity levels in the polymer solutions. When three volumes of ethanol were added to the broth, the recovered EPS amounts were comparable with two volumes however any further increase in ethanol volume resulted in lower polymer yields. Hence these results together with economic considerations suggested that two volumes of ethanol was the optimum volume for the recovery of EPSs from the broth.

Table 4.9. Effect of ethanol volume on polysaccharide recovery

Ethanol:Broth, v/v	EPS (g/L)
1:1	4.890
2:1	6.086
3:1	6.060
4:1	5.438

4.1.1.8. Effect of Color Removal by Repeated Activated Carbon Treatment on Cellular Growth and EPS Production. In order to investigate the effect of color removal and some trace metals (Fe, Ni, Zn) in beet molasses on biomass growth and EPS production, clarified sugar beet molasses (BM CpH) was subjected to one, two, three and four runs of AC pretreatment as described in Methods 3.2.1.4 with the utilized AC amount reduced from 3% to 2% (w/v). Color removal was followed by measuring the optical density of the pretreated molasses samples at 395nm (Ahmadi et al., 2006) and results were shown in Figure 4.7. As expected

with repeated cycles of AC treatment, coloring substances were effectively adsorbed on AC resulting in up to 90% color removal.

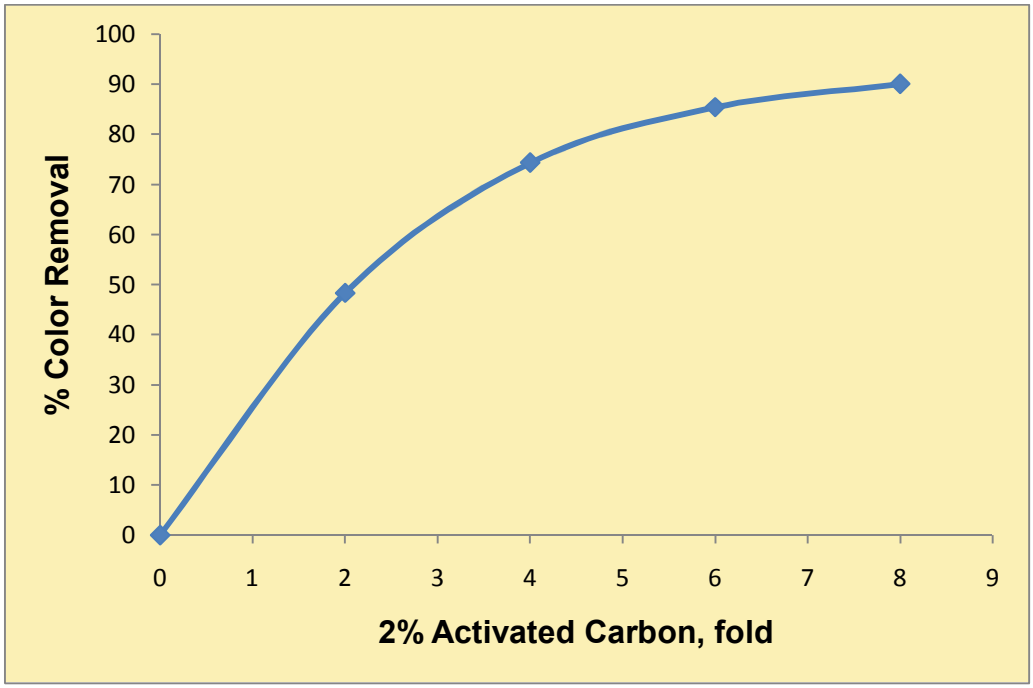


Figure 4.7. The effect of activated carbon treatment on the color removal of the beet molasses solution

To investigate the effect of repeated AC pretreatment on the heavy metal composition of sugar beet molasses, samples were analyzed by using Perkin Elmer Aanalyst 300 Atomic Absorption Spectrophotometer for their iron, zinc and nickel content and results are tabulated in Table 4.10. The percent changes in the metal content were also indicated in paranthesis.

In accordance with earlier results reported in 4.1.1.2., dissolved iron (Fe^{+2}) concentration increased with number of cycles however with decreasing efficiencies so that three and four cycles of pretreatment did only result in 38% and 10% increases in iron concentration, respectively. Nickel concentration was found to change ±5% only resulting in only minor changes in its consentration which in fact was similar to previous observations. Zinc was effectively removed up to three cycles of pretreatment however an additional fourth cycle did not result in a detectable change in zinc content.

Table 4.10. The effect of activated carbon on the removal of trace metals

Run	Total AC utilized (w/v)	Fe (mg/L)	Ni (mg/L)	Zn (mg/L)
	-	16.005	6.240	24.030
1xAC	**4%**	36.930 (+131%)	6.030 (-3.37%)	21.210 (-11.4%)
2xAC	**8%**	92.640 (+151%)	5.685 (-5.72%)	11.550 (-45.5%)
3xAC	**12%**	128.100 (+38.3%)	5.505 (-3.17%)	8.910 (-22.90%)
4xAC	**16%**	141.375 (+10.4%)	5.775 (+4.91%)	8.985 (+0.842%)

To investigate the effect of repeated AC treatment on cellular growth and EPS production, samples were added to chemical media at 10 g/L final carbohydrate concentration. Growth media were inoculated with 1% (v/v) of fresh preculture and samples were taken from *Halomonas* sp. AAD6 shake flask cultures at certain times, analyzed for biomass and EPS production. Growth was followed by measuring the optical densities at a wavelength of 660 nm using Lambda35 UV/Vis spectrophotometer. Time dependent growth profiles of the cultures were given in Figure 4.8.

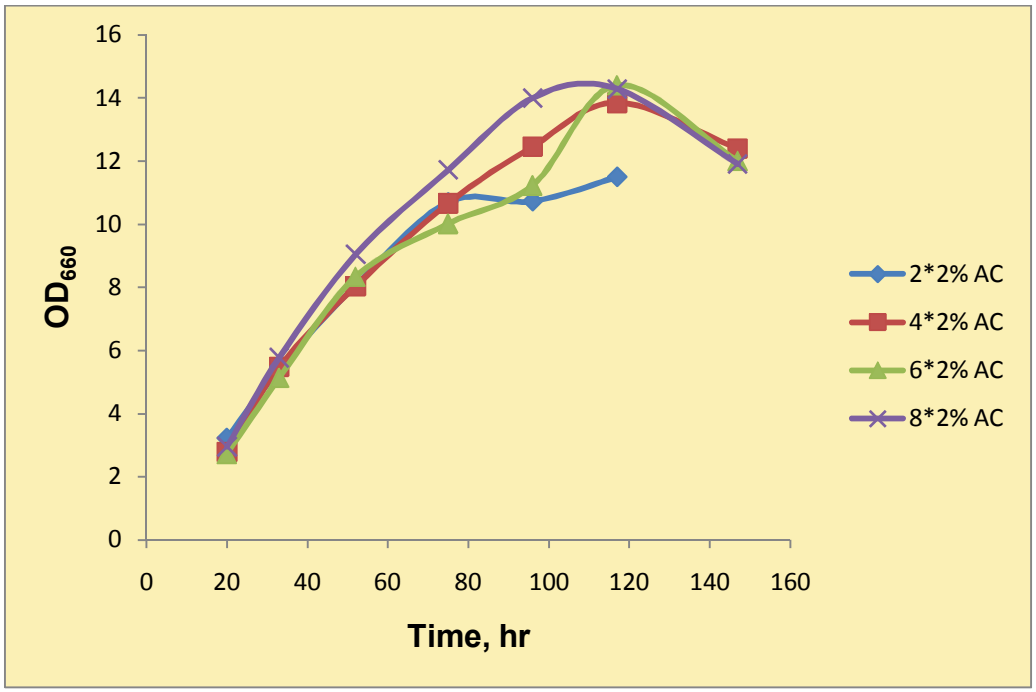


Figure 4.8. Growth profiles of *Halomonas* sp. AAD6 cultures grown on beet molasses subjected to repeated AC treatment

Cultures grown on BM pretreated with only 4% AC reached stationary phase after 75 hours in good agreement with growth profile in Figure 4.3 where a total of 6% (w/v) AC was used for the pretreatment. Since the additional 40 hours of fermentation did not result in any change in culture turbidity, fermentation was terminated and the cell-free broth was treated with equal volume of ethanol for EPS recovery. Other cultures were found to reach higher optical densities after 120 hours of fermentation however, within the following 30 hours, culture turbidity was found to decline and hence fermentation was terminated for EPS recovery. For the biomass yields, stationary phase cultures were centrifuged and the cell pellets were dried in oven until constant weight. The biopolymers in the clear supernatants were recovered as explained in 3.2.7. The carbohydrate, protein and DNA content of the samples were also analyzed to evaluate their composition. Results were summarized in Table 4.11.

Table 4.11. Effect of activated carbon pretreatments on biomass and EPS production

Run	Cells (g DCW/L)	EPS (g/L)	DNA (%)	Protein (%)	Carbohydrate (%)	Yield (EPS/Cells)	Final pH
1	8.447	2.265	7.17	21.1	71	0.268	7.06
2	8.158	1.205	7.59	29.6	46	0.148	7.68
3	7.169	1.000	5.28	33.1	56	0.140	7.66
4	6.814	1.054	3.47	30.5	62	0.155	7.83

Decrease in the culture turbidity was also reflected in the dry cell masses obtained as DCW so that prolonged incubation had a negative effect on the maintenance of cellular integrity. This may result in cell lysis and associated lower absorbency values. This hypothesis was strengthened by the results of DNA, protein and carbohydrate assays. As shown in Table 4.11, samples have high DNA, protein and low carbohydrate contents indicating that cellular proteins and DNAs were released to the broth because of lysis and co-precipitated with EPSs. The EPS yield of the 1xAC sample with 4% AC utilized (2.265 g/L) was very close to the 2.780 g/L yield reported in Table 4.3 where 6% AC was utilized. However both samples differ in their fermentation times (Figure 4.3 and Figure 4.8) and whereas a prolonged incubation of about 30 hours resulted in some cell lysis and associated decrease in the purity of recovered EPSs. The detrimental effect of prolonged incubation became more evident in the other samples where growth reached higher values and lysis was more apparent by the decrease in the OD values.

It is known that trace elements like Fe, Ni, Zn are essential for microbial growth (Zhang et al., 2003; Patidar et al., 2006; Müller, 2009). Since exopolysaccharide production process in this microbial system is growth associated, it is highly linked to the growth conditions of the cultures.

Kotzamanidis et al. (2002) showed that maximum lactic acid production was higher for untreated molasses than that of obtained from activated carbon pretreated colorless beet molasses. Jiang et al. (2009) obtained same amount of butyric acid from untreated and activated carbon pretreated molasses. Teichel et al. (2009) indicated that this pretreatment may remove important nutrients from the substrate, affecting growth of the microorganism.

In this study, removal of heavy metals and increases in iron concentration most probably affected the cellular metabolism that did not result in any growth impairment but rather in diminished maintenance of the cellular integrity. The lower EPS yields can be attributed to the prolonged fermentation periods as well as the differences in the heavy metal composition of the

fermentation media. To elucidate the effect of AC treatment on EPS production, further experiments should be conducted where EPS production profiles should also be considered and fermentation time should be decided accordingly.

4.1.2. Cheese Whey Powder

Cheese whey powder (CWP) is mainly composed of lactose. However, rather than a carbon source, CWP may be considered as a good nitrogen source because of its proteins with a high biological value and high content of sulfur-containing amino acids such as cysteine and methionine. Moreover, whey is a good source of electrolytes including sodium and potassium, minerals such as calcium, magnesium, zinc and phosphorus and also vitamins such as B12, B6, pantothenic acid, riboflavin, biotin, thiamine, nicotinic acid, folic acid, and ascorbic acid (Goyal et al., 2008). CWP was purchased from the wholesaler market and it was diluted to appropriate concentration and autoclaved to denature and precipitate large proteins. Total carbohydrate content of the clarified supernatant was determined by the phenol/sulphuric acid method, autoclaved for sterilization and then it was added to the optimum medium to keep the final carbohydrate concentration between 30 g/L to 105 g/L. Biomass and EPS concentrations of the stationary phase *Halomonas* sp. AAD6 shake flask cultures were determined and summarized in Table 4.12.

Table 4.12. Effect of CWP on cellular growth and EPS production

Carbohydrate conc. (g/L)	Biomass (gDCW/L)	EPS (g/L)	Yield (gEPS/gCells)
30	1.8530	0.0000	0.0000
45	4.1917	0.0275	0.0066
60	4.7797	0.0559	0.0117
75	7.0855	0.0726	0.0103
90	10.1371	0.0815	0.0080
105	14.6771	0.0000	0.0000

Biomass concentrations were found to be approximately constant within 30-60 g/L wheras after that range, it increased with increasing carbohydrate concentration. However, the EPS production yields were considerably low and even undetectable at 105 g/L concentration. This result is in good aggreement with earlier observations. As shown in Table 4.1., when lactose was used as the sole carbon source in chemical media, poor cellular growth (0.103 gDCW/L) and low EPS yields (0.060 g/L) (Poli et al., 2009). Moreover, low yields of EPS (0.830 g/L) from lactic acid bacteria *Lactobacillus delbrueckii* subsp.*bulgaricus* (Shene et al, 2007) and also even lower EPS yields (0.106 g/L) from an extremophilic microorganism, *Streptococcus thermophilus*, were reported (Shene et al., 2008).

In order to evaluate the potential use of CWP as nitrogen source for EPS production, it has been mixed with BM HAC at different ratios and then used as substrate in shake flask experiments conducted under the same conditions. Biomass and EPS yields obtained from the stationary phase cultures were summarized in Table 4.13. As the concentration of beet molasses decreased in the mixture of beet molasses and cheese whey powder solution, both the cellular mass and the amount of exopolysaccharide produced decreased as well. It can be concluded that there is no synergistic effect in the combinations of sugar beet molasses and cheese whey powder solution.

Table 4.13. Effect of CWP and BM HAC combinations on cellular growth and EPS production

BM HAC:CWP (g/L)	Biomass (gDCW/L)	EPS (g/L)	Yield (gEPS/gCells)
30:0	23.903	7.610	0.318
25:5	17.224	6.707	0.389
20:10	11.746	5.525	0.470
15:15	10.664	4.622	0.433
10:20	10.333	1.676	0.162
5:25	8.4470	0.268	0.032
0:30	1.8530	0.000	0.000

4.1.3. Sugar Beet Pulp

Sugar beet pulp is a cheap, abundant and carbohydrate rich substrate and in order to evaluate its potential use as a carbon and energy source for EPS production, the dry beet pulp obtained from Kütahya Sugar Factory was used as a solid substrate for *Halomonas* sp. AAD6 batch cultures. Optimum media containing 20-100 g/L pulp were inoculated with preculture and then incubated in the orbital shaker at 37^{o}C and 180 rpm rotating speed. Cells, pulp and particles were removed from the solid state fermentation cultures by centrifugation and the EPSs from the clear supernatants were recovered by ethanol precipitation, dialyzed against distilled water and then lyophilized. The EPS yields and their protein and DNA contents were summarized in Table 4.14.

Table 4.14. Cellular growth and EPS production from sugar beet pulp

Beet Pulp (g/L)	EPS (g/L)	DNA (%)	Protein (%)
20	0.683	1.793	7.196
40	1.431	3.140	8.235
60	1.609	3.830	11.995
80	2.204	7.216	10.162
100	2.582	9.783	14.979

There was a direct relationship between the amount of beet pulp in the media and EPS yield. The heterogeneous nature of the fermentation medium caused by the suspended particles and beet pulp fibers prevented the spectrophotometric analysis of turbidity. Therefore, cell concentration could only be assumed to be increasing with increasing beet pulp concentration as also reflected by the increasing EPS production.

The quantities of DNA and protein found in exopolysaccharide samples increase with increasing amounts of beet pulp. This could be because of the difficulty in separation of the liquid and solid parts of the fermentation culture resulting in some cellular or pulp remains in the clarified media which in turn might interfere with the subsequent ethanol precipitation step.

4.1.4. Olive Mill Wastewater

Olive mill wastewater (OMW) supplied from Verde Olive Oil Factory (İzmir, Turkey) was first clarified by centrifugation at 10000 rpm for 10 minutes to eliminate the coarse particles which might inhibit the cellular growth and then analyzed for its total carbohydrate content by the phenol-sulphuric acid method. OMW with 28 g/L total carbohydrate concentration was diluted to different volumetric concentrations (%40-%90 v/v) so as to use in the fermentation

experiments. Having adjusted the initial pH of the fermentation medium to 7, it was inoculated with a freshly prepared preculture and the *Halomonas* sp. AAD6. Batch cultures were incubated in the orbital shaker which was set to 37^{o}C and 180 rpm. Samples were taken from shake flask cultures at certain times, analyzed for cellular growth by reading their OD_{660}. For 140 hours of incubation, no increase in turbidity was observed in any of the cultures suggesting the improper composition of the fermentation media that hampered not only the growth of microorganisms but also any biopolymer production. The conditions used in these preliminary experiments should be improved to make them suitable for the metabolic needs of *Halomonas* sp. AAD6 cells. Hence more studies should be done to make OMW a suitable fermentation substrate for EPS production by the halophilic cells.

4.1.5. Orange Bagasse

Orange bagasse was considered as a suitable waste resource because of its composition rich in soluble carbohydrates, particularly fructose, glucose, sucrose, and pectins, as well as insoluble cellulose. Therefore, orange shells obtained from Starbucks coffee shops (Istanbul, Turkey) were chopped to small and equal pieces, weighed and then incubated overnight in an oven at 80°C to dry until constant weight. From the weight measurements, the shells were found to contain 61 % moisture by mass.

In order to compare the cellular growth and EPS production capability of *Halomonas* sp. AAD6 cultures grown in optimum medium containing wet and dry orange shells, solid state cultures were prepared by weighing 5 grams and 0.5 grams of dry shells and their equivalent wet shells in each Erlenmeyer flask containing 100 ml optimum medium. The media were steam sterilized, inoculated and cultures were incubated in the orbital shaker at 37ºC and 180 rpm. After 120 hours of solid state fermentation, shells and particulate matter were separated by centrifugation and the EPSs from the clear supernatants were recovered by ethanol precipitation, dialyzed against distilled water and then lyophilized. The EPS yields and their carbohydrate, protein and DNA contents were summarized in Table 4.15.

Table 4.15. Dry and wet orange bagasse on cellular growth and EPS production

Orange Shells (g/L)	Wet/Dry	EPS (g/L)	Carbohydrate (%)	DNA (%)	Protein (%)
50	Dry	1.4033	43	9.30	8.495
128	Wet	0.4022	68	-	8.645
5	Dry	0.0831	28	8.19	-
12.8	Wet	0.0653	33	-	7.081

As shown in Table 4.15, highest EPS yields were obtained from 50 g/L orange shells with more than 10-fold decrease in the polymer yield when the shell concentration decreased to 5 g/L on dry basis. Total carbohydrate analysis showed that all the four samples consisted of low amounts of carbohydrate. Mass based composition of the samples were similar in terms of DNA and protein so that each sample had almost 10% DNA and 10% protein which are considerably high. These results suggested that these samples were most likely to be a mixture of exopolysaccharides, proteins and DNA, also called extracellular polymeric substances, which may originate from the shells as well as from the cellular metabolism.

4.2. Structural Characterization of Exopolysaccharides

Fourier Transform Infrared Spectroscopy (FT-IR) is a fast method that shows the absorption of infra red light by molecular bonds at a given wavelength. Light absorption is closely associated with the molecular vibrations whose frequency changes with the masses of the atoms forming the bond as well as the strength of the bond. Hence a FT-IR spectrum shows the IR-bands characteristic for specific functional groups present in a sample analyzed.

FT-IR does have potential as a diagnostic tool for EPS identification. It was also used for the chemical characterization of EPS produced by halophilic *Halomonas* sp. AAD6 cells growing in chemical medium containing pure sucrose (Poli et al., 2009).

FT-IR spectra of EPSs produced in this study were measured for their preliminary identification and to elucidate any structural differences from the fructan-type EPS, namely levan, which was reported to be produced by *Halomonas* sp. AAD6 on pure sucrose (Poli et al., 2009). For the analysis, only EPSs produced by cultures growing on pretreated sugar beet molasses were used and the results were compared with the spectra of commercial Levan produced by Zymomonas mobilis (Sigma no: 66674) as well as with the spectra of Levan produced by *Halomonas* sp. AAD6. FT-IR spectra were shown in Figures 4.9-4.11.

Major peaks associated with levan were summarized in Table 4.16.

Table 4.16. Peaks originating in levan (Barone et al., 2007)

Peak position (cm^{-1})	**Peak assignment**
1398	δ(CH), δ(OH)
1370	δ(CH), δ(OH)
1265	ν(CH)
1248	ν (CH)
1165	ν (CO) linkage
1093	ν (CO) linkage
945	ν (CO) furanose ring
813	ν (CO) furanose ring

Vibrations: ν = stretching, δ = bending

In these spectra, the strong bands around 3200 cm^{-1} was assigned to the hydroxyl (OH) stretching vibration of the polysaccharide and the two bands around 2880 cm^{-1} 2920 cm^{-1} in all figures were because of carbon-hydrogen (C-H) stretching vibration which indicate the existence of fructose residue (Liu et al. 2010). The bands around 1640 cm^{-1} was because of the residual water (Barone et al., 2007). The bands in the region of 1430 cm^{-1} and 1200 cm^{-1} were assigned to C-H plane deformation vibration combined with aromatic skeletal vibrations (Schwanninger et al., 2004; Liu et al. 2010). The bands between 1120 cm^{-1} and 1020 cm^{-1} were dominated by the stretching vibrations of the glycosidic linkage contributions of C-O-C and C-O-H (Wu et al., 2009). A characteristic absorption around 930 cm^{-1} was resulted from the stretching vibration of pyran ring (Schwanninger et al., 2004).

The high level of resemblance observed in the spectra of the EPS samples suggested the fact that these polymers were in fact Levan type polysaccharides.

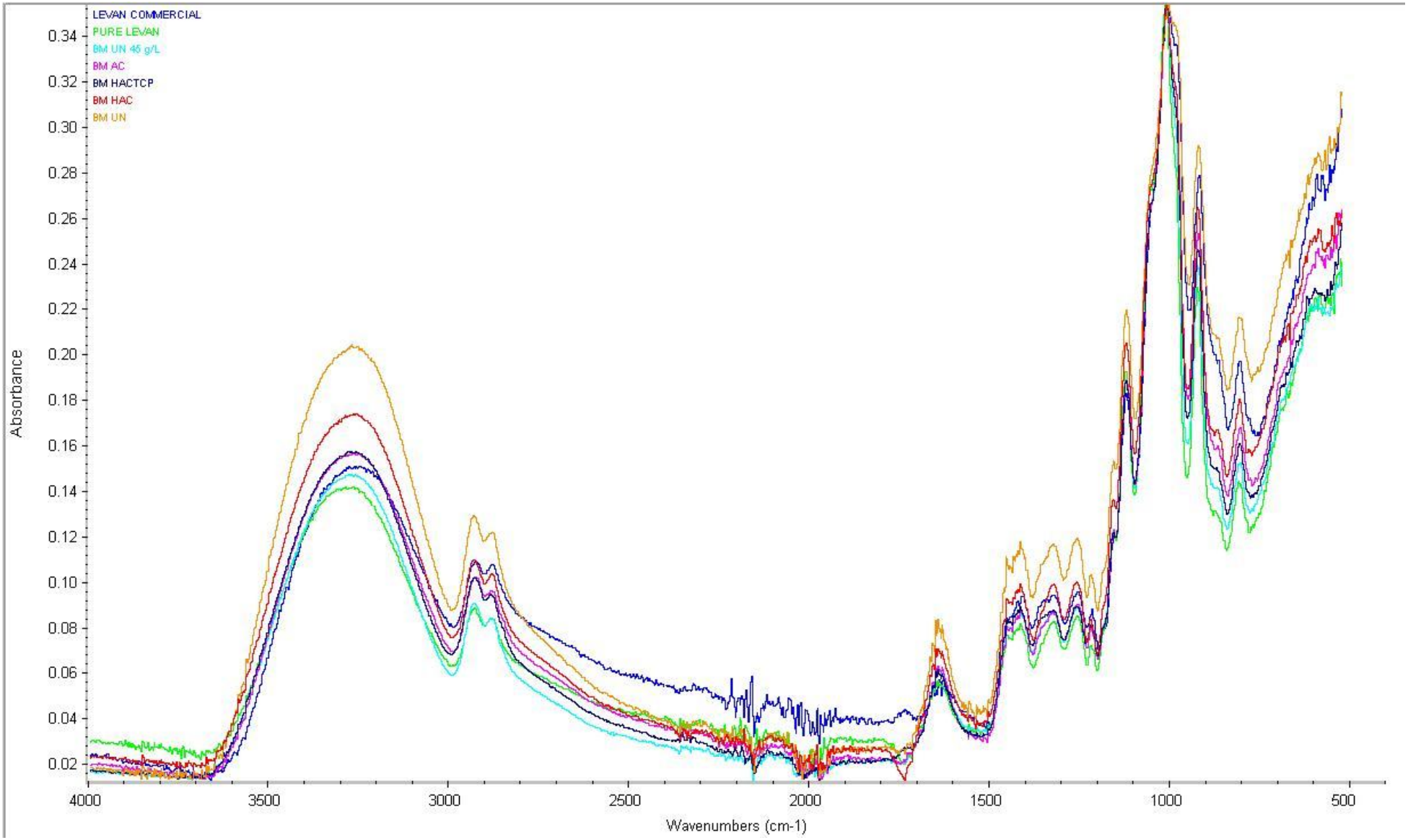


Figure 4.9. FT-IR spectra of commercial levan, pure levan, and EPSs produced from pretreated BM

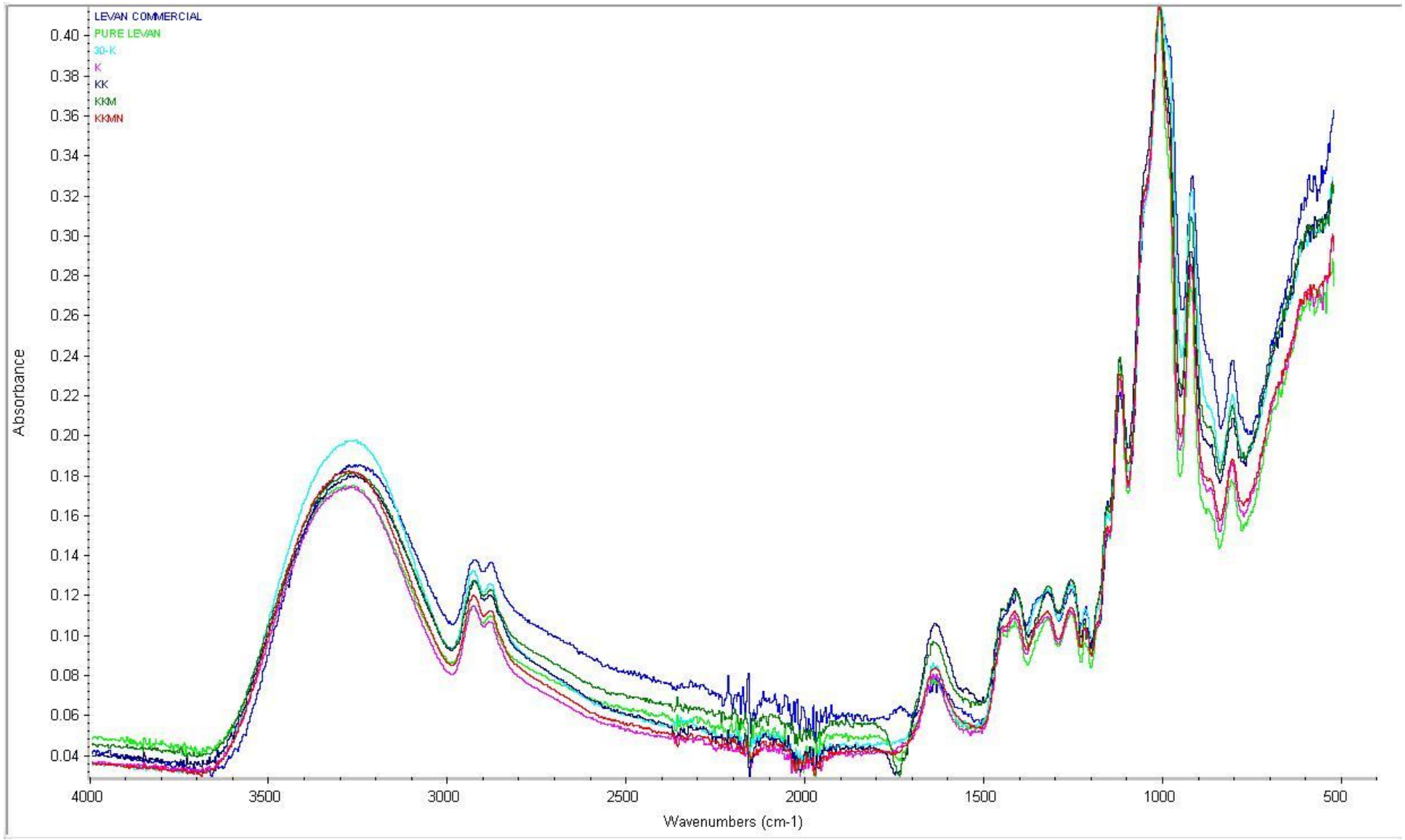


Figure 4.10. FT-IR spectra of commercial levan, pure levan, and EPSs produced from pretreated BM with salt drop out

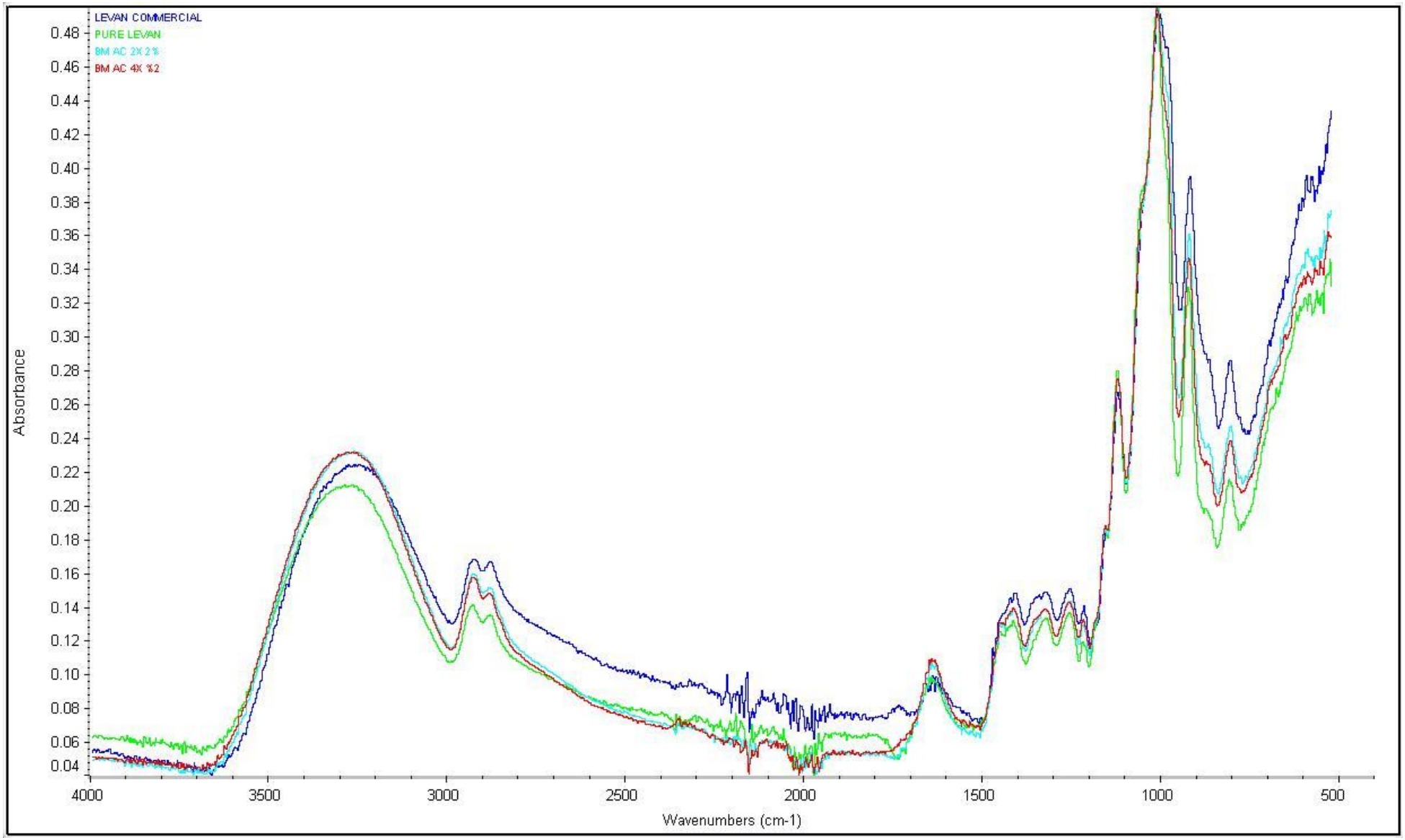


Figure 4.11. FT-IR spectra of commercial levan, pure levan, and EPSs produced from BM subjected to different runs of AC pretreatment

4.3. Rheological Analysis of Exopolysaccharides

In order to understand rheological characteristics of the biopolymers, the flow behaviors of EPSs produced from pretreated sugar beet molasses were used at 1% concentration. Samples including BM HAC (10g/L), BM TCPHAC (10 g/L, 30 g/L, 45 g/L), different mineral salts (K, KK, KKM, KKMN) of BM TCPHAC (30 g/L) at two different temperatures (20 ^{o}C and 37 ^{o}C) and their %10 salt concentrations at these temperatures were measured. To understand the thixotropic and shear thinning properties of the samples, steady shear stress was measured over a range of shear rates of 10-200 s^{-1} at aforementioned temperatures and salt concentration.

The rheological characterizations of the EPSs were carried out and compared with those of alginate, pullulan and xanthan. All EPSs have shown the characteristic of low viscosity in the experiments performed at two different temperatures and their %10 salt concentrations (Figures 4.12-4.15.) These results were in good agreement with earlier reports where the intrinsic viscosity of levan biopolymer was between 0.13-0.38 dL/g (Bae et al., 2008). These intrinsic viscosity values were quite low compared with typical intrinsic viscosities ranging from 1 dL/g for compact coil or flexible chains (dextran) to 20 dL/g for extended chains (alginate) and up to 50 dL/g (xanthan).

The flow behaviours of all polysaccharides at two different temperatures and their %10 NaCl solutions were investigated and represented in Figures 4.16-4.19. The shear stress measurements for the biopolymer solutions indicated that the shear stress versus shear rate curves were characterized by increased shear stress with increasing shear rate. As a consequence, all biopolymer samples appeared to exhibit pseudoplastic behaviours.

Furthermore, the shear stress versus shear rate curves were fitted into the Oswald–de Waele model or also called the power law model given in the equation below:

$$\sigma = K^{*}\dot{\gamma}^{n} \qquad (4.1)$$

Where σ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (1/s), K is the consistency coefficient (Pa s^n), and n is the flow behaviour index.

The flow behaviour indices, n, which provide the degree of pseudoplasticity were determined to be less than 1, which verified the pseudoplastic behaviours of the polymer samples. Oswald–de Waele model parameters for all exopolysaccharides were given in Table 4.17 together with those of xanthan, pullulan and alginic acid.

Moreover, the consistency coefficient (*K*) which is a measure of viscosity, was found to vary considerably with the type of sample. As expected, its value for xanthan was highest within the range 2.7-5.3 Pa s^n. The corresponding values for the samples were closer to pullulan. Also the viscosity profiles of the samples were very similar to that of pullulan up to a shear stress of 10 s^{-1} (Figures 4.12-4.15).

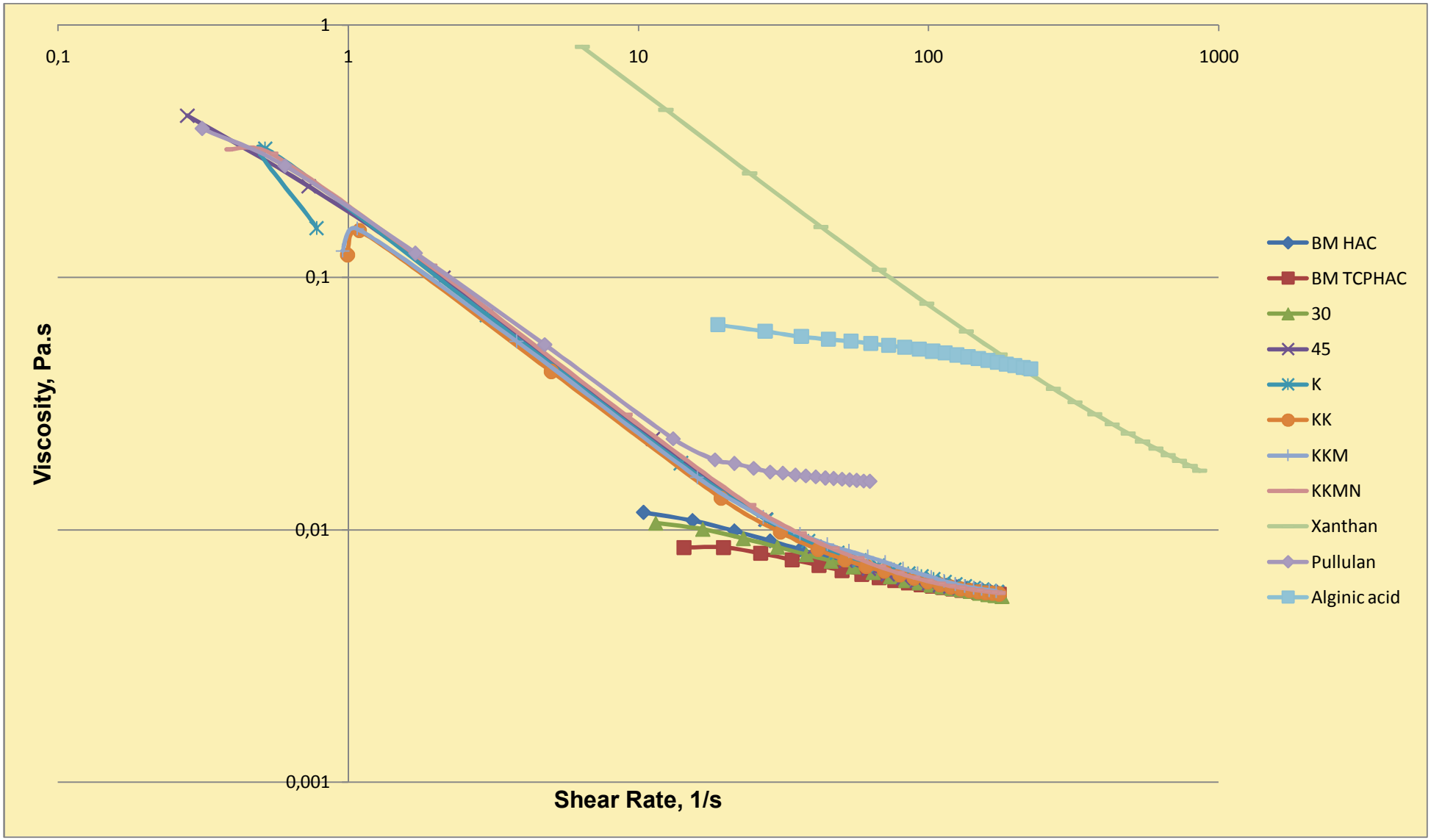


Figure 4.12. Viscosity versus shear rate curves of the %1 (w/v) exopolysaccharide solutions at 20 ^{o}C

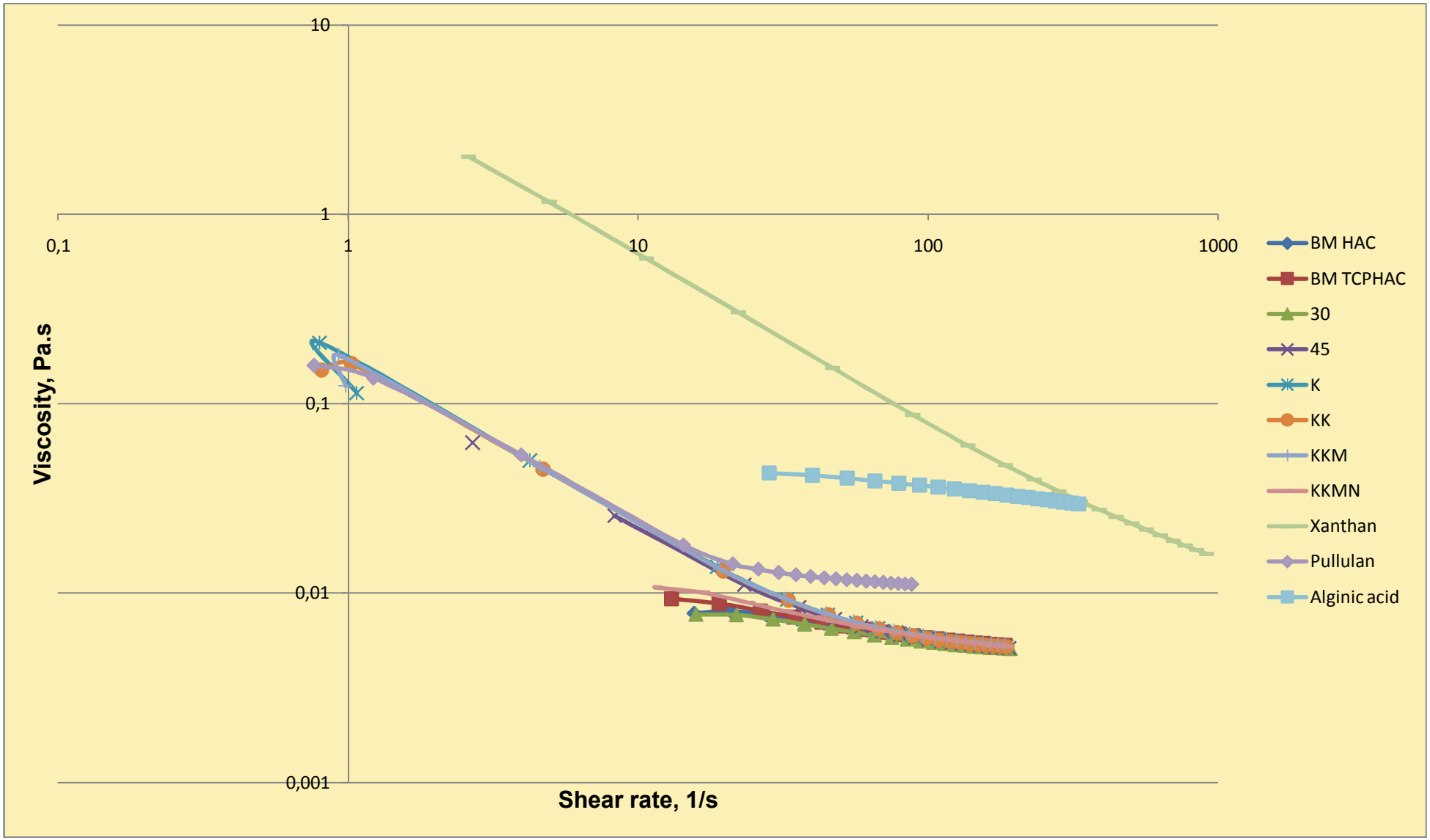


Figure 4.13. Viscosity versus shear rate curves of the %1 (w/v) exopolysaccharide solutions at 37 ^{o}C

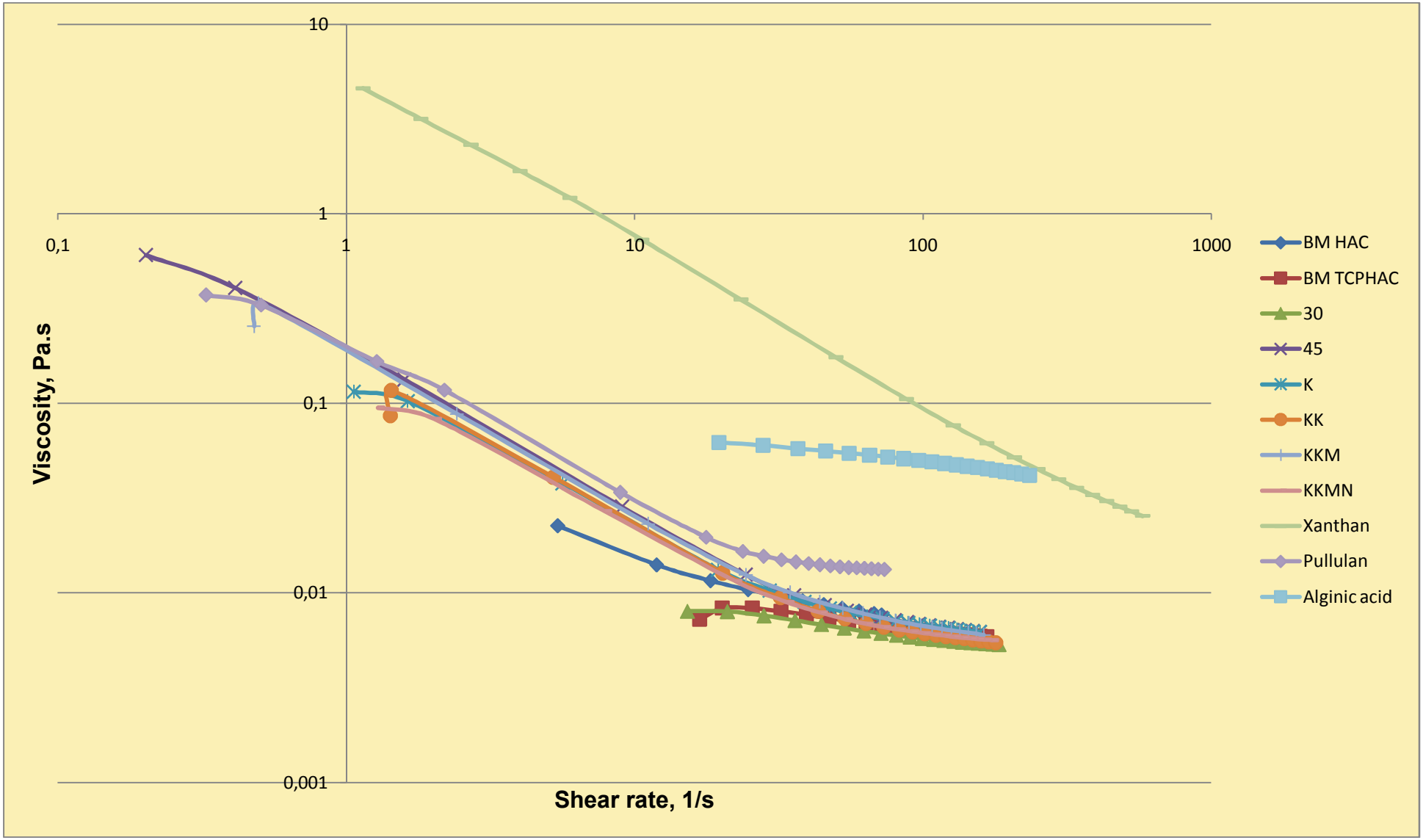


Figure 4.14. Viscosity versus shear rate curves of the %1 (w/v) exopolysaccharide solutions at 20 ^{o}C and in %10 NaCl solution

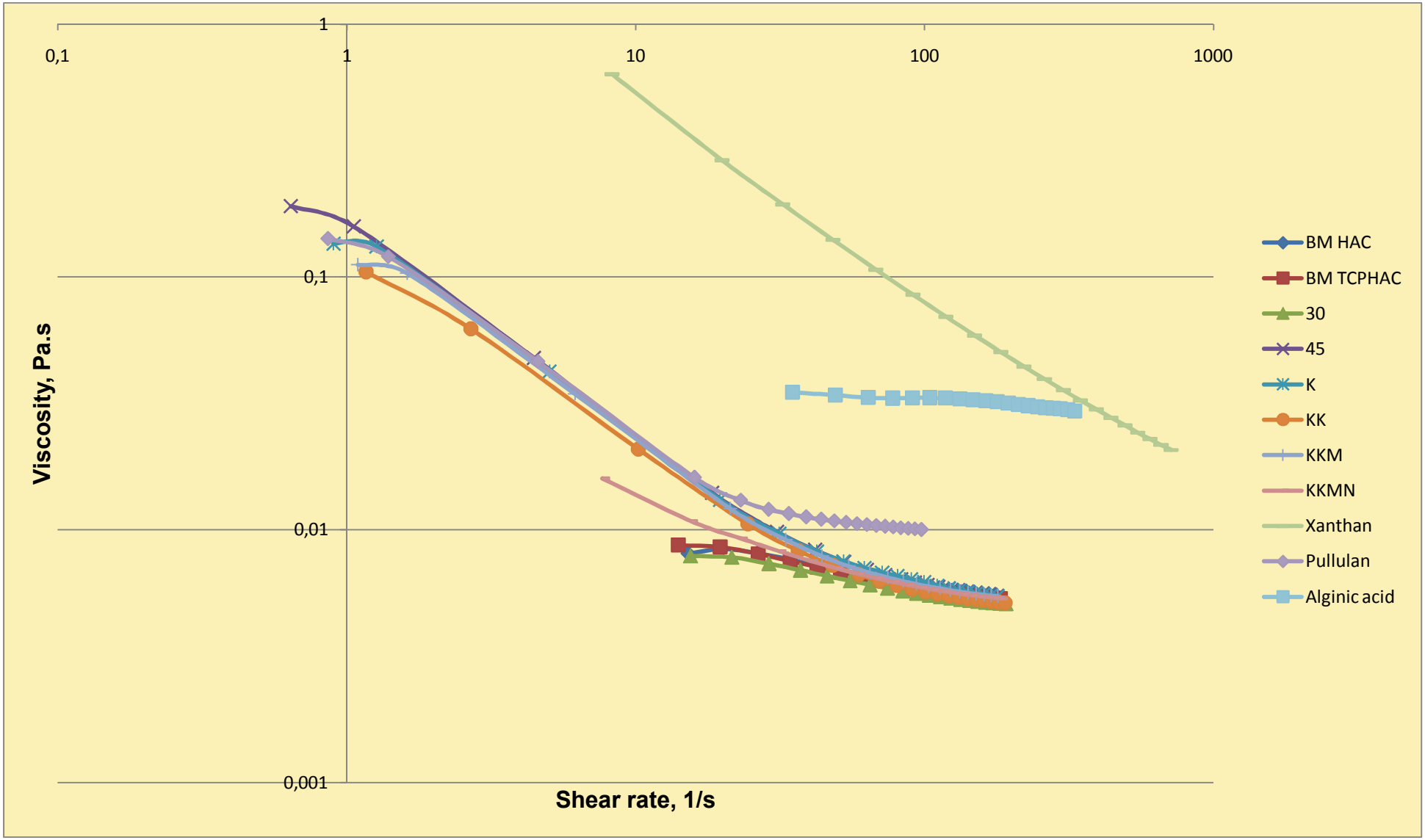


Figure 4.15. Viscosity versus shear rate curves of the %1 (w/v) exopolysaccharide solutions at 37 ^{o}C and in %10 NaCl solution

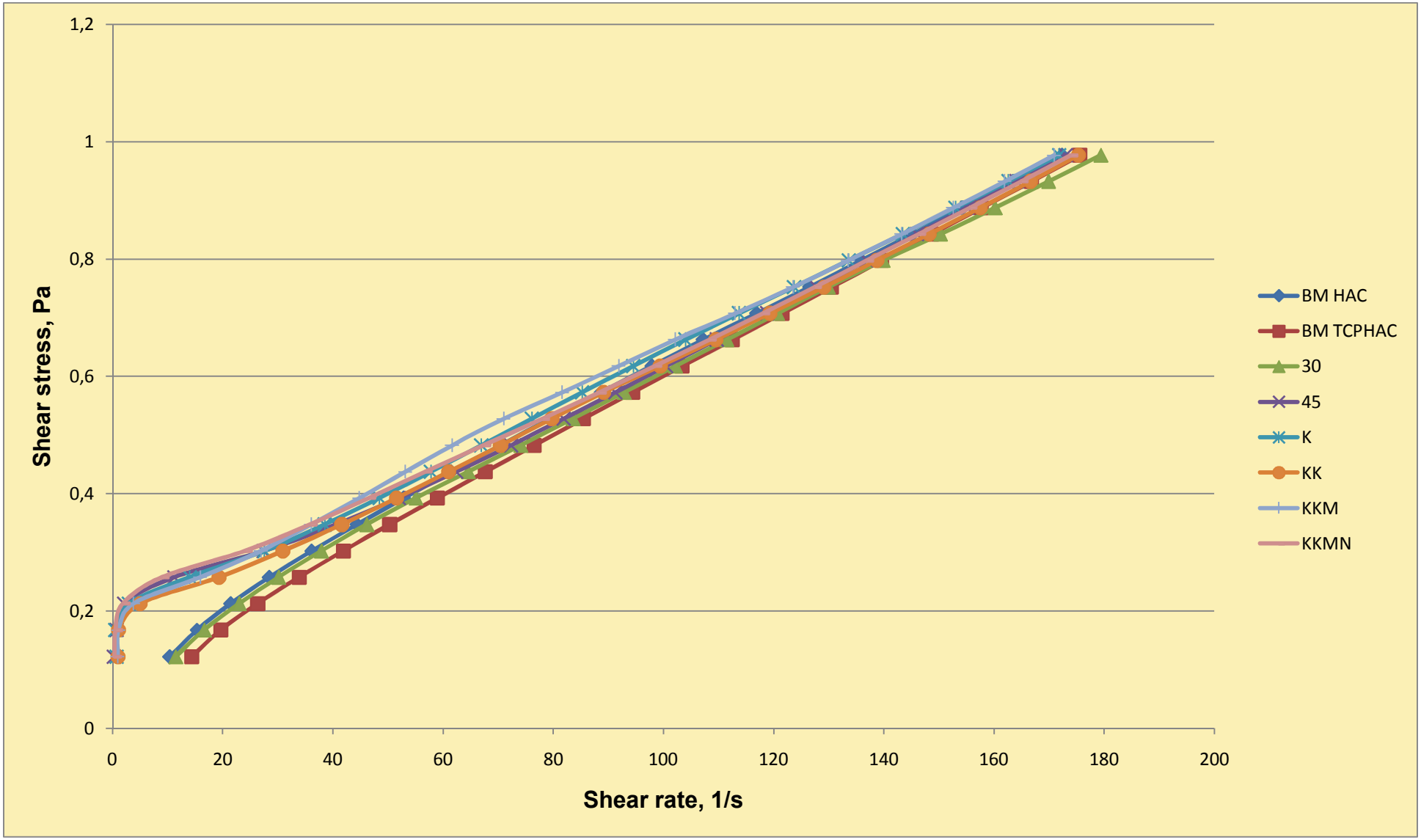


Figure 4.16. Rheograms of the %1 (w/v) exopolysaccharide solutions at 20 ^{o}C

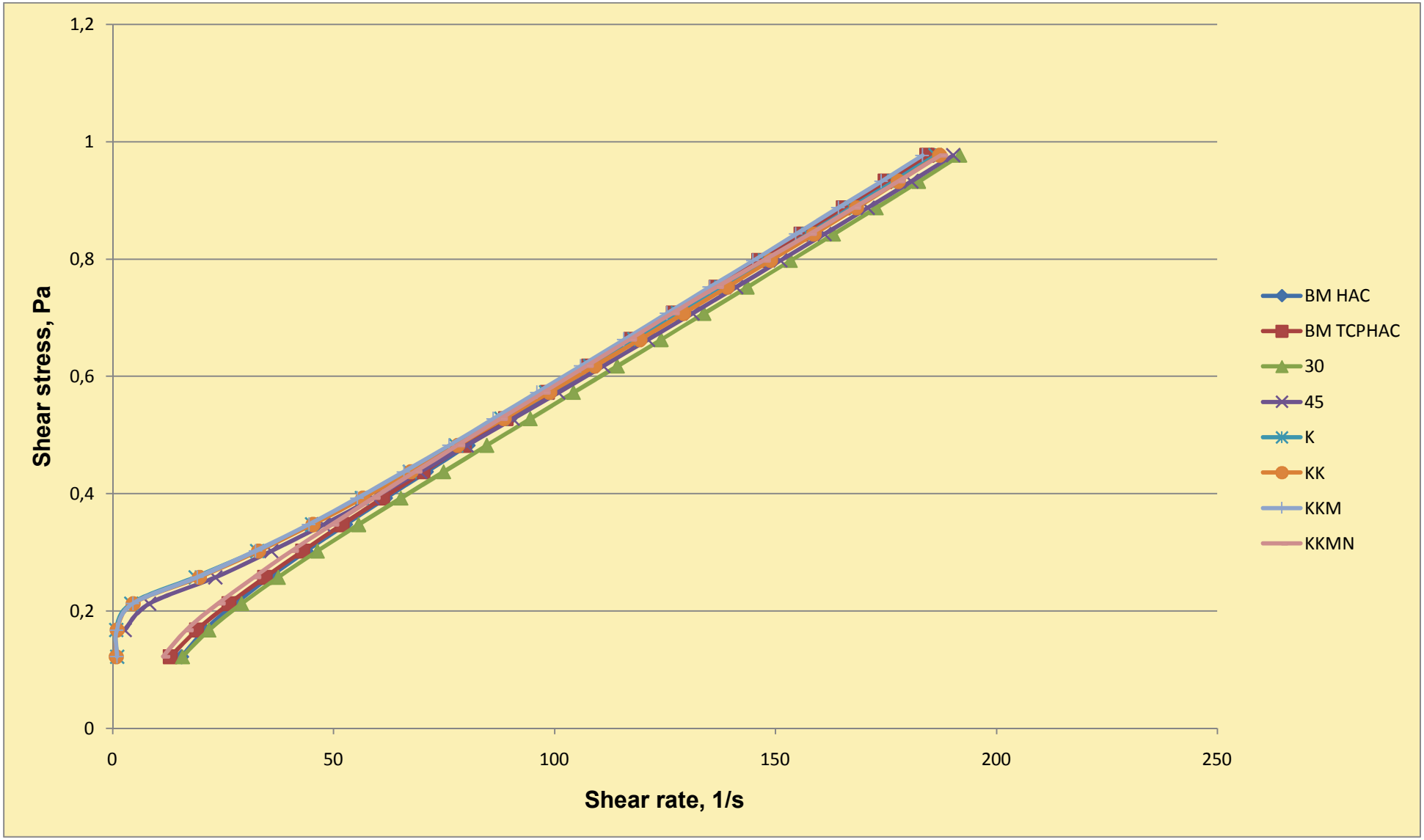


Figure 4.17. Rheograms of the %1 (w/v) exopolysaccharide solutions at 37 ^{o}C

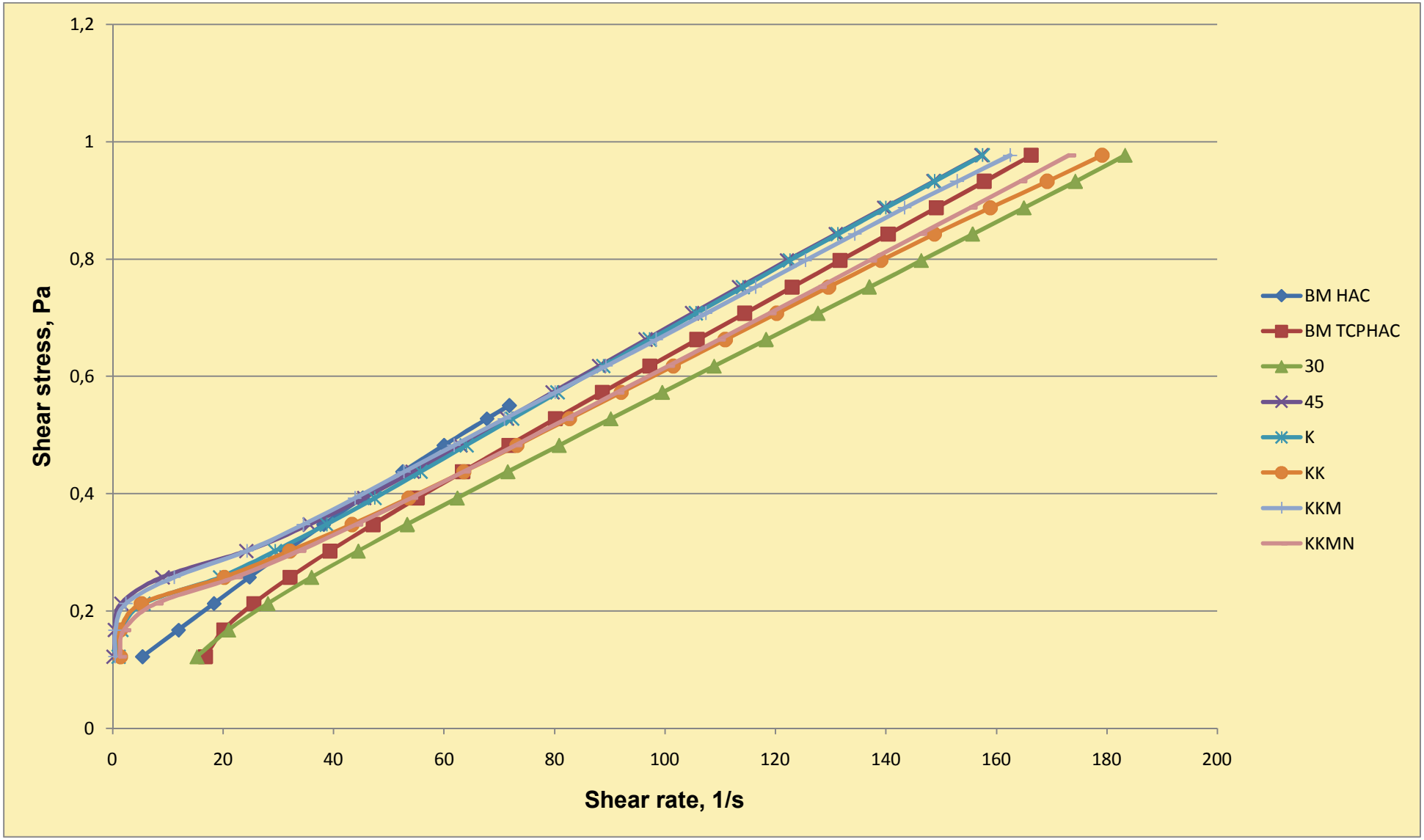


Figure 4.18. Rheograms of the %1 (w/v) exopolysaccharide solutions at 20 ^{o}C and in %10 NaCl solution

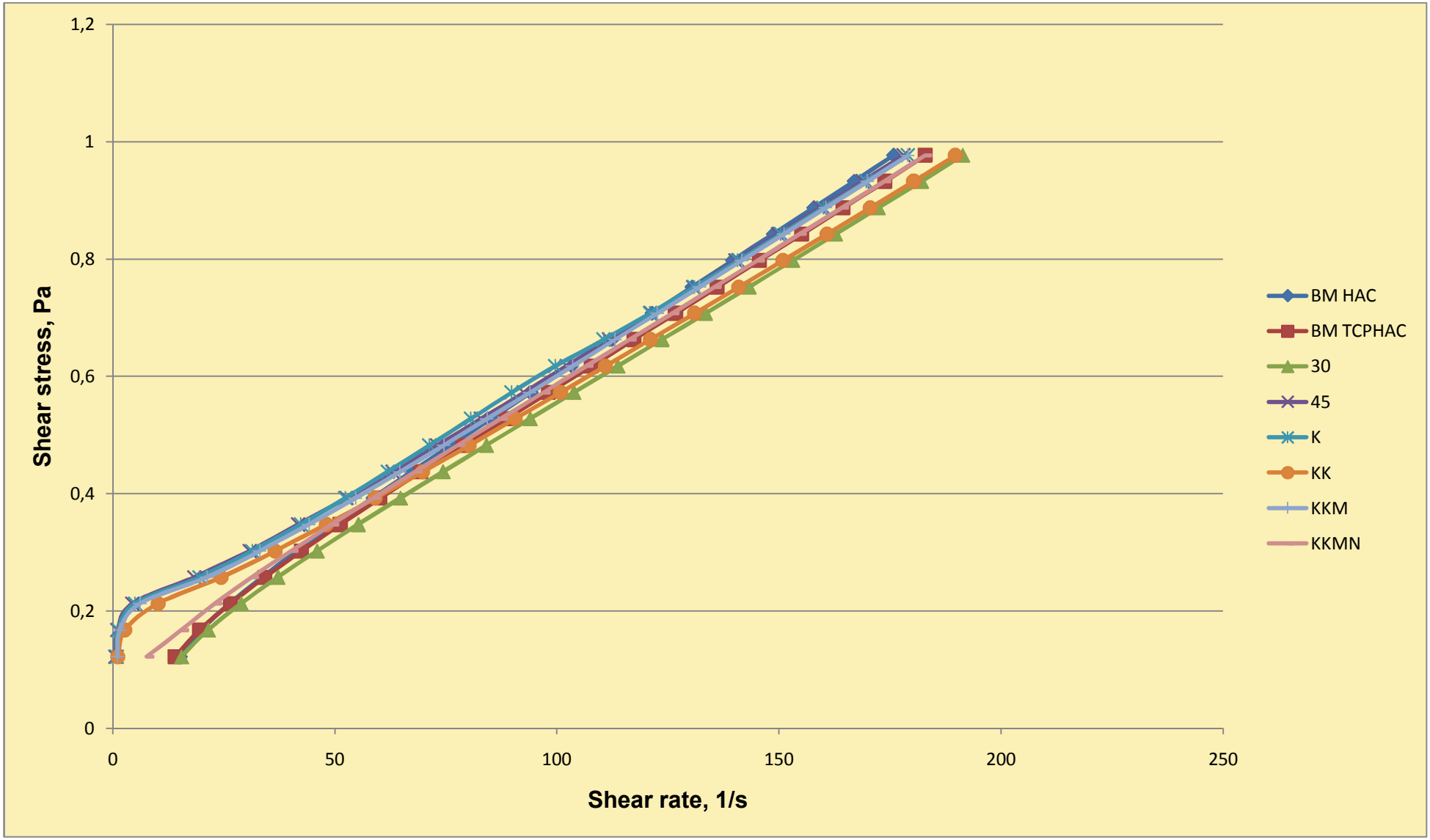


Figure 4.19. Rheograms of the %1 (w/v) exopolysaccharide solutions at 37 ^{o}C and in %10 NaCl solution

Table 4.17. The power law parameters of the EPS solutions

	Aqueous solution						Aqueous %10 NaCl solution					
	20 °C			37 °C			20 °C			37 °C		
	K (Pa s^n)	n	R^2	K (Pa s^n)	n	R^2	K (Pa s^n)	n	R^2	K (Pa s^n)	n	R^2
BM HAC	0.0223	0.7278	0.9991	0.0138	0.8123	0.9990	0.0392	0.6055	0.9864	0.0142	0.8166	0.9987
BM TCPHAC	0.0146	0.8096	0.9993	0.0164	0.7779	0.9991	0.0130	0.8457	0.9955	0.0155	0.7902	0.9992
30	0.0203	0.7417	0.9992	0.0133	0.8127	0.9992	0.0136	0.8165	0.9993	0.0137	0.8081	0.9993
45	0.1547	0.3034	0.8918	0.0780	0.4434	0.9049	0.1725	0.2870	0.8852	0.1254	0.3468	0.9000
K	0.1400	0.3278	0.8897	0.1230	0.3468	0.8896	0.1054	0.3956	0.9151	0.1148	0.3667	0.9084
KK	0.1159	0.3656	0.9047	0.1214	0.3491	0.8971	0.1047	0.3867	0.9110	0.0909	0.4087	0.9157
KKM	0.1201	0.3650	0.9212	0.1205	0.3527	0.8952	0.1508	0.3170	0.8990	0.1050	0.3841	0.9106
KKMN	0.1564	0.3044	0.9048	0.0204	0.7327	0.9985	0.0954	0.4070	0.9153	0.0257	0.6839	0.9905
Alginic Acid	0.0105	0.8418	0.9995	0.0076	0.8393	0.9997	0.0105	0.8356	0.9995	0.0046	0.9279	0.9993
Pullulan	0.1649	0.3671	0.9025	0.1184	0.4168	0.8967	0.1781	0.3381	0.9176	0.1121	0.4196	0.8984
Xanthan	3.1366	0.2135	0.9566	4.0284	0.1707	0.9294	5.2724	0.1452	0.9661	2.6966	0.2464	0.9697

4.4. Bioflocculation Properties of Exopolysaccharides

Exopolysaccharides which were produced from six different pretreated and untreated beet molasses and starch molasses, namely BM CpH, BM HAC, BM TCPHAC, SM CpH, SM HAC, SM TCPHAC, were evaluated for their bioflocculation properties.

Synthetic water (100 mg $CaCO_3$/L hardness and alkalinity, pH 7.3, 100 NTU), synthetic sea water (2.40 g/L NaCl, 0.35 g/L $MgSO_4 \cdot 7H_2O$, 0.30 g/L $MgCl_2 \cdot 6H_2O$, 0.07 g/L KCl, $CaCl_2 \cdot 2H_2O$, pH 7.3, 100 NTU), and real sea water (pH 7.3, 100 NTU) were used in the experiments. Commercial cationic (Magnafloc LT 22), nonionic (Magnafloc LT 20), and anionic (Magnafloc LT 25) polyelectrolytes were utilised also in order to compare with the results of bioflocculants.

Each of the six EPS samples were dosed at 100 mg/L concentration into synthetic water and synthetic sea water media containing 50 mg/L kaolin at 100 NTU turbidity. Jar test experiments were conducted where the state of aggregation was followed dynamically by a photometric dispersion analyser (PDA 2000). At the end of 20-minutes settling period, samples were withdrawn from the supernatant for turbidity measurement and results are summarised in Table 4.18.

Table 4.18. Residual turbidity values of EPS specimens in synthetic water and synthetic sea water media (dose 100 mg/L)

	Residual Turbidity (NTU)					
	Sugar Beet Molasses			**Starch Molasses**		
Pretreatment	**CpH**	**HAC**	**TCPHAC**	**CpH**	**HAC**	**TCPHAC**
Sample	**EPS1**	**EPS2**	**EPS3**	**EPS4**	**EPS5**	**EPS6**
Synthetic Water	93	82	96	91	32	74
Synthetic Sea Water	34	29	38.5	35	6.5	19

Since exocellular polymeric substances in the form of polysaccharides usually have very low or no charge density they exhibit higher performance in waters with high ionic strength. Considering the halophilic nature of the producer strain, EPSs were also evaluated in synthetic sea water medium. As expected, all the EPS samples showed considerably higher flocculating activity in synthethic sea water/kaolin suspension rather than synthethic water/kaolin suspension. Among the six biopolymers, as can be seen from the Table 4.18, the biopolymer forming the largest flocs and showing the lowest residual turbidity was found to be EPS5, which was obtained from starch molasses subjected to acid hydrolysis followed by activated carbon pretreatment (SM HAC). In synthethic water/kaolin suspension, almost no floc formation was detected in the experiments with the other five polymers, leading to very low turbidity removals. On the other hand, particle removal with SM HAC was remarkably discernible therefore it was chosen for further studies.

These findings correlate with the Flocculation Index values (relative sizes of flocs) formed with SM HAC as depicted in Figure 4.20. In synthetic sea water larger flocs were formed with SM HAC compared to those in synthetic water, thus better removal of particulate matter was obtained, as confirmed with lower residual turbidity value (Table 4.18). Because of higher ion concentration in synthetic sea water, particle aggregation started sooner and steady-state floc size distribution was reached much earlier (~90 min). Following (Yukselen et al, 2002) the breakage and reformation of flocs under cycled shear conditions were quantified by a ‘strength factor’ and a ‘recovery factor’ in terms of relative floc sizes (FI values), for fully grown (FI_1), ruptured (FI_2) and regrown (FI_3) flocs. With this terminology, the two factors (%) are defined as:

$$\text{Strength factor (\%)} = (FI_2/FI_1) \times 100 \quad (4.2)$$

$$\text{Recovery factor (\%)} = [(FI_3 - FI_2) / (FI_1 - FI_2)] \times 100 \quad (4.3)$$

The strength factors with SM HAC in synthetic water and synthetic sea water were calculated from Figure 4.20 as 1% and 13%, respectively. Higher floc strength in synthetic sea water suggests enhanced aggregation with less fragile

structure. Unlike hydrolyzing metal coagulants in sweep flocculation regime, SM HAC exhibited complete (95.2% recovery factor in synthetic water/kaolin suspension) or almost complete (82.2% recovery factor in synthetic sea water/kaolin suspension) size recovery after floc breakage, which is peculiar to synthetic organic polyelectrolytes (Yukselen et al., 2004).

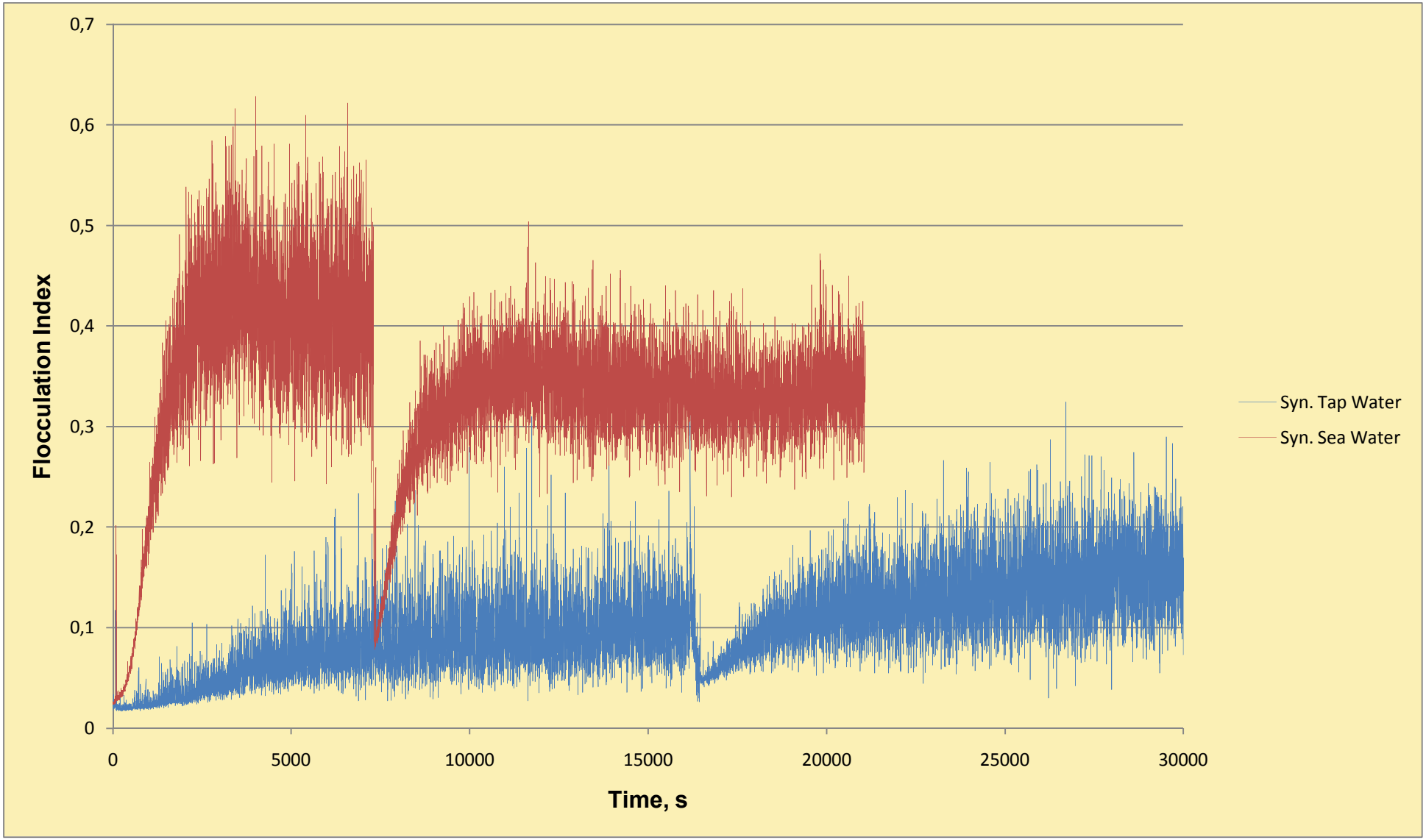


Figure 4.20. Relative floc sizes of the flocculation experiments done with SM HAC (100mg/L) in synthetic tap and sea water

In order to find the optimum dose of SM HAC, i.e. a lower dose that could achieve particle removal similar to that with 100 mg/L dose, several experiments were conducted within the range of 10-100 mg/L SM HAC dose. Also, for comparison reasons, real sea water obtained from Seraglio Point was clarified by filtration and used in these optimization studies. No significant differences in the flocculating properties were detected when the dose was decreased from 100 mg/L down to 20 mg/L, however, a further decrease to 10 mg/L resulted in diminished flocculation performance (Figures 4.21 and 4.22) as reflected by increased residual turbidity presented in Table 4.19.

Table 4.19. Residual turbidity values of SM HAC in synthetic and real sea water media

	10 mg/L SM HAC dose		**20 mg/L SM HAC dose**	
	Synthetic Sea Water	**Real Sea Water**	**Synthetic Sea Water**	**Real Sea Water**
Residual Turbidity (NTU)	16	9	8.5	5
Recovery Factor (%)	56	81	60	85
Strength Factor (%)	0.5	6.9	2.2	7.4

As shown in Table 4.19, lower residual turbidities because of enhanced particle removal were observed as the SM HAC dose was increased from 10 mg/L to 20 mg/L. However, a minute reduction in residual turbidity was observed when the dose was further increased to 100 mg/L. 20 mg/L was determined as the optimum SM HAC dose for sea water medium. This is a typical dose for biopolymers which indicates that the EPS produced by *Halomonas* sp. AAD6 is an effective flocculating agent with an average removal efficiency of 93%, in good aggrement with other reports (Yokoi et al., 1997; Salehizadeh et al., 2000; Li et al., 2008; He et al., 2009; Freitas et al., 2009). Examination of the FI values in Figure 4.21 suggests that the beginning of flocculation was retarded and smaller flocs were formed in synthetic sea water. By increasing the EPS dose, larger flocs with better settlability were formed, resulting in better particle removal correlating with the residual turbidity data in Table 4.19. Increasing the SM HAC dose lead to

formation of stronger flocs with better size recovery after breakage. The observed enhanced floc strength with polymer dose may be attributed to increased number of bonds in flocs since polymer addition provides more contact between clay particles and polymer molecules.

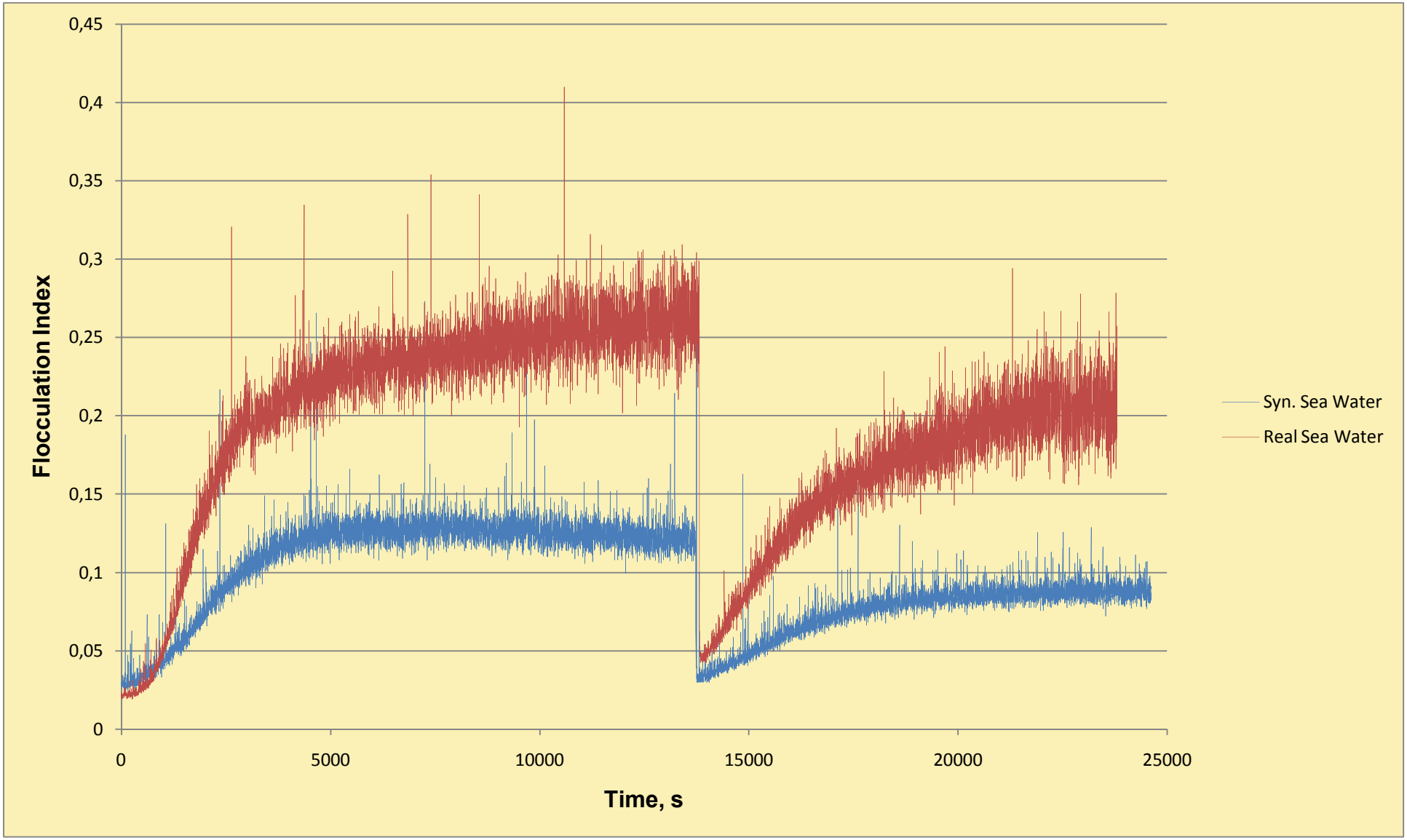


Figure 4.21. Relative floc sizes of the flocculation experiments done with SM HAC (10mg/L) in synthetic sea water and real sea water

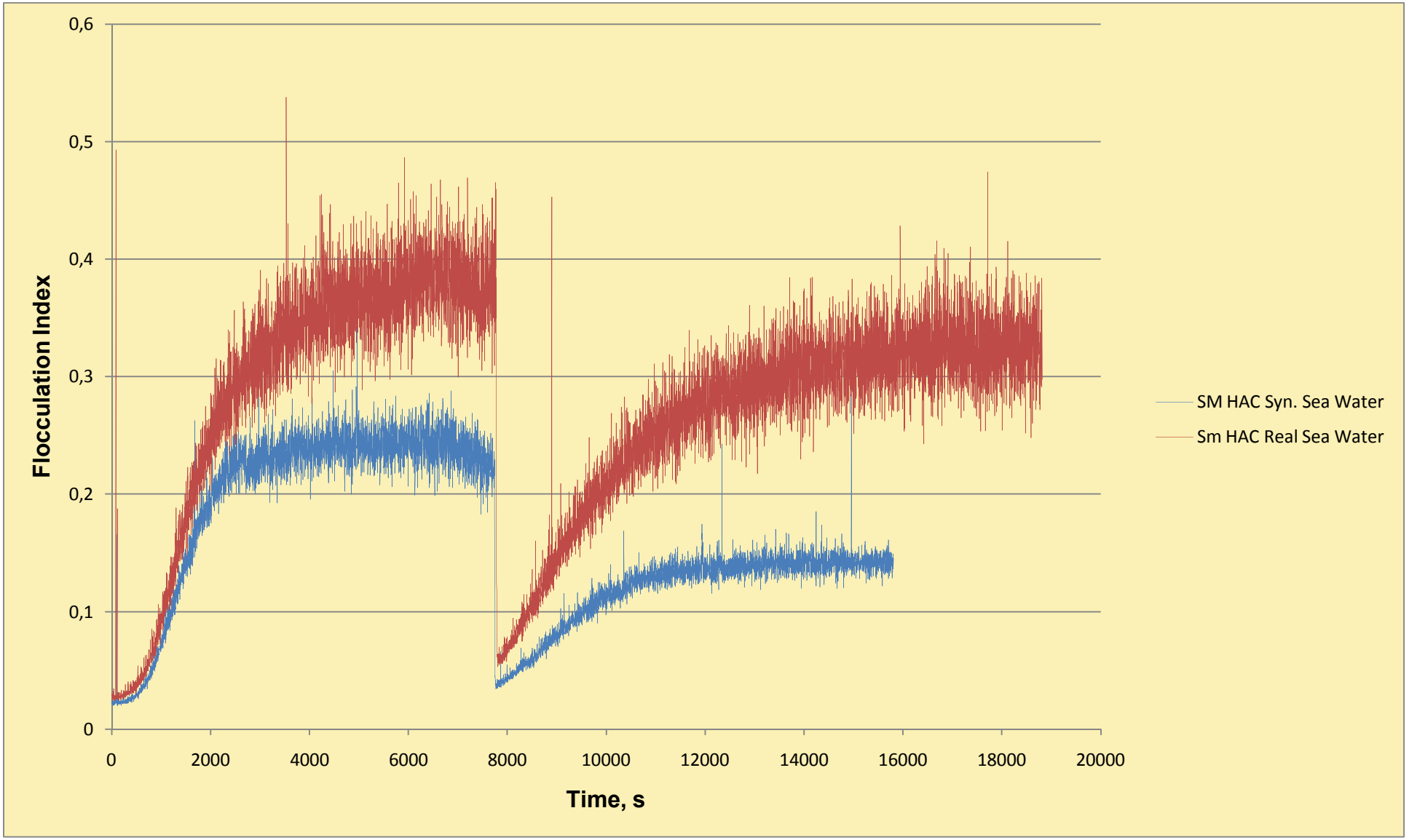


Figure 4.22. Relative floc sizes of the flocculation experiments done with SM HAC (20mg/L) in synthetic sea water and real sea water

In order to compare the performance of the biopolymer SM HAC, same experimental procedure was followed with commercial cationic (Magnafloc LT 22), nonionic (Magnafloc LT 20), and anionic (Magnafloc LT 25) polyelectrolytes. The doses of these synthetic polyelectrolytes which could yield with relative floc sizes and particle removals similar to those with 20 mg/L SM HAC dose were investigated.

Different concentrations of cationic polyelectrolyte Magnafloc LT 22 (125 μg/L to 2.5 μg/L) were used in the flocculation experiments and their flocculation behaviours in the synthetic sea water and real sea water were shown in Figure 4.23 and Figure 4.24. As the polyelectrolyte dose was decreased, onset of flocculation was more delayed and the steady-state floc size got smaller. The residual turbidities of this polyelectrolyte in synthetic and real sea water for the concentration range given above were tabulated in Table 4.20. LT 22 exhibited slightly better performance in synthetic sea water so that a 7.5 μg/L dose formed flocs with similar sizes to those with SM HAC in sea water.

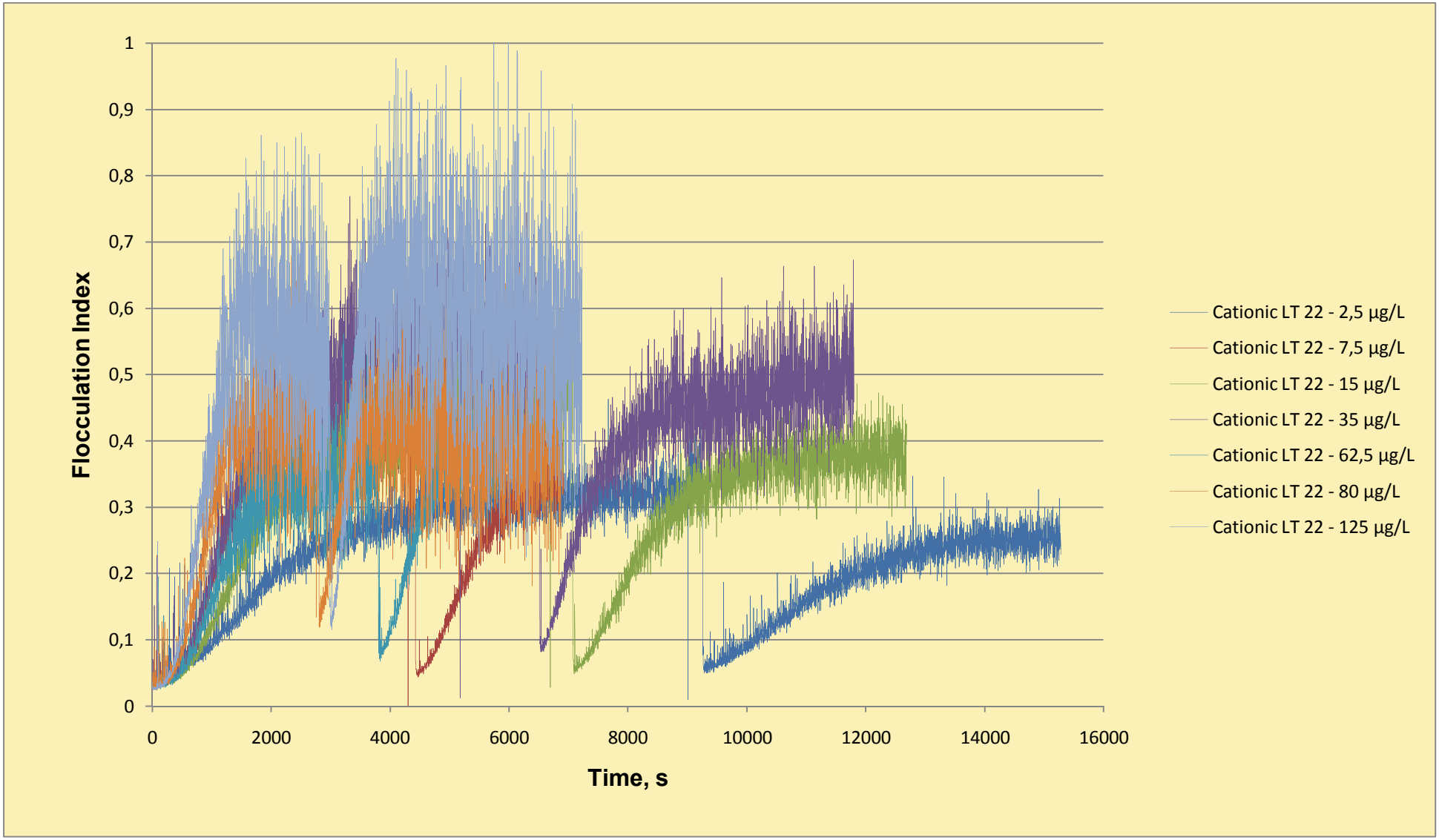


Figure 4.23. Relative floc sizes of the flocculation experiments done with different concentrations of cationic polyelectrolyte Magnafloc LT 22 in synthetic sea water

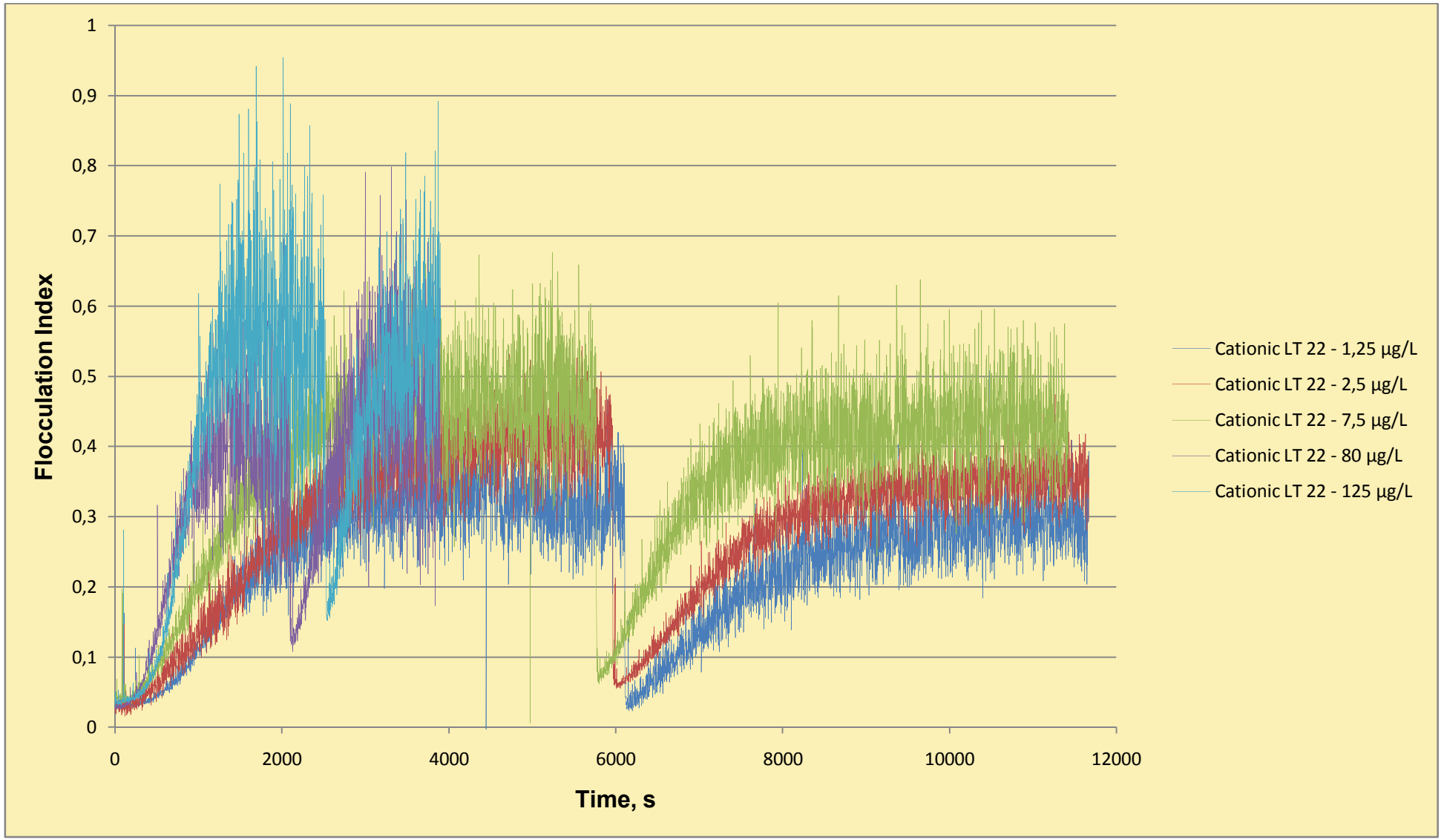


Figure 4.24. Relative floc sizes of the flocculation experiments done with different concentrations of cationic polyelectrolyte Magnafloc LT 22 in real sea water

Table 4.20. Residual turbidity of the different concentrations of cationic polyelectrolyte Magnafloc LT 22 in flocculation experiments done in synthetic sea water and real sea water

	Synthetic Sea Water							Real Sea Water				
Cationic Poyelectrolyte LT 22, µg/L	**125**	**80**	**62.5**	**35**	**15**	**7.5**	**2.5**	**125**	**80**	**7.5**	**2.5**	**1.25**
Residual Turbidity, NTU	2	2.5	3.5	4	5	5.5	6.5	2	2.5	4	5	5.5

Magnafloc LT 25 anionic polyelectrolyte performed similar to the other polyelectrolytes, so that earlier onset of flocculation and larger floc formation were observed with increasing polyelectrolyte dose (Figure 4.25 and Figure 4.26.). When the residual turbidity data in Table 4.21, which was consistent with relative floc sizes, was examined it was seen that the anionic polyelectrolyte dose achieving turbidity removal similar to SM HAC in sea water media was around 1.25 µg/L. This dose was lower than those of the other two synthetic polyelectrolytes which could most likely result from its charge and higher molecular weight.

Table 4.21. Residual turbidity of the different concentrations of anionic polyelectrolyte Magnafloc LT 25 in flocculation experiments done in synthetic sea water and real sea water

	Synthetic Sea Water		**Real Sea Water**	
Anionic Polyelectrolyte LT 25, µg/L	**2.5**	**1.25**	**2.5**	**1.25**
Residual Turbidity, NTU	5.5	6.5	5	5.5

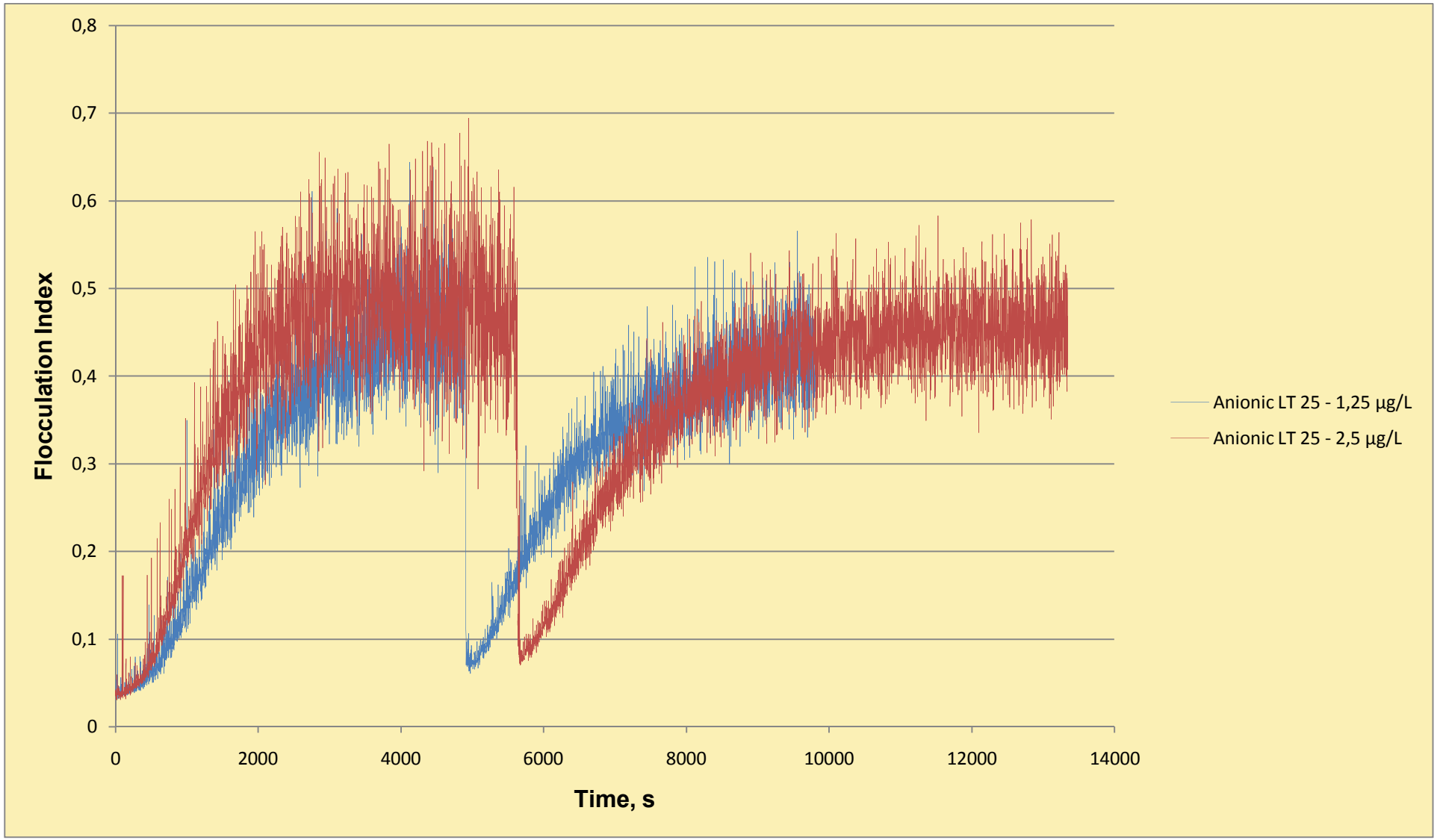


Figure 4.25. Relative floc sizes of the flocculation experiments done with different concentrations of anionic polyelectrolyte Magnafloc LT 25 in synthetic sea water

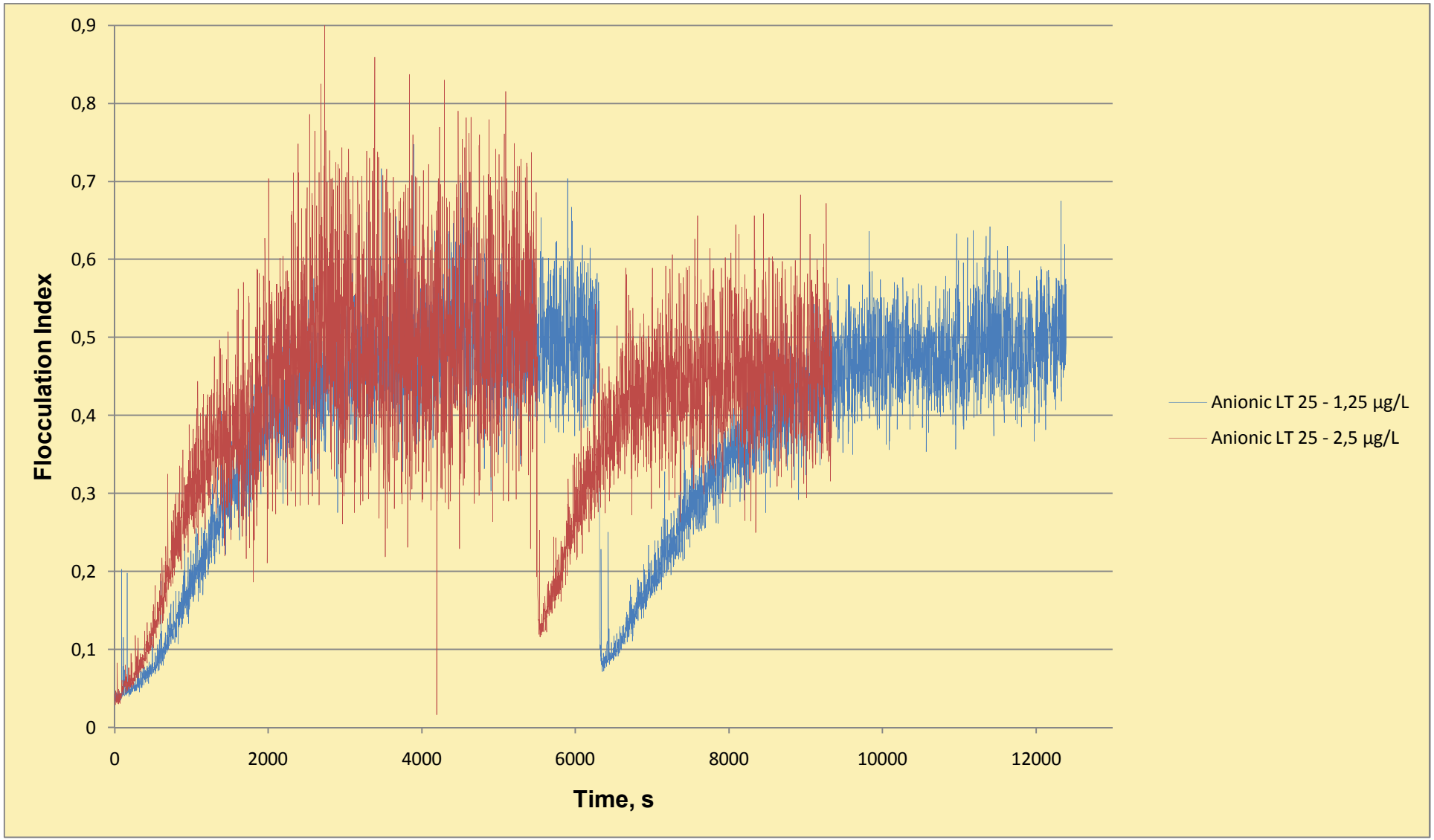


Figure 4.26. Relative floc sizes of the flocculation experiments done with different concentrations of anionic polyelectrolyte Magnafloc LT 25 in real sea water

Different concentrations of nonionic polyelectrolyte Magnafloc LT 20 were used in the flocculation experiments and their flocculation behaviours in the synthetic sea water and real sea water were shown in Figure 4.27 and Figure 4.28.

As the concentration of nonionic polyelectrolyte was increased, the floc sizes increased as well and the flocculation time decreased for both type of sea water.

The residual turbidities of each concentration of nonionic polyelectrolyte for synthetic sea water and real sea water were given in Table 4.22.

To achieve the flocculation properties of the SM HAC exopolysaccharide , the concentration between 1.25 µg/L and 2.5 µg/L of nonionic polyelectrolyte was enough for the synthetic sea water. 7.5 µg/L of nonionic polyelectrolyte provided same flocculation behaviours of SM HAC biopolymer for real sea water.

Table 4.22. Residual turbidity of the different concentrations of nonionic polyelectrolyte Magnafloc LT 20 in flocculation experiments done in synthetic sea water and real sea water

	Synthetic Sea Water			**Real Sea Water**		
Nonionic Poyelectrolyte LT 20, µg/L	**7.5**	**2.5**	**1.25**	**7.5**	**2.5**	**1.25**
Residual Turbidity, NTU	3	4.5	5.5	5	6	6.5

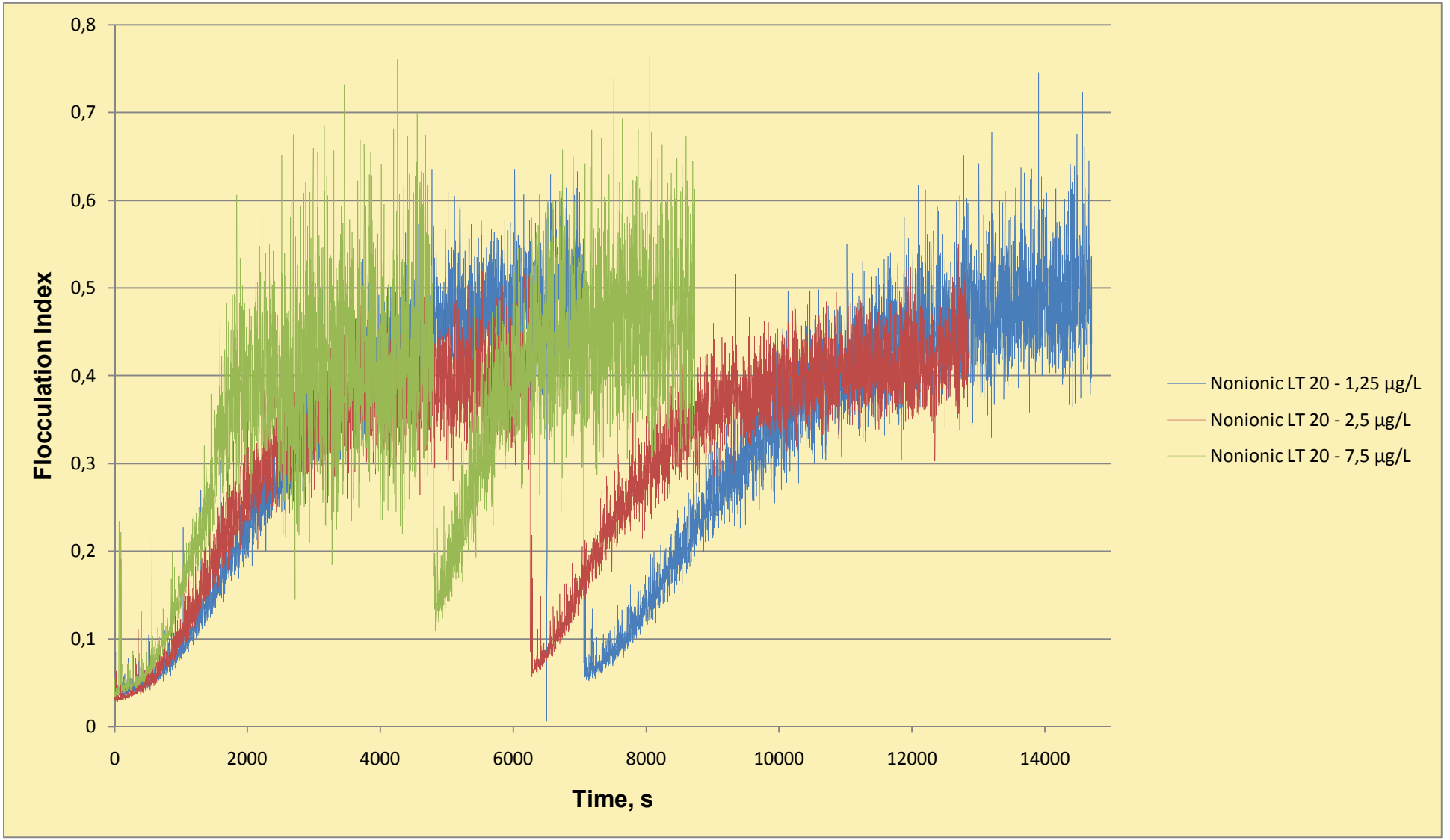


Figure 4.27. Relative floc sizes of the flocculation experiments done with different concentrations of nonionic polyelectrolyte Magnafloc LT 20 in synthetic sea water

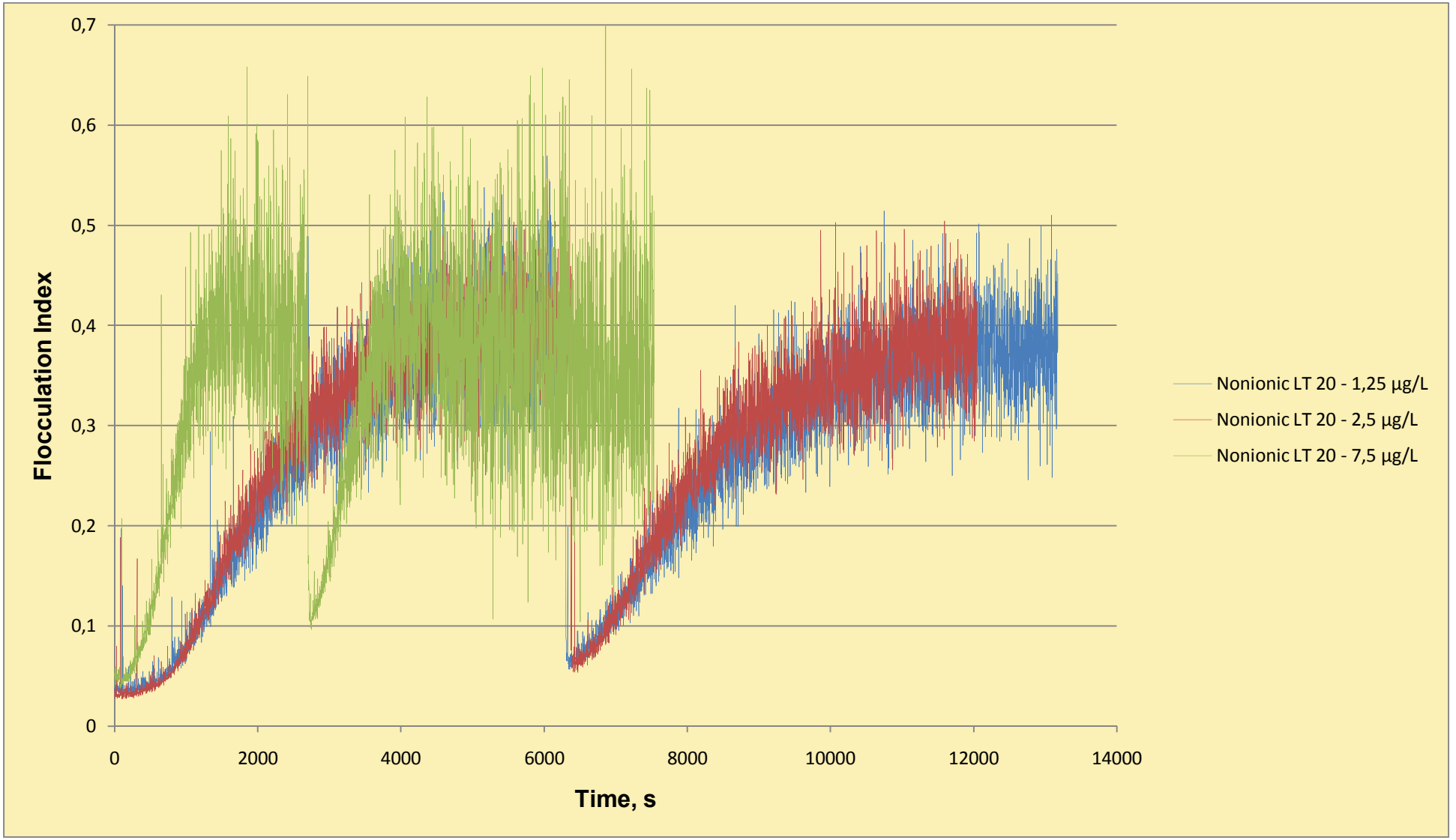


Figure 4.28. Relative floc sizes of the flocculation experiments done with different concentrations of nonionic polyelectrolyte Magnafloc LT 20 in real sea water

Microbial bioflocculants have received increasing scientific and technical attention because of their special advantages such as safety, strong effect, biodegradability, biocompatibility and the fact that they are not only nontoxic but also their degradation intermediates are not secondary pollutants (Li et al., 2009; Liu et al., 2010). For example, (Li et al., 2008) reported that EPS SM9913 produced by the deep-sea psychrophilic bacterium *Pseudoalteromonas* sp. SM9913 showed better flocculation performance than an inorganic aluminium based flocculant at low temperature (5-15°C) or in high salinity water (% 0.5-10) suggesting its probable use as an effective flocculant for wastewater treatment at low temperature and/or salinity. Hence bioflocculants may potentially be applied in drinking and wastewater treatment, downstream processing, and fermentation processes (Gao et al., 2006).

However, their industrial utilisation is hindered by their costly productions and poor yields. In order to overcome these limiting factors, studies have focused primarily on the identification of microorganisms with high bioflocculant-producing ability, mutational methods to obtain more efficient strains, rational improvement of bioflocculating efficiency and search for low cost feedstocks (Wang et al., 2007).

EPSs investigated in this study were produced by halophilic bacteria grown on pretreated molasses which was used as a cheap fermentation substrate. All the six EPSs were found to perform better in saline water with SM HAC's performance standing out. Moreover, flocculation activity of SM HAC was comparable to those of commercial synthetic polyelectrolytes which suggests its high potential for its use in treatment of waters with high ionic concentration such as sea water, bilge and industrial wastewaters.

4.5. Economic Advantage Provided

In a biopolymer production process, the carbon source in the fermentation medium represents up to 50% of the total cost of the fermentation process. Expensive nutrients such as yeast extract, peptone and salts add more to

the total cost making the process highly unattractive from the economical point of view (Van Hoek et al., 2003).

In order to assess the economical advantage provided with this work, the costs of fermentation media before and after this work were calculated and compared. For that, first of all, the prices of medium constituents were found and used to calculate the cost of the optimum medium based on its composition as given in Methods 3.1.5 (Table 4.23)

Table 4.23. Price list of the constitutents used in the fermentation medium (Sigma-Aldrich, Rodrigues et al., 2006)

		Poli et al., 2009		This study	
Constituent	**Price (€/kg)**	**Amount (g/L)**	**Price (€/L)**	**Amount (g/L)**	**Price (€/L)**
K_2HPO_4	239.2	7.0	1.674	7.0	1.674
KH_2PO_4	236.9	2.0	0.474	2.0	0.474
$MgSO_4.7H_2O$	31.6	0.1	0.003	0.1	0.003
$(NH_4)_2SO_4$	114.2	1.0	0.114	1.0	0.114
Pepton	202.5	0.5	0.101	0.5	0.101
NaCl	28.15	137.2	3.862	137.2	3.862
Sucrose	67.2	50.0	3.360	0	0
Molasses	0.12	0	0	8	0.001

According to Table 4.23, the cost of optimum medium was 9.589 €/L and in this study, the cost reduced by 35% down to 6.23 €/L. Based on the levan yields in Table 4.5 (7.56 g/L culture) and Poli et al. (2009) (1.844 g/L culture), the 5.2 € cost was reduced to 0.82 € per gram of levan produced. The catalog prices of Levan from *Erwinia herbicola* (Sigma no: L8647-1G) and Levan from *Zymomonas mobilis* (Sigma no: 66674-1G-F) are 212.20 € and 171.40 €, respectively. Commercial Sigma grade Levan is an expensive polymer when compared with

Xanthan (Sigma no: G1253, price : 0.57 €/g), Sodium Alginate (Sigma no: A2033, price : 0.64 €/g) and even with Pullulan (Sigma no: P4516, price : 61.50 €/g). Therefore any reduction in its production cost has high industrial importance. In this study, replacing pure sucrose with molasses not only increased the production yields but also resulted in five-fold lower costs of fermentation medium per gram of levan produced.

5. CONCLUSIONS AND RECOMMENDATIONS

The rapid increase in production and consumption of petroleum based polymers has led to the serious plastic waste problems, so called 'White Pollution', and landfill depletion, because of their high volume to weight ratio and resistance to degradation. Therefore it will be a must to replace these persistent nonrenewable material with biodegradable and renewable ones.

One promising potential candidate is levan which is a water soluble, strongly adhesive and film-forming biopolymer with many valuable properties like low viscosity, high solubility in oil, compatibility with salts and surfactants, stability to heat, acid and alkali, high holding capacity for water and chemicals and good biocompatibility.

As part of an on-going research, this thesis work was mainly focused on developing a cost-effective and environmentally friendly biopolymer production process using halophilic *Halomonas* sp. cultures. For this, the potential uses of different waste streams like sugar beet and starch molasses, cheese whey powder, sugar beet pulp, olive mill wastewater, and orange bagasses were investigated within the scope of this thesis.

Sugar beet molasses and starch molasses were subjected to five different physical and chemical pretreatment methods, i.e., clarification, pH adjustment, sulfuric acid, tricalcium phosphate and activated carbon treatment. With their combinations, a total of 16 different pretreated samples were analyzed for their iron, zinc and nickel content and then used as fermentation substrates for *Halomonas* sp. AAD6 batch cultures. Whereas highest EPS concentrations were reached by cultures grown in tricalcium phosphate-sulphuric acid-activated carbon (TCPHAC) pretreated sugar beet and starch molasses, generally, biopolymer amounts that were produced from pretreated sugar beet molasses were higher than those produced from starch molasses. Moreover, 30 g/L initial concentration

of TCPHAC pretreated beet molasses was found to yield highest EPS concentration.

Salt drop-out experiments indicated the importance of $(NH_4)_2SO_4$ as a nitrogen source in the medium and also pointed to the fact that high yields could be attained by using a simple and economical medium. Additionally, EPS yields were found to increase with initial pH of the culture broth and two volumes of ethanol was the optimum volume for the recovery of EPSs from the broth.

To understand the effect of color and some trace metals on EPS production, the activated carbon pretreatment was applied consecutively until the color of the molasses was almost bleached. Clarified sugar beet molasses (BM CpH) subjected to multiple AC treatment resulted in not only effective removal of coloured substances from the broth and improved growth profiles but also variety in some trace metals (Fe, Ni, Zn) composition. Loss of cell integrity and associated poor EPS yields and low carbohydrate content could be because of prolonged cultivation which called for further studies.

Fermentation substrates other than molasses were also investigated. Cheese whey powder (CWP) which is mainly composed of lactose as well as its combination with sugar beet molasses was found to result in low EPS yields. Similar results were also obtained from preliminary experiments conducted with sugar beet pulp, olive mill wastewater and wet/dry orange shells. Hence more studies should be conducted to make them a suitable fermentation substrate for EPS production by the halophilic *Halomonas* sp. AAD6 cells.

FT-IR spectra of EPSs produced in this study were measured for their preliminary identification and the results were compared with the spectra of commercial Levan and Levan produced by *Halomonas* sp. AAD6. The high level of resemblance observed in the spectra of the EPS samples produced from pretreated BM suggested the fact that these polymers were in fact Levan type polysaccharides.

The rheological characterizations of the EPSs were carried out and compared with those of alginate, pullulan and xanthan. All EPSs have shown the characteristic of low viscosity and appeared to exhibit pseudoplastic behaviours.

The potential use of these EPSs as easily biodegradable, natural alternative for the widely used synthetic polyelectrolytes containing toxic and carcinogenic monomers was investigated by monitoring their flocculating activity in synthetic water, synthetic sea water and real sea water as model raw waters with a photometric dispersion analyser. SM HAC, which formed the largest flocs and yielded with lowest residual turbidity, exhibited flocculation performance and particles removal efficiency comparable with commercial cationic, nonionic and anionic synthetic polyelectrolytes.

Finally, a rough economic analysis revealed that replacing pure sucrose with molasses not only increased the production yields but also resulted in five-fold lower costs of fermentation medium per gram of levan produced.

In the view of the findings of this research, the following recommendations are made for future researches:

1. In the present study, all experiments were performed using batch shake flask cultures without any control over culture pH and aeration. However, pH and dissolved oxygen are two important parameters for EPS production and therefore, fermentations should be performed using bioreactors under a well controlled environment of pH, temperature and aeration. Such experiments could reveal the importance of these parameters and provide useful data to design new fermentation strategies for improved yields.

2. There are many other organic wastes to be used as carbon source (e.g., apple pomace, grape pomace). Their potentials in the production of exopolysaccharides can be investigated.

3. Distilled water and salt used in the fermentation media make up a considerable fraction of the total cost. Further experiments can be conducted where distilled water could be replaced with Tap water and crude sea salt or table salt could be used instead of pure NaCl.

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APPENDIX A

TOTAL CARBOHYDRATE CONCENTRATION CALIBRATION CHART

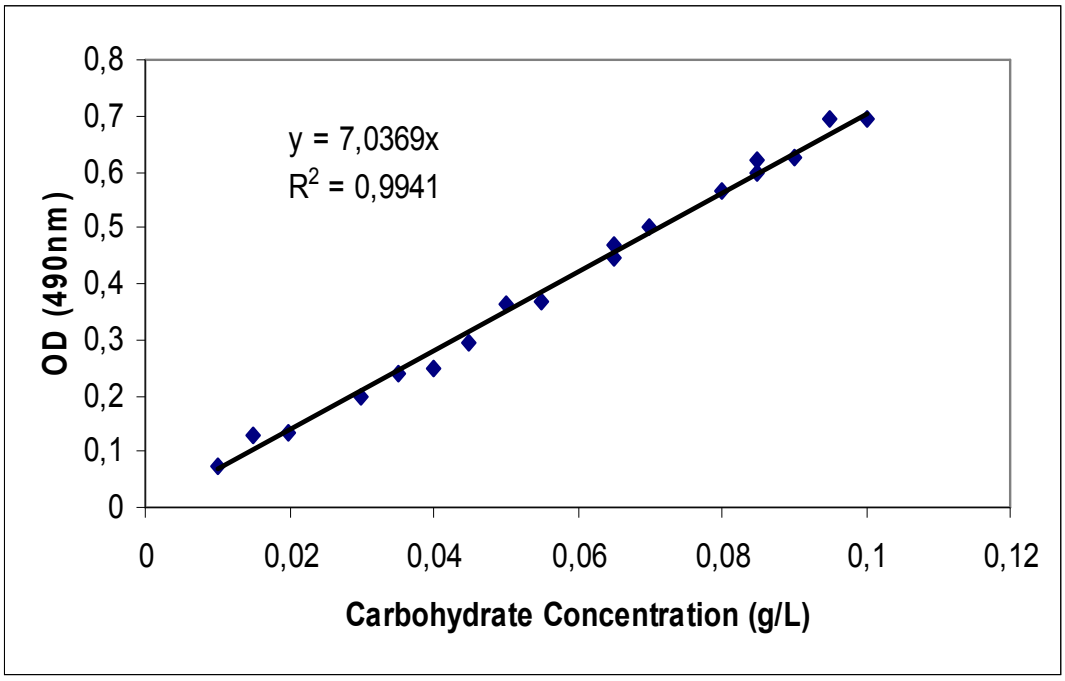

Figure A.1. Total carbohydrate concentration calibration chart

APPENDIX B

PROTEIN CONCENTRATION CALIBRATION CHART

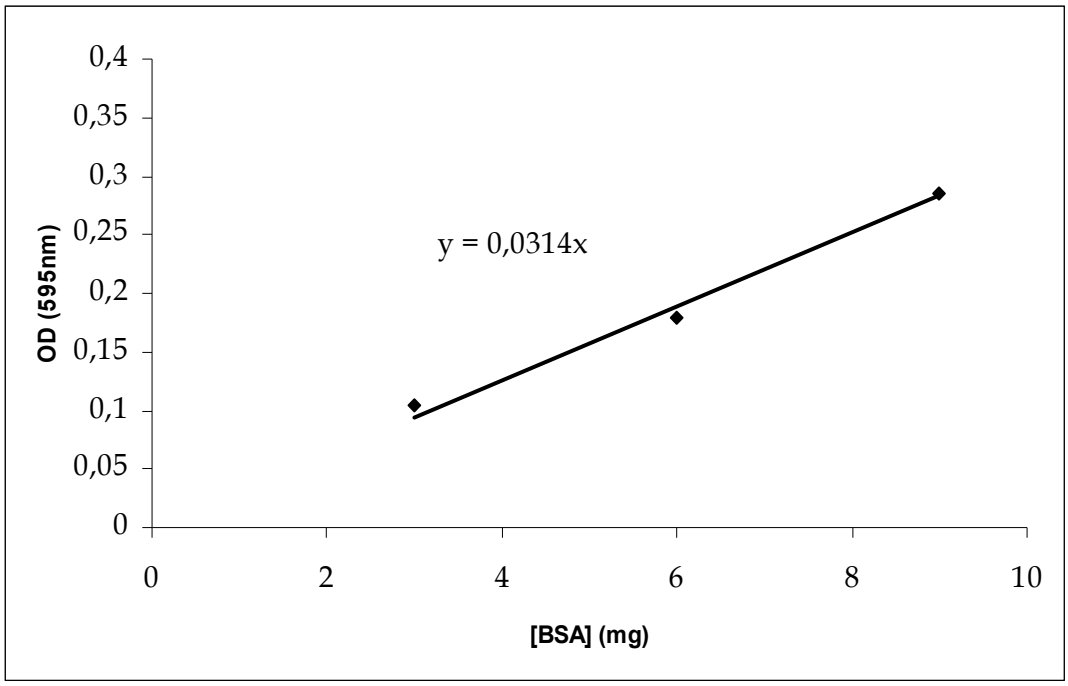

Figure B.1. Protein concentration calibration chart