T.C.
MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES IN
PURE AND APPLIED SCIENCES

EXOPOLYSACCHARIDE PRODUCTION BY *HALOMONAS*
STRAINS ISOLATED FROM TURKEY

Hande KAZAK

THESIS
FOR THE DEGREE OF MASTER OF SCIENCE
IN
BIOENGINEERING

SUPERVISOR
Assoc. Prof Ebru TOKSOY ÖNER

İSTANBUL 2009

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ABSTRACT

EXOPOLYSACCHARIDE PRODUCTION BY *HALOMONAS* STRAINS ISOLATED FROM TURKEY

Biopolymers are superior to petrochemical-derived polymers in several aspects that embrace biocompatibility, biodegradability, and both environmental and human compatibility. 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. Due to their many interesting physicochemical and rheological properties with novel functionality, the microbial exopolysaccharides (EPS) act as new biomaterials and find wide range of applications in many industrial sectors like textiles, detergents, adhesives, microbial enhanced oil recovery (MEOR), wastewater treatment, dredging, brewing, downstream processing, cosmetology, pharmacology, and food additives. Xanthan, dextran and pullulan are examples of microbial polysaccharides with a considerable market due to their exceptional properties. Biopolymers with properties superior to the commercial ones could not preserve their functions under industrial process conditions like extremes of temperature, salinity or pH. Hence, most research is focused on the identification of EPS producing extremophiles with the idea that as these microorganisms survive environmental extremes of desiccation, temperature, pressure, salinity, acidity, heavy metals, and radiation, it is to be expected that their biopolymers will also have some unique properties to adapt to such extreme conditions.

Considering the widespread use of microbial polysaccharides in various industrial applications, production of EPS by *Halomonas* sp AAD6 bacteria isolated from Çamaltı Saltern Area in Izmir was used within the scope of this study. The fermentation conditions were optimized for high-yield biopolymer production by this microorganism. Also within the scope of this study, this novel exopolysaccharide was produced under controlled bioreactor conditions, purified with high yield and chemically characterized.

Key Words: Extremophiles, Halophilic microorganisms, Exopolysaccharide production

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ÖZET

TÜRKİYE'DEN İZOLE EDİLEN *HALOMONAS* SUŞLARINDAN EKZOPOLİSAKKARİT ÜRETİMİ

Biyopolimerler, biyouyumluluk, biyobozunurluk, çevre ve insana uyumluluk gibi bir çok açıdan petrokimyasal türevli polimerlere göre daha üstün olmalarından dolayı doğal ve çevreye dost birçok uygulamada yaygın olarak kullanılabilir duruma gelmişlerdir. Yeni biyomalzemeler olarak kabul edilen mikrobiyal ekzopolisakkaritler (EPS), farklı fizikokimyasal ve reolojik özellikleri sayesinde tekstil, deterjan, tutkal, petrol ve türevleri atıklarının mikrobiyal yıkımı, atıksu arıtma, kozmetik, ilaç ve gıda gibi çok farklı endüstriyel sektörlerde uygulama alanı bulmuş biyopolimerlerdir. Dekstran, ksantan ve pullulan gibi çok geniş pazara sahip mikrobiyal ekzopolisakkaritlerden daha üstün özelliklere sahip olduğu düşünülen biyopolimerlerin bu özelliklerini, endüstriyel uygulamaların gerektirdiği ekstrem sıcaklık, tuzluluk ve pH koşullarında koruyamadıkları görülmüştür. Bu sebeple, ekstrem ortamlarda yaşayabilen ekstremofilik mikroorganizmaların ürettiği biyopolimerlerin karakterizasyonu çok önem kazanmıştır.

Mikrobiyal polisakkaritlerin çeşitli endüstriyel alanlardaki yaygın kullanımı da göz önünde bulundurularak, İzmir Çamaltı Tuzlası'ndan izole edilen *Halomonas* sp. AAD6 halofilik bakteri izolatu ile polisakkarit üretimi bu çalışma kapsamında araştırılmıştır. Bu doğrultuda, *Halomonas* sp. AAD6 mikroorganizmasının üretim koşulları optimize edilmiştir. Kontrollü biyoreaktör koşullarında üretilen polisakkarit saflaştırılmış ve karakterizasyonu yapılmıştır.

Anahtar Kelimeler: Ekstremofiller, Halofilik mikroorganizmalar, ekzopolisakkarit üretimi

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LIST OF SYMBOLS AND ABBREVIATIONS

Da	: dalton
g	: gram
h	: hour
kDa	: kilodaltons
L	: liter
M	: Molar
min	: minute
Mpa	: Mega Pascal
nm	: Nanometers
rpm	: Round per minute
U	: Unit
vvm	: volume air/volume medium/min
v/v	: Volume per volume
w/v	: Weight per volume
X	: Microbial biomass concentration
X₀	: Initial microbial biomass concentration
μ	: Specific growth rate
μ_{max}	: Maximum specific growth rate
μL	: Micro liters
μg	: Micro grams
BSA	: Bovine Serum Albumin
CPS	: Capsular polysaccharides
DCW	: Dry cell weight
DMSO	: Dimethyl sulfoxide
DNA	: Deoxy ribonucleic acid
DNS	: 3,5-dinitrosalicylic acid
EPS	: Exopolysaccharide
FBS	: Fetal Bovine Serum
GRAS	: Generally Recognized As Safe

HPAE-PAD	: High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection
LAB	: Lactic Acid Bacteria
LD₅₀	: Median Lethal Dose
LPS	: Lipopolysaccharides
MEOR	: Microbial Enhanced Oil Recovery
OD	: Optical density
PAC	: Polyaluminum Chloride
PBS	: Phosphate Buffered Saline
PGA	: Poly Glutamic Acid
PHA	: Polyhydroxyalkanoates
PHB	: Poly(3-hydroxybutyrate)
PVP	: Polyvinyl-pyrrolidone
RNA	: Ribonucleic Acid
RSM	: Response surface methodology
SMCs	: Smooth Muscle Cells
sp.	: Species
TFA	: Trifluoroacetic Acid
TLC	: Thin Layer Chromatography
UV	: Ultraviolet

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PART I

I. INTRODUCTION AND OBJECTIVES

Biopolymers produced by diverse groups of microorganisms are rapidly emerging as a new and industrially important source of polymeric materials, which are gradually becoming economically competitive with natural gums produced by a variety of microbes (W.F. Fett, 2000).

Biopolymers are superior to petrochemical-derived polymers in several aspects that embrace biocompatibility, biodegradability, and both environmental and human compatibility. Due to their many interesting physicochemical and rheological properties with novel functionality, the microbial exopolysaccharides (EPS) act as new biomaterials and find wide range of applications in many industrial sectors like textiles, detergents, adhesives, microbial enhanced oil recovery (MEOR), wastewater treatment, dredging, brewing, downstream processing, cosmetology, pharmacology, and food additives (Martinez-Cánovas et al., 2004; Iyer et al., 2006). Xanthan, dextran and pullulan are examples of microbial polysaccharides with a considerable market due to their exceptional properties.

Biopolymers with properties much better than the commercial ones could not preserve their functions under industrial process conditions like extremes of temperature, salinity or pH (Lee et al., 2002). Hence, most research is focused on the identification of EPS producing extremophiles with the idea that as these microorganisms survive environmental extremes of desiccation, temperature, pressure, salinity, acidity, heavy metals, and radiation, it is to be expected that their biopolymers will also have some unique properties to adapt to such extreme conditions.

Since 1991, EPS producing extremophilic microorganisms have been isolated from deep-sea hydrothermal vents characterized by extreme pressure and temperature, high concentrations of H₂S and heavy metals. These include some

Bacteria such as *Alteromonas macleodii* sub sp. *fijiensis*, *Vibrio diabolicus*, *Alteromonas infernos* (Raguénès et al., 1997; Roger et al., 2004) and *Thermotoga maritima* (Rinker et al., 2000) and *Archaea* such as *Thermococcus litoralis* (Rinker et al., 2000). EPS producing bacteria from marine Mediterranean shallow vents have also been described (Maugeri et al., 2002; Schiano Moriello et al., 2003). Hypersaline environments are also known to harbor a variety of EPS producing strains such as moderately halophilic bacteria of the genus *Halomonas* (Bouchotroch et al., 2000), *Halomonas maura* (Arias et al., 2003), *Halomonas alkaliphila*, strain CRSS (Poli et al. 2004), *Halomonas ventosae* and *Halomonas anticariensis* (Mata et al., 2006), *Halomonas cerina* (González-Domenech et al., 2008), *Aphanocapsa halophytica* (Matsunaga et al., 1996), haloalkalophilic *Bacillus* sp. I-450 (Kumar et al., 2004).

With the ultimate goal of finding EPS with novel and valuable properties, halophilic microorganism isolated from Çamalti Saltern area in Turkey, *Halomonas* sp. AAD6, was investigated in terms of its carbon source requirements for EPS production. EPS production was investigated both in shake flask cultures and under controlled bioreactor conditions and it is reported along with the chemical and rheological characterization of the polymer.

PART II

GENERAL BACKGROUND

II.1. MICROBIAL 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 providing 350 billion pounds per year of materials valued at over \$250 billion. Yet the vast majority of these materials are based upon the extraction and processing of fossil carbon, typically oil and gas, leading ultimately to increases in greenhouse gases in the atmosphere and the accumulation of persistent plastic materials in the environment.

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).

Biopolymers are superior to petrochemical-derived polymers in several aspects that embrace biocompatibility, biodegradability, and both environmental and human compatibility. They include various polysaccharides, polyamides (proteins and poly- γ -glutamic acid (γ -PGA)), nucleic acids (DNA and RNA), polyesters (polyhydroxyalkanoates, PHAs), polyphosphates, and polyisoprenoids (natural rubber). Today, some of these biopolymers are produced by bacterial fermentation and are used commercially in a wide range of applications such as foods, pharmaceuticals, plastics and agriculture. Not only refined carbohydrates but also agricultural and dairy byproducts can be used as substrates for the production of these biopolymers by fermentation processes. Some important biopolymers, for example cellulose and starch, are obtained directly from plant materials. Hence, it is

unnecessary to produce these by fermentation due to the higher production costs, except for certain materials such as bacterial cellulose which has been produced by fermentation and used for specialty applications (Lee et al., 2002).

II.2. EXOPOLYSACCHARIDES

Exopolysaccharide (EPS) is a term first used by Sutherland (1972) to describe high molecular weight carbohydrate polymers produced by many marine bacteria. Since that time, EPS also has been used to indicate more broadly defined extracellular polymeric substances (Nichols et al., 2005).

Exopolysaccharides exist in a wide variety of unique and often complex chemical structures and they are believed to provide self-protection against antimicrobial substances, antibodies and bacteriophages and/or afford adherence to other bacteria, animal and plant tissues or inert surfaces, thus forming biofilms. They are also proving to have interesting bioactive functions and an extensive range of potential applications in industry, pharmacy, agriculture and various other areas.

During the past 50 years, a considerable number of bacterial EPSs have been described, but few have achieved great commercial success due either to their being unable to offer better properties than those already on the market or to difficulties in finding new practical applications (Mata et al., 2006).

II.2.1. Biochemistry and Genetics of EPS

Microbial polysaccharides can be present as constituents of cell walls, as part of lipo-polysaccharides (LPS), often referred to as O-antigens, as capsular polysaccharides (CPS) covalently associated with the cell surface, or secreted as exopolysaccharides (EPS) into the extracellular environment. Cell-associated polysaccharides like CPS and LPS are of considerable medical importance since they contribute to the virulence of their producers (Yoshida et al., 2003; Boels et al., 2001).

EPSs are long-chain polysaccharides consisting of branched, repeating units of sugars or sugar derivatives. These sugar units are mainly glucose, galactose and rhamnose, in different ratios. They are secreted into their surroundings during growth and are not attached permanently to the surface of the microbial cell. This distinguishes them from the structurally similar capsular polysaccharides, which do remain permanently attached to the surface of the cell. EPSs from microbial sources can be classified into two groups: homopolysaccharides (e.g. cellulose, dextran,

mutan, alternan, pullulan, levan and curdlan) and heteropolysaccharides (e.g. gellan and xanthan). Homopolysaccharides consist of repeating units of only one type of monosaccharide (D-glucose or D-fructose) and can be divided into two major groups: glucans and fructans. By contrast, heteropolysaccharides have repeating units that demonstrate little structural similarity to one another. The molecular mass of these polymers ranges between 4.0×10^4 and 6.0×10^6 Da. Heteropolysaccharides are constructed from multiple copies of oligosaccharides, which contain between three and eight residues. Two or more different monosaccharides are usually present in each repeating unit and show different linkage patterns (Welman et al., 2003).

Bacterial polysaccharides have been proposed to protect cells from desiccation or other environmental stresses, including immune responses, and also to enhance the colonization of various ecological niches.

Since bacterial polysaccharides have various rheological properties, they are of industrial interest (Akçelik et al., 2002; Stingelet al., 1999). And because the interest in EPS has increased over the last decade in industry, several EPS structures have been elucidated (Stingelet al., 1996; Stingelet al., 1999).

Different classes of EPS can be distinguished based on the mechanism of biosynthesis and the precursors required. The first class comprises the extracellularly produced homopolysaccharides like dextran, levan, and mutan. The polymerization reaction in these cases proceeds via extracellular glycosyltransferases, which transfer a monosaccharide from a disaccharide to a growing polysaccharide chain (Fig. II.1). The other categories comprise homo- and hetero-polysaccharides with (ir)regular repeating units that are synthesized from intracellular sugar nucleotide precursors (Fig. II.1). Some of these sugar nucleotides serve as precursors for EPS biosynthesis. However, they are also involved in the biosynthesis of several cell wall components and can therefore be considered essential for growth.

Figure II.1 Schematic representation of pathways involved in sugar fermentation via glycolysis to lactate and/or other acids and biosynthesis of exopolysaccharides (EPS) from intracellular sugar nucleotides (EPS^a) and extracellular EPS biosynthesis (EPS^b) (Boels et al., 2001).

While investigation of polysaccharide gene clusters in gram-negative bacteria began over 20 years ago, research on those of gram-positive microorganisms has advanced only lately. Recent reports include the characterization of genes involved in polysaccharide biosynthesis from the pathogens *Staphylococcus aureus* and *Streptococcus pneumoniae* (Kolkman et al., 1997; Lin et al., 1994; Morona et al., 1997) and from the food microorganisms *Streptococcus thermophilus* and *Lactococcus lactis* (Stingele et al., 1996; van Kranenburg et al., 1997). The general organization of these clusters seems to be conserved: a central region with similarity to glycosyltransferase genes is flanked by two regions exhibiting similarity to genes involved in polymerization and export, and a putative regulatory region can be found at the beginning of each cluster. Even though this organization is not always conserved among genes involved in O-antigen synthesis, their homology points to similar biosynthetic pathways. The repeating unit is first assembled by the sequential transfer of sugar residues onto a lipophilic carrier by specific glycosyltransferases. Unlike the other glycosyltransferases, the first glycosyltransferase does not catalyze aglycosidic linkage but transfers a sugar-1-phosphate onto a lipophilic anchor, such as undecaprenylphosphate. Subsequently, the completed repeating unit is exported

and polymerized. In the case of cell surface polysaccharides, it is anchored to a cell envelope component while secreted polysaccharides are released (Stingele et al., 1999).

The mechanisms involved in assembly, polymerization and translocation across the outer membrane in Gram-negative bacteria follow a similar pathway to that of some capsule EPSs in *Escherichia coli* groups 1 and 4 (Rahn et al., 1999) and *Klebsiella pneumoniae* (Arakawa et al., 1995) and of extracellular polysaccharides, including those produced by *Erwinia* spp. (Bugert et al., 1995), *Methylobacillus* sp. strain 12S (Yoshida et al., 2003), *Rhizobium* spp. (Reuber et al., 1993) and *Xanthomonas campestris* (Katzen et al., 1998). Undecaprenol-pyrophosphate-linked oligosaccharide repeating units are formed at the cytoplasmic face of the inner membrane, transported through this membrane by a Wzx-protein-dependent process, and then polymerized by a mechanism involving a Wzy protein. Some of the polysaccharide-biosynthesis gene clusters have been cloned and sequenced, and found to form long operons with similarities in their genetic organization. In general, the first three genes, which are necessary for high-level polymerization and surface assembly, are conserved in the abovementioned micro-organisms; the genes are *wza* (encoding an outer-membrane protein), *wzb* (encoding an acid phosphatase) and *wzc* (encoding an inner-membrane tyrosine autokinase). It has been suggested that this high level of conservation is the result of lateral transfer events occurring between some of these species. The rest of the genes included in the operon encode glycosyltransferases and components of a Wzy-dependent polymerization system (Arco et al., 2005).

II.2.2. Rheological properties of EPS

There is great diversity in the composition and structure of EPSs, which results in different properties (Levander et al., 2002).

The rheological behaviour of polysaccharide 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, textural analysis. Rheological characteristics depend on a large number of factors, as observed in Figure II.2.

Figure II.2 Factors influencing the rheological characteristics of biopolymeric aqueous solutions (Mironescu, 2003)

For example, if the polysaccharide is a polyelectrolyte, viscosity can be controlled through electrostatic repulsion, ionic strength or addition of divalent and polyvalent cations (making possible the formations of gel through ionic bounds).

The molecules size influences the rheological behaviour at various shear stresses.

For the rheological analysis of EPS, two measuring systems are recommended: parallel plate and cone-plate geometries. Using these geometries, steady state and oscillatory (or dynamic) measurements can be performed; the first one type of analyses gives information on the flow behaviour of polysaccharides and the second one characterises the viscoelastic behaviour (Mironescu, 2003).

Lambo-Fodje et al. (2007) studied the rheological properties of an exopolysaccharide from *Pediococcus damnosus* 2.6. They compared the rheological behaviour of the EPS to that of a commercial cereal β -glucan ($0.359 \times 10^6 \text{ g mol}^{-1}$). The maximum storage modulus, $G'_{\max}$ for EPS solution was lower than that for the cereal β -glucan at all concentrations, while the relaxation time was higher. And this EPS displayed viscoelastic properties typical of a macromolecular solution with structural entanglements and thus, could be used as a thickener in industrial applications.

El-Tayeb et al. (2007) determined the shear stress (culture viscosity) using various concentrations of purified biopolymer produced by *Pseudomonas* sp. UBF 2

at increasing shear rates. Its shear stress increased according to the decrease of the shear rate. However, the degree of the increase was not linear and declined at higher shear rates, suggesting that the biopolymer showed characteristics of a typical non-Newtonian pseudoplastic fluid. Its shear stress also increased in solutions with higher biopolymer concentration.

II.2.3. Microbial EPS Production Process

Many microorganisms produce exopolysaccharides as an extracellular or cell surface-attached material in the form of amorphous slime. In microorganisms, EPSs may be associated with virulence in some cases, and these molecules are believed to be produced by the bacteria as means of protection against hostile conditions, such as desiccation, osmotic stress, low temperature and bacteriophage infection (Hugenholtz et al., 2000; Lee et al., 2002).

EPSs play a major structural role in the formation of biofilms, in which various prokaryotic and eukaryotic microorganisms grow while attached to solid-liquid interfaces. Several of these microbial polysaccharides are now widely accepted industrial products, while others are in various stages of development (Lee et al., 2002; Sutherland, 1998).

At present, very few EPSs are available commercially, but the number and their applications are gradually increasing (Lee et al., 2002). 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 (Sutherland, 2002).

Some of the microbial polysaccharides such as xanthan (the EPS from *Xanthomonas campestris* pv. *campestris*) are already well established by modern biotechnology and have a sizable market. Others such as pullulan possesses potentially useful chemical and physical properties, and consequently are receiving much attention from industrial areas. Microbial EPSs are promising substitutes for traditional plant polysaccharides because of their unique and superior physical properties (Lee et al., 2002).

The EPSs can be produced on agar plates or in liquid culture (Yang et al., 2001).

Production of most microbial polysaccharides involves growth in stirred tank fermenters using media with glucose or sucrose as the carbon and energy source. Synthesis is often favoured by high C:N ratios. Because of the high viscosity of the

fermentation broths, efficient mixing and aeration are required together with considerable energy input (Sutherland, 2002).

The most used cultivation systems are batch, fed-batch and continuous. Some examples of industrial cultivation systems for polysaccharide production are shown in Table II.1.

Table II.1. Industrial production of principal exopolysaccharides (Mironescu, 2003)

Exopolysaccharide	Cultivation System	Cultivation Conditions	Cultivation Medium
Alginate	Continuous	30°C; pH 7.2; Aeration (oxygen in excess does not stimulate the synthesis of EPS); Agitation.	Carbon source: sucrose (20 g/l); Higher production speed through molybdenum and phosphorus limitation or through smaller dilution rates.
Dextran	Batch or fed-batch	25°C (30°C optimal for microorganism, 23°C optimal for EPS); pH 6.0 (optimal for EPS production); No aeration in the phase of EPS stimulation.	Carbon source: sucrose (5-10g/l) with sucrose conversion yield of 90%. High levels of nitrogen and CaCl ₂ stimulate the EPS production.
Xanthan	Batch	28°C; pH 7.0; Aeration (0.5-0.75 l/min. at the beginning, 0.75 1.5 l/min. after 2 days because of the increase in viscosity). Agitation.	Carbon source: glucose (2-3.5 g/l)
	Continuous		Dilution rate control. Nitrogen, sulphur or potassium limitation.

Fed-batch fermentations may be preferable to the use of high initial sugar concentrations. After pasteurization of the broth, recovery by precipitation with isopropanol is followed by drying and grinding to yield a fine powder. Filtration or centrifugation and other downstream processing add to the final cost (Sutherland, 2002).

A process for the isolation and purification of pullulan is shown in Figure II.3, the main steps being centrifugation, evaporation, precipitation, ultrafiltration, and drying.

Figure II.3. Downstream process flowsheet for isolation and purification of pullulan.

II.3. IMPORTANT APPLICATIONS OF EPS

The industrial uses of polysaccharides are based until recently on materials extracted from plants (starch, cellulose, pectins, galactomanans, gums) or algae (carragenan, alginates, agar). In the last 30 years, biotechnologies for the microbial production of extracellular polysaccharides were developed. The polysaccharides of microbial origin are largely used in food industry, biotechnology, medicine and pharmacy. The success is due to their properties and to the diversity of producing microorganisms and synthesized polysaccharides (Mironescu, 2003; Sutherland, 2002).

Because of their many interesting physico-chemical and rheological properties with novel functionality, the microbial exopolysaccharides (EPS) act as new biomaterials and find wide range of applications in many industrial sectors like textiles, detergents, adhesives, microbial enhanced oil recovery (MEOR), wastewater treatment, dredging, brewing, downstream processing, cosmetology, pharmacology, and food additives (Kumar et al., 2004). Moreover, there are numerous studies on their participation in pathogenic and symbiotic processes in plants and animals and on the general interactions between microorganisms and their environment (Loaëc et al., 1998; Nicolaus et al., 1999). Polysaccharides also contribute to various physiological activities in human beings as anti-tumor, anti-viral, and anti-inflammatory agents, and can act as inducers for interferon, platelet aggregation inhibition, and colony stimulating factor synthesis (Kumar et al., 2004).

Many bacteria secrete polysaccharides that can have diverse functions, such as virulence factors, signalling molecules in bacterium-plant interactions, or protective agents. Some of these polymers have unique properties for food applications and are used as viscosifiers, stabilizers, emulsifiers or gelling agents (Table II.2). Commercially, the most important microbial exopolysaccharide (EPS) is xanthan gum, a complex polymer that is very efficiently produced by the phytopathogen *Xanthomonas campestris* and has been used for decades in food and non-food applications. Acetan is structurally related to xanthan and is produced by *Acetobacter xylinum*, a strain that is used in the food industry for the production of a sweet confectionery (Nata) and vinegar. Sphingans, capsular polysaccharides secreted by *Sphingomonas* strains, including gellan, wellan, rhamsan, and sphingan S-88, have special rheological properties and can be used in food as gelling agents, stabilizers or suspending agents. In the dairy industry, EPS-producing lactic acid bacteria (LAB), including the genera *Streptococcus*, *Lactobacillus*, and *Lactococcus*, are used *in situ* to improve the texture of fermented dairy products such as yoghurt or cheese. This group of food-grade bacteria produces a wide variety of structurally different polymers with potential use for new applications. In addition, for example, replacement of presently applied thickeners produced by non-food-grade bacteria. In addition, it has been suggested that EPSs produced by LAB may confer health benefits as studies with mouse models indicate that EPSs have immunostimulatory, antitumoral, or cholesterol-lowering activity. Furthermore, LAB are used for the

production of simple polymers such as dextrans, which are used for their rheological properties, by the activity of extracellular glycosyltransferases (van Kranenburg et al., 1999).

Table II.2. Food applications for complex microbial EPSs (van Kranenburg et al., 1999).

Polymer	Bacterial source	Use	<i>In situ</i> EPS production
Xanthan	<i>X. campestris</i>	Viscosifier, stabilizer , emulsifier and suspending agent	–
Acetan	<i>A. xylinum</i>	Viscosifier and gelling agent	–
Gellan	<i>Sphingomonas</i>	Gelling agent, stabilizer and suspending agent	–
EPSs of LAB	<i>S. thermophilus</i> , <i>Lactobacillus ssp</i> , <i>L. lactis</i>	Viscosifier and hydrating agent	+

II.3.1. Emulsifier

Chemically synthesized and plant derived emulsifiers are used in the food, pharmaceutical, cosmetic and petroleum industries. However, emulsifiers from microbial sources have attracted attention because of the several advantages they offer over the synthetic products such as lower toxicity, higher biodegradability, better environmental compatibility, higher foaming, high selectivity and specific activity at extreme temperature, pH, salinity and ability to be synthesized from renewable feed stocks (Iyer et al., 2006).

Bacteria are capable of making two kinds of polymers, low molecular weight molecules that efficiently lower surface tension and interfacial tensions and high molecular weight polymers that bind tightly to surfaces. These high molecular weight biosurfactants are highly efficient emulsifiers that work at low concentrations and they exhibit considerable substrate specificity, they are produced by a large number of bacteria and they are composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins, etc. (Martínez-Checa et al., 2007).

Dextran, synthesized by certain lactic-acid bacteria such as *Leuconostoc mesenteroides* and *Streptococcus mutans*, was the first microbial polysaccharide that

was approved for its use in food industry and became commercialized in early 1950s (Sutherland 2002). Xanthan gum (the EPS from *Xanthomonas campestris* pv. *campestris* bacterium) entered the market in 1967 and then received its approval for food use in 1969. Xanthan is already well established by modern biotechnology and have a sizable market due to its exceptional qualities as a rheology control agent in aqueous systems and as a stabilizer for emulsions and suspensions (Rosalam and England, 2006). Another example is pullulan (the fungal EPS produced by *Aureobasidium pullulans*) that is used extensively in food industry and as a pharmaceutical bulking agent since more than 20 years.

Numerous studies have been conducted for developing and implementing innovative technology to clean up contamination with petroleum hydrocarbons (King et al., 1998). Bioemulsifiers are able of emulsifying these pollutants much more effectively than do chemical surfactants. Halophilic EPS producers are considered as an interesting source for microbial enhanced oil recovery (MEOR) where polymers act as emulsifiers and mobility controllers. Active emulsification of petroleum has been noted for six strains, close to *Halobacterium salinarium*, *Haloferax volcanii*, and *Halobacterium distributum* (Kulichevskaya et al., 1998).

Martínez-Checa et al. (2007) studied the characteristics of the bioemulsifier V2-7 synthesized by strain F2-7 of *Halomonas eurihalina* and they found that it has the ability of emulsifying a wide range of hydrocarbons i.e. *n*-tetradecane, *n*-hexadecane, *n*-octane, xylene mineral light and heavy oils, petrol and crude oil.

Iyer et al. (2006) studied the emulsifying ability of the exopolysaccharide, EPS 71a, produced by a marine bacterium, *Enterobacter cloacae*. They found that EPS 71a emulsified hexane, benzene, xylene, kerosene, paraffin oil, cottonseed oil, coconut oil, jojoba oil, castor oil, groundnut oil and sunflower oil and the emulsions stabilized by EPS 71a had a good shelf life under standard conditions. In addition, the emulsions were stable under stress conditions like pH and salinity, similar to those often encountered in the development of food and pharmaceutical formulations.

The emulsifying activity of several biopolymers produced from different bacterial isolates were examined comparing with other pure gums by El-Tayeb et al. (2007). The highest biopolymer concentration (8.6 g/L) was obtained by an isolate which was identified as *Pseudomonas* sp. UBF 2. This biopolymer exhibited the

highest lipid emulsifying capacity (100 %) when compared with that of xanthan gum (90 %). The minimum concentration of biopolymer solution to obtain a 100 % emulsion stable for 10 days against all oils was 0.2%, while against cotton seed oil was 0.5%.

II.3.2. Biofloculants

Flocculating agents are generally categorized into three major groups, namely, inorganic flocculants such as aluminum sulfate and polyaluminum chloride (PAC); organic synthetic polymer flocculants such as polyacrylamide derivatives and polyethylene; and naturally occurring biopolymer flocculants such as chitosan and algin. Among these, organic synthetic polymers are the most widely used flocculants, since they are the most economical and highly effective. However, the use of these flocculants sometimes causes environmental and health problems, because they are not readily biodegradable and some of their degraded monomers, such as acrylamide, are neurotoxic and even potent human carcinogens. On the other hand, naturally occurring biopolymers (biofloculants), which are produced by microorganisms during their growth, have special advantages such as safety, strong effect, biodegradable and harmlessness to humans and the environment, so they may potentially be applied in drinking and wastewater treatment, downstream processing, and fermentation processes (Lu et al., 2005; Gao et al., 2006). Microbial flocculants have been suggested to replace organic high-polymer flocculants, such as polyacrylamide, which are inexpensive and highly effective but not easily degraded and can be harmful to humans. Among the microbial flocculants reported previously, protein polysaccharide, glycoprotein, polyglutamic acid and xanthan have been investigated (Prasertsan et al., 2008).

In recent years, many biofloculant-producing microorganisms including bacteria, fungi and actinomyces have been reported to produce extracellular polymeric substances, such as polysaccharides, functional proteins and glycoprotein, which function as biofloculant. Flocculants produced by a marine dinoflagellate *Gyrodinium impudicum* KG03, *Bacillus subtilis* DYU1, *Vagococcus* sp. W31, Poly- γ -glutamic acid (γ -PGA) produced by *Bacillus subtilis* F-2-01, *Enterobacter aerogenes* W-23, a haloakalophilic *Bacillus* sp. I-471, *Alcaligenes cupidus* KT201 and *Bacillus subtilis* IFO3335 are polysaccharides. However, only few of these biofloculants has been practically applied in industry because of their low

flocculating capability and large dosage requirement. To utilize bioflocculants widely in industrial fields, it is desirable to find various microorganisms with high bioflocculant-producing ability and improve the flocculating efficiency of the bioflocculant (Yükselen et al., 2004).

Li et al. (2008) studied the flocculation behavior and mechanism of the exopolysaccharide secreted by *Pseudoalteromonas* sp. SM9913 (EPS SM9913). They found that EPS SM9913 could flocculate various suspended solids such as kaolin clay, activated carbon, soil, MgO and Al₂O₃. The flocculating activities of this EPS could be enhanced by the addition of bivalent cations such as Ca²⁺ and Fe²⁺ because of these cations' mediating function. Results indicated that EPS SM9913 delivers better flocculation performance than alum at low temperature (5-15°C) or in high-salinity (5–100‰) water. Such findings suggested the probable use of this EPS SM9913 as an effective flocculant for wastewater treatment at low temperature and/or salinity.

El-Tayeb et al. (2007) studied the flocculating activity of several biopolymers produced from different bacterial isolates comparing with other pure gums such as xanthan, pullulan, dextran, rhizobial exopolysaccharide and arabic gum. The highest flocculating activity was observed with the biopolymer produced by *Pseudomonas* sp. UBF 2 followed by xanthan gum, biopolymer No. 1, biopolymer No. 5 and pullulan. However, dextran, rhizobial exopolysaccharide and arabic gum showed poor flocculating effect. The unique flocculating activity of the biopolymer produced by *Pseudomonas* sp. UBF 2 suggested that it has a great potential as a flocculating agent.

II.3.3. Heavy Metal Biosorption Ability

EPS are metabolic products of bacteria but they also result from organic matter of the effluent and from microbial lysis or hydrolysis and serve as a protective barrier for cells against the harsh external environment. EPS also have the function of sorption of inorganic ions which constitute metabolic elements for bacteria. Their composition is complex but EPS are mainly composed of polysaccharide, protein, humic substances, uronic acid, nucleic acid and lipids, containing ionisable functional groups such as carboxyl, phosphoric, amine, and hydroxyl groups. These functional groups represent potential binding sites for the sequestration of metal ions. It is assumed that metal biosorption involves a physicochemical interaction between

the metal and functional groups on the cell surface, based on physical adsorption, ion exchange, complexation and precipitation. Moreover, metal biosorption performance depends on external factors, such as pH, other ions in bulk solutions (which may be in competition), organic material in bulk solution and temperature. These properties are potentially of great importance in sewage treatment processes for the removal of toxic heavy metal pollutants (Comte et al., 2008; Liu et al., 2001; Guibaud et al., 2003; Thomas et al, 2003).

II.3.4. EPS in Pharmacy and Medicine

Polysaccharides constitute a structurally diverse class of biological macromolecules with a wide range of physicochemical properties, which are the basis for the different applications in the broad field of pharmacy and medicine.

A more generally inclusive and descriptive name for the group that are abundant in nature, present in many plant sources are "polysaccharide hydrocolloids"; only a restricted number is of commercial importance of substances. Functional properties of polysaccharide hydrocolloids are; stabilizer (suspending agent), thickener (film-forming agent), coagulant (water retention agent), and colloid (lubricant or friction reducer).

The importance of various polysaccharide hydrocolloids for pharmaceutical purposes has a long historical background and increased considerably during the last decades. Many interesting areas have been opened in the past which include their role in drug delivery, in wound treatment, in cancer therapy, and the diagnosis, prevention, and treatment of bacterial and viral diseases.

Compendial standards and governmental regulations require that even the natural polysaccharides as well as their derivatives meet the strict standards of identity, potency, and purity, in order to define the necessity of drug quality (Table II.3). Some polysaccharides may undergo chemical changes with time, resulting in a loss of biological and therapeutical activity.

Table II.3. Criteria for the pharmaceutical utilization of polysaccharides.

Polysaccharides must have:	Polysaccharides must not have:
<ul style="list-style-type: none">▪ High purity▪ Chemical, physical, and mechanical properties for the proposed functions▪ High stability▪ Easy fabricability	<ul style="list-style-type: none">▪ Inactivate enzymes▪ Cause cancer▪ Produce toxic or allergic reactions▪ Deplete electrolytes

It is important to note that in almost all commercial products the polysaccharide hydrocolloids never occur alone but are part of a more or less complex formulation with other plant constituents or chemical compounds (Franz, 1989).

Some polysaccharides form integral components of vaccines, usually when coupled to a suitable protein. Thus, meningitis vaccines have been prepared in this way and multivalent polysaccharide vaccines have been formulated against *Streptococcus pneumoniae* and *Klebsiella* spp. However, these are expensive to prepare and only use very small amounts of material. Possibly of much greater significance is the role of certain microbial polysaccharides in tumour suppression and immunestimulation. A homopolymer named ‘Scleroglucan’ or schizophyllan, from several fungal species, appears to be very effective against some cancers when it is applied in the ordered, triple helical form. These β -linked glucans are therefore the subject of much current study and have already been tested clinically in Japan, proving effective against certain types of tumor. Dextran, although no longer used as a food ingredient, is the base from which the ‘Sephadex’ range of biochemical adsorbents is prepared. Dextran solutions can also be used as a plasma substitute, being very poorly antigenic and having the correct physical properties (Sutherland, 2002).

Polysaccharides also contribute to various physiological activities in human beings as antitumor, antiviral, and antiinflammatory agents, and can act as inducers for interferon, platelet aggregation inhibition, and colony stimulating factor synthesis (Kumar et al., 2004).

Within the field of pharmacologically active polymers, the area of immune-stimulating polysaccharides is a rather new and attractive field of tumor therapy. The interest in polysaccharides as antitumor substances came from the dissatisfaction

with current cancer chemotherapy. Countless numbers of chemical compounds have been tested as cytostatic agents with possibly a high specificity for the cancer cell. However, unfortunately in many cases, this specificity is rather low, and therefore many chemotherapeutic antitumor substances have considerable side effects. Extensive studies have been carried out on nontoxic antitumor substances and most interestingly, some polysaccharides were shown to be most useful. Mainly fungal polysaccharides have been reported to be active against a series of allogeneic and syngeneic tumors. These antitumor polysaccharides, in most cases, were shown to be glucans with different types of glycosidic linkages. It is interesting to note that besides these very active glucans, several fructans were shown to exhibit pronounced activity against a series of different tumor types (Franz, 1989).

Three examples of polysaccharides in clinical use are Schizophyllan, Lentinan and Krestin. The clinical usefulness of these polysaccharides was demonstrated with patients having head and neck cancer, lung cancer, gastric cancer, and cervical cancer. These polymers were either used alone or in combination with chemotherapy and/or radiotherapy, but in most cases after the surgical removal of the primary tumor (Franz, 1989).

In the past few years, some studies revealed that polysaccharides from mushrooms have shown direct antiproliferative effect on cancer cells. Chen et al. (2008) studied the antitumor effect of a medicinal mushroom *Fomes fomentarius* isolated from the fruiting body of a wild *F. fomentarius*. To evaluate the effect of growth inhibition, various concentrations of EPS produced by *Fomes fomentarius* in submerged culture were added to culture medium of SGC-7901 human gastric cancer cells. EPS induced a distinct dose- and time-dependent diminution of cell viability. It was also reported that polysaccharide at low dose could sensitize doxorubicin (Dox) - induced growth inhibition and apoptosis of some cancer cells. Therefore, Chen et al. (2008) evaluated whether EPS at noncytotoxic concentration (0.125-0.25 mg ml⁻¹) could sensitize Dox-induced growth inhibition of SGC-7901 cells.

Recently there is an attempt to explore pullulan for various biomedical applications including targeted drug and gene delivery and tissue engineering due to its non-toxic, non-immunogenic, non-mutagenic and non-carcinogenic nature. The early observation on this exopolymer was made by Bauer in 1938 and this exopolysaccharide was named as pullulan by Bender et al in 1959. Pullulan and its derivatives can be used as a denture adhesive. It can also be used for pharmaceutical

coatings, including sustained release formulations. Novel preparations such as tablets, pills, granules or the like, which contain pullulan in the sugar layer serve the purpose of preventing brownish color change of the composition. Oral care products have been commercialized based on pullulan films. The colorless, transparent and edible pullulan film has also attracted a great deal of interest for other uses such as a non-polluting wrapping material (Singh et al., 2008; Rekha et al., 2007).

Scott et al. (2005) reported pullulan compositions to be used in pharmaceutical products preferably for predosed formulations like soft and hard capsules. Pullulan derivatives are promising as non-toxic conjugates for vaccines. Covalent attachment between the virus and pullulan remarkably enhances the inherent producibility of immunoglobulin G and immunoglobulin M antibodies and diminishes the immunoglobulin E antibody producibility as well as sufficiently inactivating and detoxifying the virus. Pullulan can provide liposome delivery. Sized pullulan fractions having molecular weight 30.000 to 90.000 Da can be used as a blood plasma expander in place of dextran. There have been several attempts to develop plasma substitutes on pullulan.

Shingel (2004) summarized all efforts that have been made so far to understand the pharmacokinetics of intravenously applied pullulan in terms of the molar mass and concentration.

Tissue engineering requires scaffold or artificial extracellular matrix that can accommodate cells and regulate their growth leading to three-dimensional tissue regeneration. Researchers developed carboxymethyl pullulan and conjugated it with heparin and investigated its properties towards tissue engineering applications. Heparin-conjugated pullulan inhibited the proliferation of smooth muscle cells (SMCs) *in vitro*. Heparin-conjugated pullulan material could thus be used for the proliferation of vascular endothelial cells and to inhibit the proliferation of SMCs (Rekha et al., 2007).

Gene therapy is another area where the application of pullulan is being explored. Gene therapy is thought to be a cure for various inherited disorders and cancer. Gene delivery is usually achieved by endocytic pathway. Efforts for gene therapy using virus have been performed but viruses are known to be immunogenic and can be hazardous. So attempts to develop non-viral vectors are taken and cationic derivatives of natural polymers are investigated towards this purpose. Recently, pullulan being biocompatible and non-toxic is investigated for gene

delivery application. Researchers developed pullulan-DTPA derivative, which has metal chelating residues and mixed with a plasmid DNA in aqueous solution containing Zn^{2+} ions to obtain the conjugate of pullulan derivative and plasmid DNA with Zn^{2+} coordination. Pullulan is also known for its specificity for liver and this property is exploited for liver targeting. The authors observed that the conjugate enhanced the level of gene expression at the liver paranchymal cells and the enhanced gene expression lasted for a period of 12 days after the injection (Rekha et al., 2007).

II.4. EXTREMOPHILES as SOURCES for EPS

For several decades, interest has been focused on the capacity of microorganisms to produce exopolysaccharides (EPS) because these biopolymers often possess advantageous qualities not shared by many polysaccharides currently in use, which are generally extracted from plants or marine algae (S Bouchotroch et al., 2000). The exopolysaccharides produced by microorganisms are widely used in the food, pharmaceutical and chemical industries, and function as biofloculants, bioabsorbents, heavy metal removal agents, drug delivery agents, etc (Prasertsan et al., 2008).

The microorganisms used as industrial or technical producers of extracellular polysaccharides are bacteria. Species of *Xanthomonas*, *Leuconostoc*, *Pseudomonas*, *Alcaligenes* which produce xanthan, dextran, gellan, curdlan are the most known and industrially used. Actually, high attention is accorded to the exopolysaccharides produced by lactic acid bacteria which are already accepted as GRAS (Generally Recognised As Safe) and the most adequate for the food industry. Another group of microorganisms producing of exopolysaccharides are the cyanobacteria. The fungal polysaccharides are limited, pullulan produced by *Aureobasidium pullulans* and scleroglucan synthesized by *Sclerotium glaucum* being the most known and already obtained at technical scale. The newest type of microorganisms, the archaea, are also presented in the literature as polysaccharides producers, as the specie *Haloferax mediterranei* (Mironescu, 2003; Sutherland, 1990).

Biopolymers produced by diverse groups of microorganisms are rapidly emerging as a new and industrially important source of polymeric materials, which are gradually becoming economically competitive with natural gums produced by a variety of microbes.

Until now, very few biopolymers have been produced on a commercial basis due to two major facts. Firstly, their production costs are much higher when compared with chemically synthesized polymers that possess similar material characteristics. Consequently, much effort has been devoted to the development of processes for biopolymer production by optimizing the upstream to downstream engineering strategies including the metabolic and cellular engineering of host cells, efficient fermentation and recovery processes, and post-production modification of the biopolymers obtained (Lee et al., 2002). The second fact is that from a variety of microbial polysaccharides characterized so far, only those with physicochemical properties similar to those of plant (cellulose, pectin and starch) and seaweed (alginate and carrageenan) are used in industry (Ruas-Madiedo and Reyes-Gavilan, 2003).

It is widely accepted that extremophiles offer important insights into biology and evolution of many organisms, and they provide a valuable resource for exploitation in novel biotechnological processes.

Although many researchers claim to have found polysaccharides ‘of superior viscosity to xanthan’, in reality few match the robustness of the *Xanthomonas* polysaccharide and few can maintain their physical properties in the presence of salts, at higher temperatures or extremes of pH (Lee et al., 2002). Therefore, most research is focused on the identification of EPS producing extremophiles with the idea that as these microorganisms survive environmental extremes of desiccation, temperature, pressure, salinity, acidity, heavy metals, and radiation, it is to be expected that their biopolymers will also have some unique properties to adapt to such extreme conditions.

A new approach to encountering polysaccharides with novel properties might entail investigating different environments such as hypersaline habitats, since most of the strains used so far for the industrial production of polysaccharides belong to a small number of taxa, which are non-halophilic, soil-dwelling diazotrophs and often plant-associated. Because of the extreme nature of hypersaline environments, they might be expected to harbor unusual microorganisms of biotechnological interest; thus, for the last few years, extensive research is being carried out in examining microorganisms from such habitats in an attempt to find new EPSs with different characteristics (Mata et al., 2006).

Since 1991, EPS producing extremophilic microorganisms have been isolated from deep-sea hydrothermal vents characterized by extreme pressure and temperature, high concentrations of H₂S and heavy metals. These include some bacteria such as *Alteromonas macleodii* sub spec *fijiensis*, *Vibrio diabolicus*, *Alteromonas infernos* (Raguénès et al., 1997; Roger et al., 2004) and *Thermotoga maritime* (Rinker et al., 2000) and archaea such as *Thermococcus litoralis* (Rinker et al., 2000). EPS producing bacteria from marine Mediterranean shallow vents have also been described (Maugeri et al., 2002; Schiano Moriello et al., 2003). EPS produced by moderately halophilic bacteria of the genus *Halomonas* (Bouchotroch et al., 2000), *Halomonas maura* (Arias et al., 2003), *Halomonas ventosae* and *Halomonas anticariensis* (Mata et al., 2006), *Aphanocapsa halophytica* (Matsunaga et al., 1996) and recently by haloalkalophilic *Bacillus* sp. I-450 (Kumar et al., 2004) and *Salinivibrio costicola* subsp. *alcaliphilus* (Romano et al., 2005) has also been reported.

II.5. EPS PRODUCTION by *Halomonas* sp.

Microorganisms that produce exopolysaccharides are to be found widely in nature and thus, in theory, large numbers of bacterial polysaccharides should be available to industry. Nevertheless, after a great deal of experimental work and examination of their physical properties, only a few have in fact proved to have any real potential for biotechnological purposes. In this category are xanthan and gellan, which have found various applications in the food industry and other fields. Some other EPSs are at different stages of development and may be on the market in the next few years. Nevertheless, industry is constantly looking for EPSs with novel functional properties to satisfy the needs of modern technology and help provide manufacturing with new finished products. Further, there is a pressing demand for industry to resort to non-toxic, biodegradable, environmentally friendly substances.

Extreme environments are proving to be a valuable source of microorganisms that secrete interesting new molecules, including exopolysaccharides. Halophilic bacteria are just such extremophiles and the properties of their extracellular polysaccharides seem to offer numerous applications in various fields of industry (Arias et al., 2003).

Halophilic bacteria are members of the archaeobacteria that require extremely high concentrations of NaCl to grow and survive. Although classically halobacteria

were considered organisms of very slow growth able to utilize only a restricted range of organic compounds, mostly amino acids, as energy sources, some groups described more recently utilize a wide range of substrates, including sugars, and grow more rapidly.

The genus *Halomonas* was originally proposed to accommodate one species, *Halomonas elongata*. It now contains a large number of recently discovered species, as well as already known bacteria which were initially assigned to other genera, such as *Deleya*, *Volcaniella* and *Halovibrio*, but have since been included in *Halomonas*.

Halomonas species are widely distributed throughout hypersaline environments. Some of them have been recognized for their potential use in biotechnology (Martínez-Checa et al., 2004). They are of great interest, as these bacteria bear great biotechnological potential for the production of compatible solutes, hydrolytic enzymes, bacteriorhodopsin, bioplastic and exopolymer (Lungmann et al., 2007; Yeon et al., 2005).

Moderate halophiles, the group to which *Halomonas* species belong, already have numerous applications in various fields of industry. The genus *Halomonas* contains about 30 species of halophilic gram negative bacteria, most of which have been isolated from saline environments and grow best in media containing 0.5 to 2.5 M NaCl. Four of these species, *Halomonas eurihalina*, *Halomonas maura*, *Halomonas ventosae* and *Halomonas anticariensis*, all isolated from the rhizosphere of xerophytic plants, have the ability to produce large quantities of EPSs with novel physical and chemical characteristics. Among these, mauran, the EPS produced by strain S-30 of *H. maura*, is notable for its high viscosifying capacity, similar to that of xanthan, and for the pseudoplastic and thixotropic behaviour of its solutions. In addition, the stability of its functional properties under a wide range of pH, saline and freezing–thawing conditions makes this polymer a good candidate for use in foodstuffs, pharmaceutical products and other fields of biotechnology. Although extensive studies have been made into the biotechnological applications of these halophilic EPSs, little is known about the molecular mechanism of their biosynthesis or how they are regulated (Arco et al., 2005).

PART III

EXPERIMENTAL WORK

III.1. MATERIALS

III.1.1. Microorganism

Halomonas sp AAD6 strain (GenBank accession number DQ131909) used in this study was isolated from Çamaltı Saltern Area in Turkey.

III.1.2. Chemicals

All chemicals and solutions used in this study were supplied by MERCK (Germany), SIGMA (USA), DIFCO (USA) and FLUKA (Switzerland). All experiments were carried out in the Biotechnology Laboratories of Bioengineering Department at Marmara University.

III.1.3. Culture Media

Culture media used for the growth of microorganisms and for the production of EPS are described below.

Minimal Medium

K ₂ HPO ₄	7 g
KH ₂ PO ₄	2 g
MgSO ₄ ·7H ₂ O	0.1 g
(NH ₄) ₂ SO ₄	1 g
Pepton	0.5 g
NaCl	100 g
Carbon source	10 g

pH = 7.0

per liter of distilled water.

Optimum Medium

K ₂ HPO ₄	7 g
KH ₂ PO ₄	2 g
MgSO ₄ ·7H ₂ O	0.1 g
(NH ₄) ₂ SO ₄	1 g
Pepton	0.5 g
NaCl	137.2 g
Sucrose	50 g
pH = 7.0	

per liter of distilled water.

The pH was adjusted to pH 7 by using 1 M NaOH or with 1 M HCl. While preparing both media, NaCl and the rest of the medium components were dissolved in 500 mL and 400 mL distilled water, respectively and then sterilized separately by autoclaving at 1.06 bar and 121°C for 15 minutes. Carbon sources were also sterilized separately in the same manner but for 3 minutes to avoid their degradation. Sterile salt and carbon source solutions were combined to one liter final volume. 18 g/L Agar-Agar was added to both formulations to prepare solid media in petri dishes.

To determine the effects of various carbon sources on growth and EPS yield, *Halomonas* sp AAD6 was grown in Minimal Medium supplemented with glucose, sucrose, maltose, lactose, raffinose, arabinose, xylose, fructose, galactose, mannose, acetate, glycerol, trisodium citrate, and rhamnose at the concentration of 10 g/L.

III.1.4. Buffers and Standard Solutions

III.1.4.1. Solutions Used in Phenol/Sulfuric Acid Method

Phenol Solution (80 % by weight)

Phenol	80 g
dH ₂ O	20 g

III.1.4.2. Solutions Used in Preparing Stock Culture

Sea Water Stock Solution (30%)

NaCl	240 g
MgSO ₄ ·7H ₂ O	35 g
MgCl ₂ ·6H ₂ O	30 g
KCl	7 g
pH = 7.0	

After the contents were dissolved in 1000 mL distilled water, 5 mL of sterile $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (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.

III.1.4.3. Buffers Used for pH Adjustment

NaOH Buffer (1M)

NaOH	40 g
dH ₂ O	1000 mL

NaOH was dissolved in 1000 mL distilled water. The buffer was sterilized at 121°C 1.06 bar for 3 minutes and stored at +4°C.

HCl Buffer (1M)

HCl (37%)	83 mL
dH ₂ O	917 mL

83 mL of HCl (37%) was added to 917 mL of distilled water. The buffer was sterilized at 121°C 1.06 bar for 3 minutes and stored at +4°C.

III.2. LABORATORY EQUIPMENT

<i>Analytic Balances</i>	Ab204-S, Pg403-S, Pg40002-S
(0.1 Mg, 1 Mg, 10 Mg)	(Mettler Toledo, Sweden)
<i>Autoclaves</i>	Mod3870 Elv (Systec, Germany)
	OT 032 Nüve, Turkey
<i>Automatic Pipettor Sets</i>	4500080, (Finipipette, Finland)
	4500110, (Finipipette, Finland)
<i>Centrifuges</i>	Highspeed Centrifuge 3K30
	Benchtop Refrigerated
	Centrifuge, 8K10 (Sigma, Germany)

<i>Deepfreezes</i>	-80°C, (Heto Holten, Denmark) -20°C, (Arçelik, Turkey) -20°C, (Heto Holten, Denmark)
<i>Fermenter</i>	Multi-Fermenter, Biostat Q, (B.Braun-Sartorius, Germany)
<i>Heating Magnetic Stirrer</i>	Mr3003s, (Heidolph, Germany)
<i>Laminar Flow Cabinet</i>	Class II Safety Cabinet, Safe 2010 0.9, (Heto Holten, Denmark)
<i>Orbital Shaker</i>	Certomat BS1 (B. Braun, Germany) New Brunswick Scientific, Excella E24 (Edison, New Jersey, USA)
<i>Ovens</i>	Oven Bd115 Pasteur Oven, Ed115 (Binder, Germany)
<i>pH Meter</i>	Mp220k, (Mettler Toledo, Germany)
<i>Refrigerators</i>	+ 4°C, (Arçelik, Turkey)
<i>Spectrophotometer</i>	UV/Vis, Lambda35, (Perkin Elmer, USA)
<i>Vortex</i>	Heidolph, Germany
<i>Water Purification Systems</i>	Primary Grade Water Purification System, Purelab Prima 30 Ultrapure Water System, P.Maxima Ls, (Usf Elga, U.K.)

III.3. EXPERIMENTAL PLAN and TECHNIQUES

III.3.1. Sterilization

Sterilization is the process by which items are rendered free of almost all viable microorganisms. The purpose of sterilization is to provide the end user with a sterile product. Sterilization process must be monitored to ensure that all parameters and specifications have been met to assure sterility of items.

Throughout this study, sterilization was carried out by killing the microorganisms using steam. The culture media used were sterilized in an autoclave. This process, which is known as steam autoclave sterilization, has advantages of short cycle time and good penetration.

Sterilization of Media All media were prepared in Erlenmeyer flasks such that the total volume of the medium was always one fifth of the flask volume. The Erlenmeyer flask was sealed with cotton stopper and wrapped with aluminum foil. The chemical and optimum media were sterilized by autoclaving at 1.06 bar and 121°C for 15 minutes before the addition of carbon sources 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.

Sterilization of Fermenter Air lines and all probe cables were removed. Jacket and exhaust condenser water lines were disconnected. All the openings of the vessel were wrapped with aluminum foil. Since the vessels should not be sterilized empty, 100 ml of sterile distilled water was placed in the fermenter vessels to allow the probe tips to be moisturized during sterilization. The sterilization of the complete consisting of jar, head plate and components of head plate, was performed at 121°C and 1.06 bar for 15 minutes.

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

III.3.2. Cultivation Conditions

Microorganisms were cultured in liquid media. Shake flask cultures were grown in Erlenmeyer flasks with medium volumes up to 1/5 of the flask volume. For optimal aeration, the Erlenmeyer flask cultures were incubated in an orbital shaker at 180 rpm. For bioreactor cultivations, a 1L BIOSTAT Q multi-fermenter with an agitation rate of 200 rpm was used and the working volume was 500 mL.

III.3.3. Preparation of Preculture

50 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 (Certomat BS1, B. Braun, Germany). 1% (v/v) of this preculture was used as an inoculum.

III.3.4. Growth Conditions

III.3.4.1. Preculture Growth

The growth of preculture was carried out in an orbital shaker at 37°C with an agitation rate of 180 rpm for 24 hours.

III.3.4.2. Batch Experiments

Shake flask The batch shake flask experiments were carried out in 500 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.

Bioreactor The monitoring of various parameters such as pH, temperature and agitation were performed by the control system of the Multi-Fermenter, BIOSTAT Q. The working volume of the culture medium was kept at 500 mL in the batch fermenter experiments. The agitation speed was 200 rpm and the temperature was set to 37°C. The pH was kept constant at pH 7 by buffering the system either with 1 M NaOH or with 1 M HCl. Compressed air was sparged into the medium for aeration at a rate of 0.1 vvm.

III.3.5. Isolation and Purification of EPS

For the purification of EPS, cells were harvested by centrifugation at 10000 rpm for 20 min and the supernatant phases were treated with an equal volume of ethanol at 5°C added drop-wise under stirring, held at -18°C overnight, and then centrifuged at 12000 rpm and 4°C for 30 min using a refrigerated centrifuge. The pellets were dissolved in hot distilled water, dialyzed against several changes of distilled water for 3 days, lyophilized and then weighed. The EPS samples were analyzed for total carbohydrate, protein, nucleic acid and uronic acid contents.

III.3.6. Statistical Optimization of the Medium

Besides important fermentation parameters such as aeration, inoculum density, pH, temperature and incubation time, microbial production of EPS is extremely influenced by media components, the initial sucrose and NaCl contents.

In this study in order to optimize the initial sucrose and NaCl contents of the medium, Response Surface Methodology (RSM) was used as a statistical tool. The initial concentrations of sucrose and NaCl were varied, while the levels of Pepton, $(\text{NH}_4)_2\text{SO}_4$, KH_2PO_4 , K_2HPO_4 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were kept constant in the Minimal Medium. In order to decide on an appropriate initial sucrose and NaCl contents, the growth profiles were determined. *Halomonas* sp AAD6 was grown at pH 7 and 37°C in an orbital shaker operated at 180 rpm constant speed. At certain time intervals, samples were taken and their cell density was determined.

In accordance with the results of preliminary experiments, a second-order polynomial was used as to model the response variable (maximum biomass yield after 100 hours of fermentation) with respect to the independent factors (initial sucrose and NaCl concentrations). A 2^2 full factorial central composite design (CCD), with 4 center and 4 star points, was employed to fit this second-order polynomial model. The design contained 12 experiments and two replications of each.

III.3.7. EPS Production in Bioreactor

III.3.7.1. 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 off-line the optical densities at 660 nm using Lambda35 UV/Vis spectrophotometer (Perkin Elmer, USA). At the desired time intervals an aliquot of the cell suspension was taken and $\text{OD}_{660\text{nm}}$ 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.

In order to convert optical density values to dry cell weight (DCW), a calibration chart was prepared. For this, known volumes of culture samples at varying optical density values were centrifuged at 5000 rpm for 15 min; harvested cells were washed twice with distilled water and then dried at 100°C until constant cell dry weight was achieved. Dry weight versus optical density graph was plotted

and a line was fitted through the data points using least square method. This calibration curve given in Appendix A provides the correlation between the optical measurements obtained from the spectrophotometer and the dry cell weight of the cells that are used in this study.

III.3.7.2. Total Carbohydrate Analysis

To determine the total carbohydrate concentration, 1-2 mL samples were taken and centrifuged at 10000 rpm for 3 min to separate biomass. The supernatant was precipitated with three volumes of cold ethanol. This solution was kept at 4°C overnight before being centrifuged at 12000 rpm and 4°C for 30 min using a refrigerated centrifuge. The pellet was resuspended in distilled water and subjected to total carbohydrate analysis. 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 20 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). The net carbohydrate contents were calculated by subtracting the carbohydrate values of growth media without inoculum.

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 curves demonstrate a linear relationship between absorbance and EPS concentration, with R^2 values greater than 0.98. This calibration curve given in Appendix B was used to convert the results of phenol/sulfuric assay to carbohydrate concentration in g/L.

III.3.8. Characterization of EPS

III.3.8.1. Determination of Protein Concentration

Protein concentration was determined by the Bradford test using Bovine Serum Albumin (BSA) as standard (Bradford, 1976). The Bradford dye assay is based on the equilibrium between three forms of Coomassie Blue G dye. Under strongly acidic conditions, the dye is most stable as a doubly-protonated red form.

Upon binding to protein, however, it is most stable as an unprotonated, blue form. 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).

III.3.8.2. Determination of Quantity and Quality of DNA

Spectrophotometric measurement was used to determine the quantity and quality of DNA. DNA was diluted with distilled water and the UV absorption of DNA was read at 260nm using quartz cuvettes.

III.3.8.3 Uronic Acid Assay

The uronic acid assay of the EPS samples relies on the appearance of a chromagen when uronic acid, heated to 100°C in the presence of concentrated sulfuric/tetraborate, is treated with meta-hydroxydiphenyl. To measure the uronic acid content, 1.2 mL of the sodium tetraborate solution (0.0125 M sodium tetraborate in concentrated sulfuric acid) was added to 200 µL of sample and the mixture vortexed for 45 s before being heated for 5 min in a 100 °C water bath. Samples were then cooled on ice for 3 min before 20 µL of m-hydroxydiphenyl solution (0.15% (v/v) in 0.5% NaOH) was added. The solution was then mixed gently and the absorbance read at 520nm after 4 min (Blumenkrantz et al., 1973). The concentration of uronic acid is determined by comparison against a calibration curve prepared by similarly assaying calibration standards of a selected uronic acid (e.g., glucuronic acid, galacturonic acid, mannuronic acid, etc) (K. Mojica et al., 2007).

III.3.8.4. Rheological Studies

Measurements of specific viscosity as a function of concentration of aqueous solutions of EPS were carried out using Cannon-Ubbelohde 75 suspended level viscometers at 25 °C.

III.3.9. Biocompatibility Studies

III.3.9.1. Inhibition of Toxic Activity

The inhibition of avarol toxic activity on brine shrimp (*Artemia salina*) was performed in triplicate using 10 animals, for each dose, in artificial seawater (Meyer et al., 1982). Briefly, 10 ppm of avarol dissolved in DMSO (1% of final volume) were added to each vial containing the polysaccharides in three different doses (500, 50, and 5 ppm) and for each dose survivor shrimps were counted after 24 h and data

were statistically analyzed by the Finney program which yields IC₅₀ values (Finney, 1971).

III.3.9.2. Cell Culture

Osteoblasts were isolated from the calvaria of 1-5 days old neonatal Wistar rats. The calvaria was dissected and freed from soft tissue, cut into small pieces and rinsed in sterile PBS solution (1%). The calvaria pieces were incubated with 1% trypsin-EDTA for 15 min, followed by four sequential incubations with 2% collagenase at 37°C for 30 min each. The supernatant of the first collagenase incubation, which contains a high proportion of periosteal fibroblasts, was discarded. The other digestions produced a suspension of cells with high proportion of osteoblasts. After centrifugation at 5000 rpm for 5 min, each pellet were resuspended in 5 mL of RPMI medium supplemented with 15% FBS, 1% antibiotic-antimycotic. The cells were seeded into 10 mL tissue culture flasks, and lead to grow in a controlled 5% CO₂, 95% humidified incubator at 37°C. For experiments, only confluent cells from the 2nd passage were used.

Preparation of EPS Solution EPS was suspended in culture medium RPMI (0.5 g in 50 ml), shaken for 5 h at 37°C and sterilized by autoclaving at 1.06 bar and 121 °C for 15 minutes without 15% of FBS, which was sterilized by filtration (0.22 µm). Control medium was subjected to the same process.

Stimulation of Osteoblasts with EPS Solution Osteoblasts were plated 1x10⁵ in 24 well plates, and after 2 h, the medium was changed to medium containing EPS. After 48 h incubation, osteoblasts morphology, viability and secretion capability (alkaline phosphatase) were tested.

Cellular Viability After 48 h of incubation in the presence of EPS solution, osteoblast viability was evaluated by MTT assay, based on the reduction of tetrazolium salt to formazan crystals by living cells. About 20 µL of MTT was added to each well containing 200 µL of sample. Four hours later, the cell morphology was analysed by inverted optical microscopy and formazan salts were solubilized with 50 µL of solubilization kit. After incubation, for overnight, in a controlled 5% CO₂, 95% humidified incubator at 37°C, the optical density measurement was done at 595 nm.

Alkaline Phosphatase Activity After incubation for the established period the alkaline phosphatase production was evaluated by BCIP-NBT assay. This assay is

based on a chromagenic reaction initiated by the cleavage of the phosphate group of BCIP by alkaline phosphatase present in the supernatant. This reaction produces a proton, which reduces NBT to an insoluble purple precipitate. Briefly, 50 μ L of BCIP-NBT solution was added to 50 μ L of supernatant from each well according to the manufacturer's protocol. After overnight incubation at 37°C, the optical density measurement was done at 595 nm. Cell cultures were used without samples as control.

III.3.10. Chemical Characterization of EPS

For the sugar analysis, lyophilized samples obtained under the conditions described above were dissolved in distilled water to give 0.1% (w/v) solutions and hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120°C for 2 h. The neutral sugar composition of the EPS samples was analyzed by HPAE-PAD using standards for identification and calibration curves (Manca et al., 1996). HPAE-PAD Dionex equipped with Carbopac PA 1 column was eluted isocratically with a) 15 mM NaOH for neutral sugars; b) buffer 100 mM NaOH and 150 mM NaOAc for acidic sugars.

Fourier transform-infrared (FT-IR) spectroscopy spectra of EPS were obtained with Nicolet 6700 FT-IR Spectrometer between 400 and 4000 wave numbers (cm^{-1}). Thermogravimetric analysis of EPS was obtained with Mettler TGA apparatus where a known amount of EPS sample (5 mg in 2 mL H_2O) was heated from 30 to 400 °C at a rate of 20 °C min^{-1} under a constant flow of nitrogen.

Molecular size analyses were carried out by Gel filtration on Sepharose Cl-6B column using H_2O /Pyridine/AcOH (500/5/2, by vol.) as eluant, with a flux of 3.7 mL/h, and density gradient centrifugation method, using a sucrose gradient from 0 to 50% w/v at 13000 g for 16 h (Pazur et al., 1994). In both methods 10 mg of EPS and a mixture of dextran for calibration curves (10 mg of T-700, MW 670000; T-400, MW 410000; T- 150, MW 154000) were used.

UV spectra of EPS were obtained by reading the absorbance of aqueous solutions from 350 to 210 nm on a Varian DMS-90 instrument.

PART IV

RESULTS AND DISCUSSION

Considering the widespread use of microbial polysaccharides in various industrial applications, microbial polysaccharide production by the halophilic *Halomonas* sp AAD6 bacteria isolated from Çamaltı Saltern Area in Izmir was investigated within the scope of this study. The fermentation conditions were optimized for high-yield biopolymer production by this microorganism. In addition, this novel exopolysaccharide was also produced under controlled bioreactor conditions, purified with high yield and characterized. Finally, preliminary biocompatibility studies were performed by isolating osteoblasts from the calvaria of 1- to 5-day old neonatal wistar rats and incubating the cells in the presence of the EPS. Then, osteoblasts' morphology, viability/proliferation and secretion capability of alkaline phosphatase were tested. Also, the ability of the biopolymer to inhibit the toxic activity of avarol on brine shrimp was analyzed.

IV.1. OPTIMIZATION OF FERMENTATION CONDITIONS

The yield and quality of microbial exopolysaccharide are greatly affected by the nutritional and environmental conditions and therefore an increase in polymer production is possible by manipulating the culture conditions. Production of most microbial polysaccharides involves growth using media with glucose or sucrose as the carbon and energy source and the biopolymer synthesis is often favored by high C:N ratios (Sutherland, 2002).

IV.1.1. Effect of Different Carbon Sources on Growth and Exopolysaccharide Production

The amount of EPS produced by bacterial cultures is often linked to biomass production, but there are exceptions. Attempts have been made to improve yields by manipulating medium composition, such as the carbon source (Macedo et al., 2002).

In this study, the ability of halophilic *Halomonas* sp AAD6 cells to use different carbon sources for growth and EPS production were analyzed. For this, cells were grown in minimal medium containing 14 different carbon sources, namely, glucose, sucrose, maltose, lactose, raffinose, arabinose, xylose, fructose,

galactose, mannose, acetate, glycerol, trisodium citrate, and rhamnose at 1% concentration. Samples were taken from shake flask cultures at certain time periods and analyzed for biomass and EPS production. The results are summarized in Table IV.1.

Table IV.1. The Effect of Carbon Sources (1% (w/v)) in the Minimal Medium on Growth and EPS Production

C - Source	Biomass (g/L)	EPS Production (g/L)	Yp/x (g/g)
Glucose	1,107998	0	0
Lactose	0,103099	0,0599	0,580994
Sucrose	0,86346	1,0726	1,242212
Arabinose	0,103785	0,0273	0,263044
Xylose	0,096111	0,2665	2,77285
Maltose	1,37422	0,1893	0,137751
Raffinose	0,134516	0,2059	1,530678
Fructose	1,548088	0	0
Galactose	1,199438	0	0
Mannose	0,607132	0	0
Rhamnose	0,084974	0	0
Acetate	0,051141	0	0
Glycerol	-0,00091	0	0
Trisodium citrate	0,003102	0	0

Halomonas sp AAD6 cells grown in the presence of sucrose were found to reach highest EPS production levels of 1.0726 g/L. Although higher biomass concentrations were observed when glucose, maltose, fructose and galactose were used as carbon source, their EPS production levels (which were 0, 0.1893, 0 and 0 g/L, respectively) were very low when compared with sucrose. Acetate, trisodium citrate, rhamnose and glycerol were most probably not utilized by the microorganism metabolism resulting in very poor growth and biopolymer production levels. The product yield on biomass (Yp/x) of the cultures was calculated by dividing the net produced EPS amounts by the biomass concentration. High Yp/x value of xylose (2.77285 g/g) is due to the very low steady state biomass concentration of the

culture. Arabinose reached almost the same biomass concentrations like xylose but the cells' biopolymer production yields were about ten-fold lower than xylose.

In the light of these results, sucrose was chosen as the best carbon source for both biomass and EPS production.

In literature, there are numerous reports on the influence of various carbon sources on the biomass and EPS production.

Shih et al. (2005) studied production of levan by *Bacillus subtilis* (natto) Takahashi. A large amount of the levan was produced when the bacteria were cultivated using sucrose, but the yields varied with the sucrose concentration. The maximum levan productivity (49.4 g/L) was obtained on the medium containing 200 g/L of sucrose, whereas the yield decreased at the higher or the lower sucrose concentrations. A small amount of polysaccharide (alcohol precipitate) was also produced when the bacteria were grown on lactose and maltose, but no polysaccharide was produced using glucose or fructose.

Prasertsan et al. (2008) investigated factors affecting the production of exopolysaccharide from *Enterobacter cloacae* WD7 cultivated in basal medium. The influence of different carbon sources (glucose, fructose, galactose, sucrose and maltose at 1% w/v) and concentrations (0-4.0% w/v) of the selected carbon sources were studied. Sucrose and galactose gave similar EPS yields (2.45 and 2.50 g/L, respectively) ($p < 0.05$) and higher than those from using maltose, fructose and glucose (2.34, 2.32 and 2.23 g/L, respectively). Sucrose was chosen due to its lower cost than galactose. Increase of the sucrose concentration (testing at 1%, 2%, 3%, 4% w/v) resulted in increase in EPS yields of 2.23, 2.24, 2.72, and 2.52 g/L, respectively. The optimal sucrose concentration for EPS production from *E. cloacae* WD7 was 3% w/v while it was 2.5% w/v for *Agrobacterium tumefaciens* (Stredansky et al., 1999), 10% for *Lactobacillus* strain LB 80 (van Geel-Schutten et al., 1998) and 2% w/v for *Lactobacillus casei* CG11 (Cerning et al., 1994), giving the maximum EPS yields of 2.72, 23.5, 21.0 and 0.05 g/L, respectively.

Huang H. C. and Liu H. C (2008) studied the effects of cultural condition on the mycelial growth and exopolysaccharide (EPS) production by *Grifola umbellata* (Fries). To select the best carbon sources for mycelial growth and EPS production, various carbohydrates (2 %, w/v) such as sucrose, soluble starch, lactose, molasses and xylose were added into the basal medium, respectively. It is obvious that the highest mycelia dry cell weight and EPS production were obtained when glucose was

used. In contrast, very low cell and polysaccharide productions were observed when using lactose and xylose as carbon sources. Based on this result, glucose was selected as the carbon source for the subsequent experiments. 1–5 % glucose was added to the medium to search for the optimal glucose concentration. Both mycelia and EPS productions increased with the increase of glucose concentration from 1 to 3 %. Whereas, further increasing glucose concentration from 3 to 5% exerted a negative effect on the production.

Çelik et al. (2008) investigated the cultivation of *Pseudomonas aeruginosa* G1 and *Pseudomonas putida* G12 strains in the PAP medium containing various carbon sources (glucose, mannose, fructose and xylose) to find a suitable carbon source for the EPS production. When strains G1 and G12 were grown in the xylose containing medium, the EPS productions (335, 262 mg/L, respectively) were the highest among those tested. The EPS produced (267 mg/L) from fructose and other carbon sources by strain G11 was much lower than that from xylose. Similarly, the EPSs produced (208, 196, 242 mg/L) from glucose, mannose, fructose by strain G12 were lower than that from xylose.

E. Arsköld et al. (2007) studied the effect of several environmental conditions on the growth and EPS production in the *L. reuteri* strain ATCC 55730. The strains were grown in batch mode using a semi-defined medium with sucrose or glucose as carbon and energy source under anaerobic conditions, in 1-L fermentors. To study the influence of glucose on growth and EPS production, *L. reuteri* ATCC 55730 was grown with either sucrose or glucose and with a combination of the two sugars. The growth rate declined from the cultures on sucrose (0.72 h^{-1}), sucrose plus glucose (0.66 h^{-1}) to glucose (0.53 h^{-1}). However, in the presence of glucose the EPS production (0.14 g/g CDW) dropped by about 50% compared to the fermentation with sucrose only (0.27 g/g CDW). In the culture on glucose no EPS was produced, confirming that EPS is only formed when sucrose is present.

Poli et al. (2004) studied the influence of various carbon sources on the biomass and EPS production with newly isolated *Halomonas* strain CRSS by using batch fermentation in complex media A and B containing trisodium citrate (3g/L) and maltose (20g/L) respectively as carbon source and minimal medium with 1% (w/v) sodium acetate, D-glucose, D-galactose, lactic acid, D-maltose, D-fructose, D-cellobiose, D-ribose, D-trehalose, glycerol or sucrose. The strain CRSS grew aerobically in enrichment medium A yielding 0.5 g dry cells/L and in medium B 0.45

g dry cells/L. Among several carbon sources tested using minimal medium, the isolated CRSS utilised acetate (0.25 g dry cells/L) better than galactose, glucose, ribose, fructose sucrose or trehalose. The maximum yield of EPS for all media was reached after 48 h of incubation. The isolate grew well in complex media A and B and minimal medium plus sodium acetate producing (g EPS/g dry cells) 2.2, 2.9, 1.23, respectively.

Tallon et al. (2003) studied the influence of carbon source to improve EPS production by *L. plantarum* EP56. Five carbon sources (glucose, galactose, fructose, lactose and sucrose) were tested. Lower growth and EPS production rates, which were 0.36 h^{-1} and 46.3 mg/L, respectively, were observed when fructose was used as the carbon source. Although higher growth rates were observed when glucose, galactose and sucrose were used as the carbon source, their EPS production levels (which were 114.5, 121.1 and 129.2 mg/L, respectively) were low. Fructose and lactose were less favorable carbon sources for growth of *L. plantarum* EP56. However, lactose, which is a poor carbon source in regard to the growth, resulted in the best EPS production: 140.2 mg/L EPS.

Cerning et al. (1994) used *Lactobacillus casei* in basal minimum medium (BMM) supplemented with galactose, glucose, sucrose, maltose, or melibiose at different concentrations (2-20 g/L) to determine the effects of various carbon sources and concentrations on the yield of EPS produced. They reported that, increasing the sugar concentrations from 2 to 20 g/L did not affect growth. The lowest growth of cells was obtained with galactose and lactose, while slightly higher growth was observed with glucose, sucrose, and maltose. Even at concentrations of 20 g/L, galactose and lactose gave the lowest EPS yields (20-45 mg/L of EPS, respectively). With melibiose, the EPS yield increased dramatically from 15 to 100 mg/L of EPS when the sugar concentration was increased from 2 to 20 g/L. With glucose, sucrose, and maltose the EPS yield increased when the sugar concentration in the medium increased. The EPS yield obtained with 2 g/L of sucrose was 35 mg/L, and the EPS yield obtained with 20 g/L of sucrose was 50 mg/L. When the maltose concentration was increased from 2 to 20 g/L, the EPS yield increased from 15 to 60 mg/L. With 2 g/L of glucose, the EPS yield was 70 mg/L, which was greater than the yield obtained with any other carbon source at the same concentration. When the medium contained 20 g/L of glucose, the amount of EPS produced was 160 mg/L; thus, glucose was by far the most efficient carbon source in terms of EPS production.

To produce large quantities of an extracellular, highly viscous polysaccharide, Manresa et al. (1987) studied with *Pseudomonas* sp. GSP-910 in a simple salt medium. They tested alternative carbon sources; glucose, sucrose, manose, galactose, fructose, xylose, lactose, maltose. Among the carbon sources provided, glucose, sucrose and xylose allowed maximum EPS production (4.81, 4.43, 3.72 g/L, respectively). High yield production (g EPS/g cell dry weight) could be detected with manose and galactose as well (2, 2.15 g EPS/g cell dry weight, respectively); however, these sugars were not satisfactory as growth substrates.

IV.1.2. Statistical Optimization of the Medium

To achieve high product yields in a fermentation process, it is a prerequisite to design an optimal production medium and a set of optimal process operating conditions (Desai et al., 2006).

As the physico-chemical property of polysaccharide is influenced by culture conditions, it is very important to optimize culture conditions for the production of a specific type of polysaccharides (Lee et al., 2007).

The use of statistical instruments, such as factorial experimental planning associated to the surface-response methodology, has been used by various authors in biotechnology. Experimental planning permits product and process implementation and optimization, reducing development and production costs, because it promotes decrease in the number of experiments at the development stage, so that a considerable number of factors can be studied at the same time. The interaction among these factors can be assessed and optimum production levels obtained and hence greater precision in the results and production optimization. The response-surface methodology is a set of experiment planning and analysis techniques used in the mathematical modeling of responses that can be applied, for example, in pharmacology, food sciences and optimization of fermentative processes (de Oliveira et al., 2007).

In this study, Response Surface Methodology (RSM) was used as a statistical tool in order to optimize the initial sucrose and NaCl contents of the medium. The initial concentrations of sucrose and NaCl were varied, while the levels of other components were kept constant in the Minimal Medium. To determine the range of parameters, preliminary experiments were performed where the *Halomonas* sp AAD6 cells were cultivated in minimal medium containing different concentrations of sucrose (from 10 g/L to 50 g/L). The observed increase in EPS production with

increasing sucrose concentration was in agreement with the similar behavior reported by Prasertsan et al. (2008) for exopolysaccharide production from *Enterobacter cloacae* WD7 cultivated in basal medium. Hence the initial sucrose concentration was varied within the range of 5- 50g/L. Considering the halophilic nature of the microorganism, a NaCl concentration range of 50-200 g/L was chosen for the optimization experiments.

The design contained 12 experiments and two replications of each. The details of the design, values of the actual parameters, and results of the experiments are shown in Table IV.2.

Table IV.2. Maximum Biomass yields by shake flask cultures of *Halomonas* sp AAD6 strain grown in different media. Coded values were used during the calculations. The real values in g/l are given in parenthesis. Intermediate values are calculated assuming linear interpolation.

Sucrose (g/L)	NaCl (g/L)	Biomass (g DCW/L)
0 (27.5)	0 (125)	0.8544
1 (43.41)	-1 (71.96)	0.5139
-1.414 (5)	0 (125)	0.7561
1 (43.41)	1 (178.04)	0.7117
-1 (11.59)	1 (178.04)	0.7463
0 (27.5)	0 (125)	0.8978
-1 (11.59)	-1 (71.96)	0.6794
0 (27.5)	0 (125)	0.8571
0 (27.5)	0 (125)	0.7917
0 (27.5)	-1.414 (50)	0.2805
0 (27.5)	1.414 (200)	0.4798
1.414 (50)	0 (125)	1.3442

A second-order polynomial was constructed with the experimental results using regression analysis. The model equation obtained is given in Equation 1:

$$\text{Ln Biomass} = -0,163 + 0,147 \times \text{NaCl} + 0,06087 \times \text{Suc} - 0,401 \times \text{NaCl}^2 + 0,05792 \times \text{NaCl} \times \text{Suc} + 0,105 \times \text{Suc}^2 \quad (1)$$

where NaCl and Suc refer to the coded values of NaCl and sucrose, respectively.

The coefficient of multiple determination, R^2 was found to be 0.881, which means that the model could explain 88.1% of the total variation in the system. The relatively high value of R^2 indicates that the second-order polynomial equation is capable of representing the system under the given experimental regime. Therefore, the model equation was used for studying the effects of the medium components on the biomass yield. Figure IV.1 depicts the interactions of sucrose with NaCl, and the combined effect on the amount of biomass produced. The model equation was optimized using Nelder-Mead simplex method to maximize the biomass production within the experimental range studied. The optimum medium composition was found to be 50 g/L and 137.2 g/L for sucrose and NaCl, respectively.

Figure IV.1. The interaction of sucrose and NaCl on maximum biomass yields by shake flask cultures of *Halomonas* sp AAD6 strain.

In literature, there are numerous reports on optimization of culture conditions for the production of a specific type of polysaccharides.

Calazans et al. (2007) studied with *Zymomonas mobilis* strain ZAG-12 for optimization of levan production employing a 2^{4-1} fractional factorial design to analyze the influence of the temperature (20, 25, 30°C) agitation (50, 75, 100 rpm), and the initial concentrations of both sucrose (150, 200, 250 g/L) and yeast extract (2.0, 3.5, 5.0 g/L) on final levan concentration. The experiments showed that the final levan concentration depended on initial sucrose concentration, temperature and agitation velocity and that the initial concentration of yeast extract did not influence levan production. The best conditions for levan production occurred at 100 rpm agitation, 20 °C and 250g/L of initial sucrose resulting in 14.67g/L of levan.

Lungmann et al. (2007) studied on medium optimization for production of exopolymer by a newly isolated bacterium, *Halobacterium* sp. SM5, using the mixture design and response surface method. They designed the mixture by setting five parameters (glucose, yeast extract, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, vitamin casamino and KCl). For optimization of this product, the mixture design experiment with the observed responses of the 30 formulations and the original point were determined. The results showed that the exopolymer yields varied within the range of 1.22-2.25 g/L. The exopolymer production was highest in media which contained 7.43 g/L glucose, 12.38 g/L yeast extract, 17.33 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 9.9 g/L vitamin casamino acid and 2.48 g/L KCl.

de Oliveira et al. (2007) used the complete factorial 3^2 design to determine the optimal concentration of sucrose and fermentation time for levan production by *Z. mobilis* ATCC 31821, where the sucrose concentration (x_1) and the fermentation time (x_2) were assessed. The temperature of the fermentative process was fixed at 25°C. The optimum conditions for levan production were 250 g/L sucrose concentration and a fermentation time of 24 h. Under these conditions production reached 21.685 g/L. The results show that an increase in fermentation time caused an increase in the levan production at all concentrations of sucrose tested. However, the sucrose concentration at its upper (350 g/L) and lower (150 g/L) limits reduced levan production. An increase in fermentation time leads to an increase in consumption of the sugar present in the medium and to an increase in the levan production in all the experiments.

Cui et al. (2006) used a three-level Box-Behnken factorial design for optimizing mycelial biomass and exopolymer produced by *Grifola frondosa* GF 9801. The model estimated that, a maximal yield of mycelia biomass (17.61 g/L)

could be obtained when the concentrations of glucose, KH_2PO_4 , peptone were set at 45.2 g/L, 2.97 g/L, 6.58 g/L, respectively; while a maximal exopolymer yield (1.326 g/L) could be achieved when setting concentrations of glucose, KH_2PO_4 , peptone at 58.6 g/L, 4.06 g/L and 3.79 g/L, respectively.

Hsieh et al. (2005) used RSM for optimizing polysaccharide produced by *Cordyceps sinensis*. The maximum polysaccharide of 3.17 g/L was predicted from the regression model of RSM in the medium containing 6.17% sucrose, 0.53% corn steep powder, 0.5% $(\text{NH}_4)_2\text{HPO}_4$ and 0.15% KH_2PO_4 . When applying this optimal medium, the maximum polysaccharide production was 3.05 and 3.21 g/L in a shake flask and a 2-L jar fermentor, respectively.

Xu et al. (2003) used one-factor-at-a time and orthogonal matrix method for optimizing exobiopolymer produced by *Paecilomyces tenuipes* C240. The exopolymer 1.89 g/L was produced in the medium containing 3 g/L glucose, 0.4 g/L KNO_3 , 0.1 g/L K_2HPO_4 , and 0.1 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in shake flask.

Grothe et al. (1999) used central composite experimental design for optimizing poly (β - hydroxybutyric acid) (PHB) produced by *Alcaligenes latus*. The poly PHB 2.97 g/L was produced in the optimized medium containing 20 g/L sucrose and 1.5 g/L ammonium sulfate, which was 1.8 fold greater than the yield in the screening experiment.

From the survey of literature, it can be seen that the application of statistical experimental design techniques in fermentation process development can result in improved product yield, reduced process variability, closer confirmation of the output response (product yield or productivity) in relation to nominal and target requirements, and reduced development time overall costs (Lungmann et al., 2007).

IV.2. EPS PRODUCTION IN BIOREACTOR

Although the production yields, compositions, structures and properties of bacterial EPS are genetically determined, it is possible to influence these factors by modifying culture conditions, such as temperature, dissolved oxygen tension, and growth medium composition (i.e., the concentration of cations and the carbon source used) (Fialho et al., 1999).

In our study, bioreactor cultivations were performed in a 1L BIOSTAT Q multi-fermenter with well-controlled environment of pH and temperature. In each run, the working volume was 500 mL, the fermentation temperature and pH were

kept constant at 37°C and pH7, respectively. Aeration was provided at a rate of 0.1 vvm and agitation was set to 200 rpm. Optimum medium composition was used for the cultivations. Samples were taken at certain time periods to determine the growth and amount of EPS. Biomass concentration was measured in terms of OD_{660nm} and carbohydrate concentration of the samples was determined using phenol/sulfuric acid method using glucose as standard. Optical densities for carbohydrate concentration were measured in terms of OD_{490nm}. The net carbohydrate contents were calculated by subtracting the carbohydrate values of growth media without inoculum. In order to convert OD_{660nm} and OD_{490nm} absorbencies to grams per liter, calibration charts were prepared (Appendix A, Appendix B, respectively). Figure IV.2 shows the time course of the growth and EPS production of *Halomonas* sp AAD6 cells. Results are averages of duplicate runs for four different experiments.

Figure IV.2 Time Course of Growth and EPS Production of *Halomonas* sp AAD6

As can be seen in Fig. IV.2, biomass and EPS yields reached by the bioreactor culture after 95 hours of fermentation period were 1.1407 g/L and 4.5024 g/L, respectively.

From the survey of literature, it can be seen that bioreactor cultivations with different biomass and EPS yields were studied by several researchers who used different process conditions.

Chi et al. (2007) studied exopolysaccharide production by marine *Cyanothece* sp. 113 in 5-L fermentor under the optimal conditions; 29°C, pH 7.2 and at an aeration rate of 7.0l /min but with no agitation. Under these conditions, 22.34 g/L of exopolysaccharide was released into the medium in 11 days when cell growth reached the late stationary phase. This was the highest exopolysaccharide yield by cyanobacteria reported so far. After purification, the spectra of UV, IR, ¹H NMR, ¹³C NMR and GC–MS analysis showed that the purified exopolysaccharide was α-D-1,6 homoglucan.

Shene and Bravo (2007) studied on the continuous culture fermentations of the strain of *Lactobacillus delbrueckii* subsp. *bulgaricus* in the whey-based media at dilution rates between 0.06 and 0.8 h⁻¹ in a laboratory fermentor (Biostat M, B. Braun, Melsungen, Germany). Temperature and pH were kept at 37°C and 6, respectively. Stirring was set to 150 rpm. They used lactose from deproteinized whey as the carbon source. The highest biomass concentration (3.03 g/L) was obtained at a dilution rate of 0.21 h⁻¹. EPS were isolated and quantified from culture samples with (EPS-1) and without (EPS-2) the biomass. Highest EPS-1 (0.83 g/L) and EPS-2 (0.39 g/L) concentrations were obtained at dilution rates of 0.36 and 0.29 h⁻¹, respectively.

In order to determine the phase that EPS production is maximum, bioreactor cultivations were performed with well-controlled environment. In these experiments, EPS was purified after cells were harvested by centrifugation in certain time periods. EPS production yields obtained in exponential and stationary phases are summarized in Table IV.3.

As shown in Table IV.3, the data obtained from our experiments demonstrated that the maximum EPS yield reached by the bioreactor culture was 1.844 g/L after 41 hours of fermentation period.

Table IV.3. EPS yields in different phases in bioreactor cultivations

	17 h	21.5 h	41 h	47 h
Biomass (gDCW/L)	0.395	0.492	0.911	1.069
EPS (g/L)	0.368	0.502	1.844	1.718
EPS yield (g EPS/g biomass)	0.931	1.021	2.024	1.608

Halomonas sp AAD6 cells entered the logarithmic growth phase after 8 hours of lag time. The maximum specific growth rate ($\mu_{\max}$) of the culture was determined from the slope of the $\ln x$ versus time plots in the exponential phase using linear regression as $0.3162 \pm 0.005 \text{ h}^{-1}$. The related graph is given in the Appendix C.

IV.3. CHARACTERIZATION of EPS

Protein Concentration Protein concentration was determined by the Bradford test using Bovine Serum Albumin (BSA) as a standard (Bradford, 1976). Absorbance was measured at 595 nm. A standard curve of absorbance versus protein concentration was prepared by using known amounts of Bovine Serum Albumin (BSA). This calibration curve is given in Appendix D.

Analytical determinations carried out in this study showed that EPS produced by *Halomonas* sp AAD6 in a 1L BIOSTAT Q multi-fermenter with well-controlled environment of pH and temperature has low content of proteins (0.5125 %). Result is averages of duplicate runs for four different experiments.

Quantity and Quality of DNA Spectrophotometric measurement was used to determine the quantity and quality of DNA. DNA was diluted with distilled water and the UV absorption of DNA was read at 260nm using quartz cuvettes.

The data obtained from our experiments demonstrated that nucleic acid content of the EPS was 1.8 %. Result is averages of duplicate runs for four different experiments.

Uronic Acid Concentration The concentration of uronic acid was determined as described in Blumenkrantz (1973) by using the comparison against a calibration curve prepared by similarly assaying calibration standards of a selected uronic acid (e.g., glucuronic acid, galacturonic acid, mannuronic acid, etc) (K. Mojica et al., 2007).

In order to convert $OD_{520\text{nm}}$ absorbencies to percentage values, the following data points were considered:

- Glucuronic acids 6 micrograms read 0.6 (at $OD_{520\text{nm}}$).
- Galacturonic acids 10 micrograms read 0.8 (at $OD_{520\text{nm}}$).

As a result, uronic acid concentration of EPS from *Halomonas* sp AAD6 was found as 4.4 %. Result is averages of duplicate runs for four different experiments.

Rheological Studies The specific viscosity (η) of aqueous solution of exopolysaccharide at different concentration values was determined using Cannon-Ubbelohde 75 suspended level viscometer at 25 °C. As concentration increases coils start to overlap and become entangled, with viscosity showing a more marked dependence on concentration reaching $\eta=0.710$ at 2% (w/v) of concentration.

Martínez-Checa *et al.* (2007) studied characteristics of exopolysaccharide V2-7 produced by strain F2-7 of *Halomonas eurihalina* in MY medium supplemented with various hydrocarbons. The viscosity of solutions was measured with a Bohlin Rheometer CSR-10 at 25 °C. Rheological behavior was studied at neutral pH and at pH 3. Solutions were acidified with 1M HCl. With respect to the rheological behavior, all the EPS synthesized on hydrocarbon media originated solutions with very low viscosity and they were unable to form high viscosity solutions at pH 3, as it happens when *H. eurihalina* strain F2-7 produces the EPS with glucose as carbon source (Calvo *et al.*, 1995; Quesada *et al.*, 1993). As a result, only the EPS from glucose MY medium exhibit pseudoplastic behavior indicated when the viscosity decreases as shear rate is increased. All biopolymers synthesized in presence of the hydrocarbons originated non-viscous solutions. This is a profitable characteristic required in several industrial fields, as the cleaning of oil tankers or the recovery of solid wastes.

Dlamini *et al.* (2007) characterized physical, chemical and rheological properties of a polysaccharide produced by an isolate of *Klebsiella oxytoca* in whey based media. Viscosity was measured using a Wells-Brookfield cone and plate viscometer at 25°C. The rheological properties of the *K. Oxytoca* polysaccharide (KOP) were compared with those of gellan and xanthan gums. The results indicated that at lower gum concentrations, 0.1 to 0.5% (w/v), gellan had comparatively lower apparent viscosities than either KOP or xanthan. However, at higher concentrations (1.2%, w/v), gellan gave very high apparent viscosities. It reached over 2000 cP at 6 s⁻¹ compared to 1400 and 1100 cP reached by KOP and xanthan, respectively, at the same shear rate. The rheological behavior of the KOP resembled that of xanthan more than gellan gum. The KOP formed non newtonian fluids. The viscosity increased as the shear rates were decreased. This indicated that KOP is a pseudoplastic biopolymer and has a shear thinning effect.

Mata *et al.* (2006) studied exopolysaccharides produced by the strains of *Halomonas ventosae* (A112^T and A116) and *Halomonas anticariensis* (FP35^T and

FP36), under optimum environmental and nutritional conditions. They made rheological measurements at 25°C in a controlled-stress Bohlin CSR10 rheometer. All EPSs produced solutions of low viscosity and pseudoplastic behaviour. For comparison's sake the viscosities of the four polymers at a shear rate of 59.5 s⁻¹ are 3.13×10⁻³, 2.79×10⁻³, 3.92×10⁻³ and 2.85×10⁻³, respectively.

IV.4. BIOCOMPATIBILITY STUDIES

IV.4.1. Inhibition of Toxic Activity

The inhibitory effect of EPS on the toxic activity of the sesquiterpene hydroquinone avarol (LD₅₀ 0.18 ppm) on brine shrimp (*Artemia salina*) was tested. With decreasing doses of EPS solution (500 ppm, 50 ppm, 5 ppm, respectively), survivor shrimps increased (2, 13, 16, respectively). Activity for EPS isolated from *Halomonas* sp AAD6 strain was found that increased the value of LD₅₀ of Avarol from 0.18 ppm up to 10 ppm.

IV.4.2. Cell Culture Results

To assess the biocompatibility of EPS, basic *in vitro* tests were performed with osteoblast cells isolated from the calvaria of Wistar rats. Osteoblast cell cultures were stimulated with EPS and after 48 h incubation, osteoblasts morphology, viability and secretion capability (alkaline phosphatase) were tested. No evident change in cell morphology was detected when stimulated cells were analysed by inverted optical microscopy. As shown in Figure IV.3, comparing to control, there was no significant difference in viability and alkaline phosphatase production when the osteoblasts were incubated in the presence of EPS.

Figure IV.3. Biocompatibility results of EPS

The polysaccharide, that we produced, did not affect cellular viability and proliferation on osteoblasts, supporting the high biocompatibility of this EPS. Moreover in the Brine shrimp test the polymer, at low concentration (5 ppm), showed a protective effect against the toxic activity of Avarol, suggesting a potential anti-cytotoxic role of this polysaccharide. In the light of the results of this study, it can be concluded that the EPS produced by the *Halomonas* sp. AAD6 strain can be considered quite promising for industrial exploitation and is worthy of further investigation to determine the most suitable fields of application.

IV.5. CHEMICAL CHARACTERIZATION RESULTS

Sugar Analysis Most EPS are composed of the neutral sugars glucose, galactose and/or rhamnose. Some of them also consist of (acetylated) amino sugars. The strain, the culture conditions, and the type of carbon source influence the amount and the composition of microbial EPS that is produced by a certain species. Read and Costerton (1987) found that with glucose as a carbon source, *P. putida* and *Pseudomonas fluorescens* synthesized an EPS composed of glucose, galactose and pyruvate. Hung *et al.* (2005) found that EPS produced by *P. fluorescens* Biovar II was composed of galactose, mannose, rhamnose, glucose, fucose, ribose, arabinose and xylose. Kachlany *et al.* (2001) revealed that the purified EPS from *P. putida* G7 contained the monosaccharides, glucose, rhamnose, ribose, N-acetylgalactosamine and glucuronic acid. Similarly, some researches showed that EPS were isolated from

Pseudomonas caryophylli CFR 1705 grown on lactose containing medium was composed of rhamnose, mannose and glucose in the ratio 1:3.26:4.97, respectively. Also, EPS production and its monomeric sugar composition were dependent on the carbon source, nitrogen source and carbon/nitrogen ratio of the growth medium (Çelik et al., 2008).

In our study, sugar analysis was performed by hydrolysis of EPS with 0.5 M trifluoroacetic acid (TFA) at 120 °C for 1.5 h and was identified by HPAE-PAD using standards for identification and calibration curves. Hydrolysis of EPS showed a peak attributable to fructose presence (RT 7.8 min).

Poli *et al.* (2004) studied with a haloalkalophilic *Halomonas* strain CRSS, isolated from salt sediments in Antarctica. The growth was tested in two complex media (Medium A and B) and in the minimal medium C with 1% (w/v) sodium acetate, D-glucose, D-galactose, lactic acid, D-maltose, D-fructose, D-cellobiose, D-ribose, D-trehalose, glycerol or sucrose. The maximum yield of EPS for all media was reached after 48 h of incubation. The isolate grew well in the media A, B, and C plus sodium acetate producing (g EPS/g dry cells) 2.2, 2.9, 1.23, respectively. The sugar composition of EPS was performed by TLC and HPAE-PAD using standards for identification and calibration curves. It was yielded as principal constituents: mannose, glucose, galactose, glucosamine and galactosamine in relative proportion of 1:0.1:trace:trace:trace in the EPS from medium A; mannose, xylose, glucose, galactosamine, fructose, rhamnose and unknown component (UK 3) in a relative proportion of 1:0.7:0.3:0.2:trace:trace:0.3 in the EPS from medium B; glucose, fructose, glucosamine and galactosamine in relative proportion of 1:0.7:0.3:trace in the EPS from medium C plus sodium acetate. Two different polymers, a mannan and a xylo-mannan, were obtained from the culture supernatants using complex media A and B, respectively, while strain CRSS produced a new polysaccharide, a fructoglucan, when it was grown on acetate medium.

Thermogravimetric analysis of EPS in this study indicated a degradation temperature (Td) of 253 °C (Fig. IV.4.a). Moreover, the infrared spectrum of this biopolymer showed the characteristic peak signals of polysaccharides: broad stretching peak at around 3300 cm^{-1} , (OH stretching), a weak C-H band at around 2900 cm^{-1} , C=O stretching at 1660 cm^{-1} and several sharp peaks around 1000 cm^{-1} typical of carbohydrates (Fig. IV.4.b).

Figure IV.4. TGA thermogram (a) and Fourier transform-infrared spectroscopy spectrum (b) of the EPS from *Halomonas* sp. AAD6.

Molecular Size Analysis The molecular size of EPS, estimated using a calibration curve of standard dextrans obtained by gel filtration on Sepharose CL-6B and also by density gradient centrifugation, was greater than 1,000,000 Da.

UV Spectra Results The UV spectra of EPS, obtained by reading the absorbance of aqueous solutions from 350 to 210 nm on a Varian DMS-90 instrument, did not indicate any strong absorption peaks in that range.

PART V

CONCLUDING REMARKS AND RECOMMENDATIONS

In the light of this study, several recommendations can be drawn for future work related to the EPS production by halophilic *Halomonas* sp AAD6.

Although it is possible to influence the production yields, compositions, structures and properties of bacterial EPS by modifying culture conditions, these factors can be genetically determined. It is possible that the levels of production of exopolysaccharides by *Halomonas* sp. can be improved by metabolic modeling of exopolysaccharide production. On the other hand, composition of the EPS can be modified by metabolic engineering.

There is no published research on the use of bioflocculants of halophilic origin in high-salinity biotreatment processes. Therefore, it is of utmost importance to explore the potential of the EPS isolated from the halophilic *Halomonas* sp AAD6 as a bioflocculant agent under different conditions. Flocculating capability of this EPS should be studied. On the other hand, halophilic EPS producers are considered as an interesting source for microbial enhanced oil recovery (MEOR) where polymers act as emulsifiers and mobility controllers. It's important to study the emulsifying capacity of this EPS for future work.

The potential of this EPS in various pharmaceutical applications need to be investigated. The real potential of this biopolymer in pharmaceutical industry should be clarified.

The use of alternative regional low-cost substrates has become very interesting because in addition to the ease of acquisition it presents a relatively low cost. With the ultimate goal of developing a cost-effective and environmentally friendly biopolymer production process, the potential use of different waste streams as fermentation substrates should be evaluated for the production of EPS from *Halomonas* sp AAD6. In order to make the production process of this novel EPS cost-competitive with petrochemical-derived polymers, studies should be conducted on the potential use of cheaper fermentation substrates.

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APPENDIX A

BIOMASS CALIBRATION CHART

Figure A.1 Dry Cell Weight Calibration Chart

APPENDIX B

CARBOHYDRATE CALIBRATION CHART

Figure B.1 Carbohydrate Calibration Chart

APPENDIX C

MAXIMUM SPECIFIC GROWTH RATE (μ_{MAX}) CHART

Figure C.1 Maximum Specific Growth Rate (μ_{max}) Chart

APPENDIX D

ABSORBANCE OD₅₉₅ VERSUS PROTEIN CONCENTRATION CALIBRATION CHART

Figure D.1 Protein Calibration Chart

AUTOBIOGRAPHY

I was born on 27 July 1983 in Izmir. I graduated from Atakent Anatolian High School and I started on my undergraduate education in Ege University, Faculty of Engineering, Bioengineering Department in 2001. I graduated from Ege University Bioengineering Programme in 2006. I started master of science at Marmara University Bioengineering Department in 2006.

Presented Announcements

- Hande Kazak, Bahar Gürleyendag, Annarita Poli, Ebru Toksoy Öner, Barbara Nicolaus, “Exopolysaccharide production by *Halomonas* strains isolated from Turkey”, **13th European Congress on Biotechnology (ECB 13)**, September 16-19, 2007, Barcelona, Spain, *Poster Presentation*
- Hande Kazak, Bahar Gürleyendag, Annarita Poli, Ebru Toksoy Öner, Barbara Nicolaus, “Ekstremofilik Mikroorganizmalardan Ekzopolisakkarit Üretimi”, **15th National Biotechnology Congress**, October 28-31, 2007, Antalya, Turkey, *Oral Presentation*
- Ates, Ö., Kazak, H., Arga, K., Y., Toksoy Öner, E., “EPS Production from Proteomics Perspective”, **ICGEB International Center for Genetic Engineering and Biotechnology; Proteomic Approaches in Molecular Biology: Theory and Practice Course**, March 03-14, 2008, Buenos Aires, Argentina, *Poster Presentation*
- Ateş, Ö., Kazak, H., Arga, K. Y., Toksoy Öner, E., “Metabolic Reconstruction and Modeling of Microbial Biopolymer Production”, **9th International Conference on Systems Biology (ICSB2008)**, August 22-28, 2008, Gothenburg, Sweden, *Poster Presentation*

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ACCEPTANCE AND APPROVAL DOCUMENT

The jury established by the Executive Board of the *INSTITUTE FOR GRADUATE STUDIES IN PURE AND APPLIED SCIENCES* on 17.01.2009 (Resolution no: 2009/02-26) has accepted Ms Hande KAZAK's thesis titled "EXOPOLYSACCHARIDE PRODUCTION BY HALOMONAS STRAINS ISOLATED FROM TURKEY" as Master of Science in Bioengineering.

Advisor : Assoc. Prof Ebru TOKSOY ÖNER

1. Member of the jury: Prof. Jülide AKBUĞA

2. Member of the jury: Assist. Prof. Kazım Yalçın ARĞA

Date : 19.01.2009

APPROVAL

Ms. Hande KAZAK has satisfactorily completed the requirements for the degree of Master of Science in Bioengineering at Marmara University. The Executive Committee approves that she be granted the degree of Master of Science on 29.02.2009 (Resolution no: 2009/04-3)

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