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Joana Raquel Vicente Marques

Licenciada em Bioquímica

Exopolysaccharide production by different marine bacteria species and *Enterobacter* A47

Dissertação para obtenção do Grau de Mestre em Biotecnologia

Orientador: Doutora Cristiana Andreia Vieira Torres, Post-Doctoral Researcher, FCT-UNL

Co-orientador: Doutora Maria Filomena Andrade de Freitas, Senior Researcher, FCT-UNL

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Agradecimentos

Em primeiro lugar, gostaria de agradecer à minha orientadora, Dra. Cristiana Torres, por ter partilhado a sua experiência e pela sua disponibilidade, orientação e conselhos, sem os quais eu nunca seria capaz de realizar este trabalho.

Gostaria também de gratificar a minha co-orientadora, Dra. Filomena Freitas, pelo apoio, ensinamentos e conselhos, assim como pelo interesse em seguir este trabalho.

Não posso deixar de agradecer à Professora Maria Ascensão Reis, à Dra. Cristiana Torres e à Dra. Filomena Freitas por me disponibilizarem as condições necessárias para realizar este trabalho e, acima de tudo, por me darem a oportunidade de trabalhar com uma excelente equipa. Estou muito grata pelo acolhimento amigável que recebi por todos os membros do grupo BioEng mas, especialmente, por parte dos meus colegas de laboratório. Gostaria de agradecer à Diana Araújo, Sílvia Baptista, Inês Farinha, Sílvia Antunes, Patrícia Reis e João Pereira por me integrarem, pelos úteis conselhos e ensinamentos, e pelos momentos de diversão que partilhámos.

Por último, gostaria de agradecer aos meus colegas de mestrado, Rita Bernardino, Patrícia Freitas, Sónia Nóbrega, Letícia Fialho, Liane Meneses e David Cruz, por me ajudarem diariamente e pelo excelente ambiente de trabalho.

Palavras-chave
Exopolissacáridos, meio Schatz, bactérias marinhas, *Enterobacter* A47, metodologia de superfície de resposta, impacto do pH.

Resumo

O trabalho realizado nesta tese centra-se na produção de exopolissacáridos (EPS) bacterianos utilizando meio Schatz, suplementado com extrato de levedura e peptona, e glucose como fonte de carbono.

Foram realizados ensaios de produção em frascos de agitação com diferentes bactérias marinhas, dos géneros *Pseudoalteromonas* e *Psychrobacter*, e com a bactéria *Enterobacter* A47. De modo a otimizar a produção de EPS, as estirpes foram escolhidas para prosseguir com ensaios em bioreactor. As bactérias marinhas foram capazes de produzir 1.33-1.91 $g.L^{-1}$ de EPS, correspondendo a produtividades volumétricas de 0.67-0.96 $g_{EPS}.L^{-1}.d^{-1}$. Por outro lado, a *Enterobacter* A47 foi capaz de sintetizar 1.81 $g.L^{-1}$ de EPS, correspondendo a uma produtividade volumétrica de 0.91 $g_{EPS}.L^{-1}.d^{-1}$. Os exopolissacáridos produzidos pelas bactérias marinhas eram compostos principalmente por glucose, enquanto que o produzido pela *Enterobacter* A47 era semelhante ao exopolissacárido rico em fucose – FucoPol – tipicamente produzido por esta cultura.

De modo a otimizar a produção de EPS por *Enterobacter* A47 em condições salinas, foi estudado o impacto das concentrações de NaCl (0-40 $g.L^{-1}$), extrato de levedura (0-4 $g.L^{-1}$) e peptona (0-2 $g.L^{-1}$) no crescimento celular e produção de EPS. Para tal foram utilizadas ferramentas estatísticas, tais como a metodologia de superfície de resposta e o desenho compósito central. Os resultados mostraram que a *Enterobacter* A47 tem a capacidade de crescer e sintetizar EPS na maioria das condições experimentais testadas. As concentrações ideais de NaCl, extrato de levedura e peptona foram determinadas como sendo 8.1 $g.L^{-1}$, 0.8 $g.L^{-1}$ e 0.4 $g.L^{-1}$, respetivamente.

Posteriormente, foi avaliado o impacto do pH na produção de EPS, assim como na composição do polímero, por *Enterobacter* A47. Verificou-se que o uso do meio Schatz modificado e o controlo do pH a 7.0 levou a um aumento na produção de EPS (3.49 $g.L^{-1}$), correspondendo a uma produtividade volumétrica de 1.75 $g_{EPS}.L^{-1}.d^{-1}$. Observou-se também que a diminuição de pH 7.0 para pH 6.0 teve pouco efeito sobre a produção de EPS (3.33 $g.L^{-1}$), enquanto que a pH 8.0 foi observada uma redução significativa na síntese de EPS (0.79 $g.L^{-1}$). Os exopolissacáridos produzidos eram ricos em fucose.

Keywords

Exopolysaccharides, Schatz medium, marine bacteria, *Enterobacter* A47, response surface methodology, impact of pH.

Abstract

This thesis is focused on the production of bacterial exopolysaccharides (EPS) using a Schatz medium, supplemented with yeast extract and peptone, and glucose as carbon source.

Production assays were carried out in shake flasks with different marine bacteria, of the genera *Pseudoalteromonas* and *Psychrobacter*, and with the bacterium *Enterobacter* A47. In order to optimize EPS production, the strains were chosen to proceed with bioreactor assays. The marine bacteria were able to produce 1.33-1.91 $g.L^{-1}$ of EPS, corresponding to volumetric productivities of 0.67-0.96 $g_{EPS}.L^{-1}.d^{-1}$. On the other hand, *Enterobacter* A47 was able to synthesize 1.81 $g.L^{-1}$ of EPS, corresponding to a volumetric productivity of 0.91 $g_{EPS}.L^{-1}.d^{-1}$. The exopolysaccharides produced by marine bacteria were mainly composed of glucose, while the one produced by *Enterobacter* A47 was similar to the fucose rich exopolysaccharide – FucoPol – typically produced by this culture.

In order to optimize EPS production by *Enterobacter* A47 in salt conditions, the impact of NaCl (0-40 $g.L^{-1}$), yeast extract (0-4 $g.L^{-1}$) and peptone (0-2 $g.L^{-1}$) concentrations on cellular growth and EPS production was studied. Statistical tools, such as response surface methodology and central composite rotatable design, were used. The results showed that *Enterobacter* A47 has the ability to grow and synthesize EPS under most of the experimental conditions tested. The optimal NaCl, yeast extract and peptone concentrations were determined to be 8.1 $g.L^{-1}$, 0.8 $g.L^{-1}$ and 0.4 $g.L^{-1}$, respectively.

Afterwards, the impact of pH on EPS production, as well as on the polymer´s composition, by *Enterobacter* A47 was also evaluated. It was found that the use of the modified Schatz medium and pH control at 7.0 led to an increase in EPS production (3.49 $g.L^{-1}$), corresponding to a volumetric productivity of 1.75 $g_{EPS}.L^{-1}.d^{-1}$. It was also observed that the decrease from pH 7.0 to pH 6.0 had little effect on EPS production (3.33 $g.L^{-1}$), whereas at pH 8.0 a significant reduction in EPS synthesis (0.79 $g.L^{-1}$) was observed. The exopolysaccharides produced were rich in fucose.

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Nomenclature

Abbreviations

ANOVA	Analysis of Variance
CCRD	Central Composite Rotatable Design
CDW	Cell Dry Weight
CV	Coefficient of Variation
DO	Dissolved Oxygen
EPS	Exopolysaccharide
HPLC	High Performance Liquid Chromatography
IR	Infrared
MLR	Multiple Linear Regression
N.A.	Data not available
NDP	Nucleotide Diphosphate
OD	Optical Density
RSM	Response Surface Methodology
SLPM	Standard Liters Per Minute
TFA	Trifluoroacetic Acid
UDP	Uridine Diphosphate

Variables

b_0	Coefficient estimate for interception
b_i	Coefficient estimate for linear correlation
b_{ij}	Coefficient estimate for quadratic correlation
C:N ratio	Carbon to nitrogen ratio (w/w)
r_{p}	Volumetric productivity ($g.L^{-1}.d^{-1}$)
X_i	Experimental variables in RSM
Y_i	Observed responses in RSM

Greek letters

α	Axial level in RSM

1. Introduction and Motivation

1.1. Exopolysaccharides

Exopolysaccharides (EPS) are high molecular weight (10^4-10^7 Da) carbohydrate polymers composed of monosaccharides linked by glycosidic bonds that make up a substantial component of the extracellular polymers surrounding most microbial cells (Nichols et al., 2005; Sutherland, 2001).

Bacterial EPS are secreted by the cells, and may form a capsule that remains associated with the cell surface or a slime that is loosely bound to the cell surface (Kumar et al., 2007). The composition of these polymers includes neutral sugars, such as glucose, galactose and mannose, which are the most common monomers (Table 1.1). Other neutral sugars (e.g. rhamnose and fucose), some uronic acids (mainly glucuronic and galacturonic acids) and aminosugars (N-acetylaminosugars) are also frequently present (Table 1.1). Exopolysaccharides may be composed of a unique sort of monosaccharide (homopolysaccharides) or comprise two or more different monosaccharides (heteropolysaccharides) (Kumar et al., 2007).

Some bacterial EPS have irregular structures (e.g. bacterial alginates), despite being composed of repeating units with varying sizes and degrees of ramification. Given the huge diversity of sugar components, the range of possible molecular structures is very large (Freitas et al., 2011). Moreover, the different glycosyl linkages and configurations increase this variability (Kumar et al., 2007). Bacterial EPS can not only contain carbohydrates, but might also contain several organic ester-linked substituents and pyruvate ketals that can confer the EPS an anionic character, increase its lipophilicity, and affect its capacity to interact with other polysaccharides and cations. Chemical composition, molecular structure, average molecular weight and distribution determine the properties of bacterial EPS (Freitas et al., 2011).

Exopolysaccharides can be derived from many natural sources. They may be plant based (e.g. guar gum, starch and pectins), marine originated (e.g. carrageenan and alginate), animal originated (e.g. chitin and chitosan) or of microbial origin (e.g. xanthan, gellan and pullulan) (Kaur et al., 2012). Polysaccharides extracted from plants or algae can be directly replaced in traditional applications by some bacterial EPS, because of their improved physical properties (Freitas et al., 2011). Bacteria usually have higher specific growth rates and allow manipulation of the growth conditions for improving fermentation yields, productivity and biopolymers properties (Freitas et al., 2009). Due to this fact, several different EPS have been extensively studied over the last decades, such as xanthan, gellan and alginate.

Nowadays, xanthan, produced by *Xanthomonas campestris,* is the most widely accepted microbial polysaccharide. It is used in many manufactured foods, as well as in cosmetics and

personal care products (Freitas et al., 2011; Garcia-Ochoa et al., 2000). Gellan, synthesized by *Sphingomonas paucimobilis,* is mainly used in food applications due to its great gelling capacity that allows it to be used at a much lower concentration than agar (Bajaj et al., 2007; Freitas et al., 2011). The use of alginate, produced by *Pseudomonas aeruginosa* and *Azotobacter vinelandii*, has increased in recent years, since it can not only be used in the food and paper industries, but also in the pharmaceutical and medical industries, due to its biocompatibility (Hay et al., 2013).

Table 1.1 – Sugar and nonsugar components of bacterial exopolysaccharides (Nichols et al., 2005).

Type	Component	Example	Mode of linkage
Sugar	Pentoses	D-Arabinose D-Ribose D-Xylose	
	Hexoses	D-Glucose D-Mannose D-Galactose D-Allose L-Rhamnose (6-Deoxy-L-mannose) L-Fucose (6-Deoxy-L-galactose)	
	Amino sugars	D-Glucosamine (2-Amino-2-deoxy-D-glucose) D-Galactosamine (2-Amino-2-deoxy-D-galactose)	
	Uronic acids	D-Glucuronic acid D-Galacturonic acid	
Nonsugar	Acetic acid		O-Acyl, N-Acyl
	Succinic acid		O-Acyl
	Pyruvic acid		Acetal
	Phosphoric acid		Ester, diester
	Sulfuric acid		Ester

1.2. Microbial growth and EPS production

Maximum EPS production can be found in two different phases of microbial growth. Some studies found maximum production of EPS during the exponential phase (Bozal et al., 1994), although most bacteria produce the largest quantity of EPS during the stationary phase under nitrogen limitation, suggesting that EPS synthesis for those strains was also induced by restricted growth conditions (Samain et al., 1997).

Despite the factors leading to EPS synthesis are still not clearly elucidated, it is thought to be a response to environmental stress conditions (e.g. temperature, pH, light intensity, salinity) (Donot et al., 2012; Kumar et al., 2007). Although culture conditions generally do not affect the

type of monosaccharides in the EPS, they do affect the functional properties of the polysaccharides, such as molecular weight, conformation and monosaccharide ratios (Arias et al., 2003). Cultivation conditions for optimum cell growth will not necessarily be the same for EPS production (Kumar et al., 2007).

During most EPS production processes, changes on the rheology of fermentation broth occur. Cultivation broths containing unicellular microorganisms of simple shape should behave as Newtonian fluids. However, in a number of industrially important cultivation processes the broth develops shear thinning behaviour.

For a Newtonian fluid, there is a direct relationship between the shear stress and its shear rate. A non-Newtonian fluid is a fluid whose flow properties are not proportional to the shear rate, being non-linear and even time-dependent (Verbeeten, 2010). Shear thinning fluids are the largest and probably most important class of non-Newtonian fluids (Cross, 1965).

The viscosity of fermentation broths may be influenced by the cultivation medium, the size of both cells and cell aggregates formed, biomass concentration, morphological parameters and the products being secreted into the solution (Alves et al., 2010). It also depends on the dissolved oxygen concentration (Al-Asheh et al., 2002). However, the change in flow behaviour is generally attributed to the increasing polymer concentration being produced, with a negligible contribution from the cells (Landon et al., 1993).

1.3. Bacterial biosynthetic pathways

Bacterial EPS are synthesized by either Gram-positive or Gram-negative bacteria, by two very distinct mechanisms. EPS originated from Gram-positive bacteria are synthesized in the exterior of the cell by enzymes that were secreted by the bacteria or that are anchored to the cell surface (e.g. levan, dextrans and alternans). On the other hand, EPS originated from Gram-negative bacteria are synthesized intracellularly and then secreted to the extracellular environment (e.g. xanthan, gellan and cellulose) (Sutherland, 2001; Vanhooren et al., 1998).

The intracellular biosynthesis is regulated by enzymes that are located in various regions of the cell. In the cytoplasm, glucose-1-phosphate (G-1P) is converted to the key molecule in exopolysaccharide synthesis, uridine diphosphate glucose (UDP-Glucose) in a reaction catalyzed by uridine diphosphate-glucose pyrophosphorylase (Fig. 1.1). At this stage, intracellular enzymes catalyze the interconversion of UDP-Glucose into other sugars, such as UDP-Glucuronic acid, creating diverse sugar monosaccharides. Subsequently, in the cell periplasmic membrane, glycosyltransferases transfer the nucleotide diphosphate sugars (NDPs) to form the repeating unit attached to a glycosyl transporter lipid, an isoprenoid alcohol. Afterwards, the macromolecules

are polymerized and excreted to the exterior of the cell by one of two possible mechanisms, Wzx/Wzy- or ABC transporter dependent pathways (Kumar et al., 2007; Schmid et al., 2015).

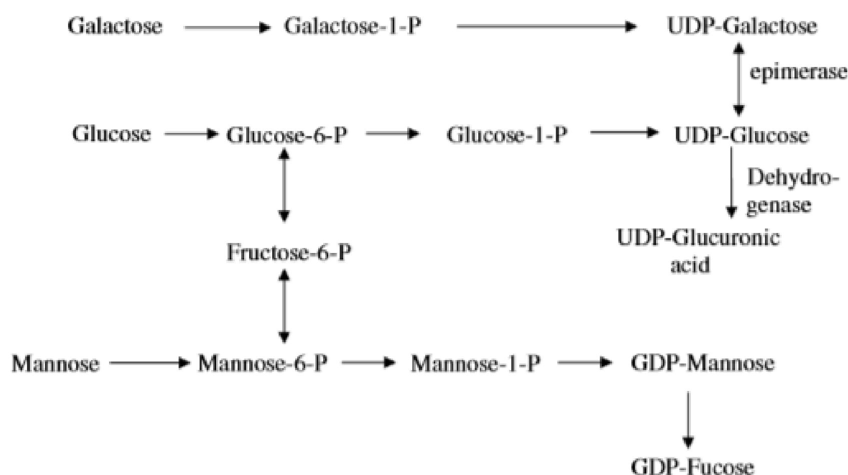

Figure 1.1 – Metabolic mechanism representing the sugar nucleoside synthesis that are involved in the biosynthesis of exopolysaccharides in Gram-negative bacteria (Kumar et al., 2007).

1.4. Marine bacteria and EPS production

1.4.1. Marine environment as potential source of research

More than 70% of the surface of the planet is covered by sea water, which contains an exceptional biological diversity, representing more than 95% of the whole biosphere (Dufourcq et al., 2014; Spízek et al., 2010). The deep sea is, therefore, the largest ecosystem of our planet: about 50% of the surface of the planet is below 3000 m depth. However, it is one of the less studied. The marine environment includes a variety of habitats, from the lower edge of the continental shelf to the deeper areas of the trenches, each of which has defined geochemical and physical characteristics that support some of the greatest biodiversity on the planet (Dionisi et al., 2012).

Marine bacteria are commonly found in intertidal zones, deep seas and extreme places, such as hydrothermal sources or polar seas. In a world where competition is very selective, this presence demonstrates their excellent ability to adapt to the environment, through the development of sophisticated metabolic pathways, which help them to survive (Dufourcq et al., 2014; Harvey, 2008). Despite the incredible capacity of marine bacteria to

develop different adaptation mechanisms, including the synthesis of EPS, not many in-depth studies have been conducted yet. Thus, the oceans are an open and very promising research field for EPS discovery (Dufourcq et al., 2014).

Recently, marine bacteria of the genera *Pseudoalteromonas*, *Alteromonas* and *Vibrio* (Nichols et al., 2005) have started to be studied as potential sources of EPS, which can be used in high-value applications, such as food, medical, pharmaceutical and cosmetics industry (Freitas et al., 2011; Poli et al., 2010). Ecologically, these polymers serve important functions in marine environments, where they may be involved in microbial adhesion to solid surfaces and biofilm formation, or mediating the fate and mobility of heavy metals and trace metal nutrients (Holmström et al., 1999; Poli et al., 2010).

So far, there are very few commercially available EPS produced by marine bacteria. One such example is HE800 EPS, an exopolysaccharide composed by equal amounts of glucuronic acid and hexosamine (N-acetylglucosamine and N-acetylgalactosamine), produced by the deep sea bacterium *Vibrio diabolicus.* It is a hyaluronic acid-like polymer and its commercial name is Hyalurift. The efficiency of this exopolysaccharide was evaluated on the restoration of bone integrity for critical size defects (Senni et al., 2011).

1.4.2. Genera *Pseudoalteromonas* and *Psychrobacter*

Members of the genus *Pseudoalteromonas* are generally found in eukaryotic hosts (Holmström et al., 1999), associated with marine animals (i.e. tunicate and mussels) (Ivanova et al., 1998) and marine algae (Egan et al., 2001). This genus was first suggested after division of the genus *Alteromonas* into *Alteromonas* and *Pseudoalteromonas*, by Gauthier et al. in 1995, based on comparative analysis of 16s rRNA sequences. Members of this genus display the following characteristics: are Gram-negative straight rods (2-3µm), isolated from sea water, sediments, sea ice, surfaces of stones, marine algae or marine invertebrates. All of them are aerobic, utilize carbon substrates as carbohydrates, alcohols, organic acids or amino acids, also are non-spore forming and non-bioluminescent. They have a single polar flagellum, sheathed or unsheathed, for mobility and require Na^+ for growth (Holmström et al., 1999).

Bacteria of the genus *Pseudoalteromonas*, which currently comprise 49 species (Duhaime et al., 2016), have recently received significant levels of attention because they are readily cultivated and are widespread in the marine environment (Ivanova et al., 2001). This genus has attracted significant interest for two reasons. First, *Pseudoalteromonas* species are frequently found in association with eukaryotic hosts in the marine environment, and studies of such associations will elucidate the mechanisms important for microbe-host interactions. Second,

numerous *Pseudoalteromonas* strains were shown to synthesize a range of bioactive molecules, that are active against a variety of target organisms (Egan et al., 2001; Hoyoux et al., 2001; Kobayashi et al., 2003). This feature appears to be important for this genus and may benefit *Pseudoalteromonas* cells in their competition for nutrients or colonization of surfaces (Bowman, 2007).

The genus *Psychrobacter* was originally described by Juni and Heym (1986). Initially the strains were referred to as "*Moraxella*-like" organisms and the strains that were competent for genetic transformation were grouped together as members of the genus *Psychrobacter* which, at present, belongs to the family *Moraxellaceae* (Bowman, 2006). *Psychrobacter* species are primarily isolated from a wide variety of sources including sea water, fish, poultry, meat products and clinical sources. Members of this genus display the following characteristics: Gram-negative, spherical to rod-shaped, non-motile, strictly aerobic, chemoheterotrophic, oxidase-positive, cold-adapted and osmotolerant (Maruyama et al., 2000; Romanenko et al., 2002). *Psychrobacter* species are neutrophilic, growing best at pH 6.0-8.0, and are able to grow in a wide range of temperatures and salt concentrations (Bowman, 2006).

In particular, the strains *Pseudoalteromonas atlantica* MD12-331 A, *Pseudoalteromonas shioyasakiensis* MD12-375, *Pseudoalteromonas mariniglutinosa* MD12-501 and *Psychrobacter submarinus* MD12-530 b, used in the present study, were isolated from a marine sediment sample collected at 15 m depth, at Desertas Islands (Roca et al., 2016).

1.5. *Enterobacter* A47 (DSM 23139)

The Gram-negative bacterium *Enterobacter* A47 (DSM 23139) synthesizes a high molecular fucose-containing EPS, named FucoPol (Alves et al., 2010). FucoPol is a heteropolysaccharide composed of fucose (32-36 mol%), glucose (28-37 mol%), galactose (25-26 mol%), glucuronic acid (9-10 mol%) and acyl groups, namely succinyl (2-3 wt.%), pyruvyl (13-14 wt.%) and acetyl (3-5 wt.%) (Torres et al., 2015). Fucose-containing polysaccharides may have biological activity, since fucose have proven to promote acceleration of wound healing and scavenge free radicals (Péterszegi et al., 2003). Moreover, it was reported that fucose has anti-inflammatory (Cescutti et al., 2005) and anti-aging properties (Fodil-Bourahla et al., 2003), enhancing its biological properties that can be incorporated into pharmaceutical and cosmetic products.

The composition of FucoPol changes depending on the physicochemical factors of the cultivation conditions, which makes this process very versatile, since the distinct EPS produced

by *Enterobacter* A47 possess different properties that can be useful in different applications (Freitas et al., 2014). This feature can be useful to obtain tailored EPS production by simply altering the cultivation conditions (Torres et al. 2012).

1.6. Motivation

Bacterial exopolysaccharides are biocompatible, non-toxic and ecofriendly macromolecules that can be easily obtained from renewable sources. Additionally, exopolysaccharides obtained from microorganisms have different structural composition, presenting a high diversity of interesting properties (More et al., 2014). Over the past years, microbial polysaccharides have been raising commercial interest in alternative to polysaccharides extracted from plants or algae. This fact is related to their use in high-value applications, such as pharmaceutical, cosmetics and biomedicine, in which traditional polymers fail to comply with the required degree of purity or lack some specific functional properties (Freitas et al., 2011).

Marine environments have been identified as an excellent source of EPS-producing microorganisms. Currently, marine bacteria are being studied more in details and are used as a source of new EPS, especially for industries looking for high added value products, due to their improved properties (Finore et al., 2014). As so, the objective of this master's thesis was to study the production of bacterial EPS synthesized by different marine bacteria, of the genera *Pseudoalteromonas* and *Psychrobacter*, and *Enterobacter* A47. This work aimed to assess the effect of a Schatz medium, supplemented with yeast extract and peptone and using glucose as carbon source, on bacterial growth. The ability of each strain to produce EPS was also evaluated, as well as the polymer´s composition.

Enterobacter A47 revealed to be one of the best EPS producers, comparing to the other strains, under the tested conditions. Moreover, the polymer produced by this bacterium presented a higher fucose content, which is very interesting since fucose is one of the rare sugars difficult to obtain in Nature. So, *Enterobacter* A47 was selected for further optimization studies.

In order to optimize EPS production by *Enterobacter* A47 in salt conditions, the impact of NaCl, yeast extract and peptone concentrations on cellular growth and EPS production was assessed. Additionally, the influence of pH on EPS production, as well as on the polymer´s composition, by *Enterobacter* A47 was also studied.

2. Materials and methods

2.1. Biopolymer production

2.1.1. Microorganisms

Pseudoalteromonas atlantica MD12-331 A, *Pseudoalteromonas shioyasakiensis* MD12-375, *Pseudoalteromonas mariniglutinosa* MD12-501, *Psychrobacter submarinus* MD12-530 b and *Enterobacter* A47 (DSM 23139) were preserved in glycerol (20% v/v) and stored at -80 °C. Culture reactivation was performed by growing the stock cultures in an agar plate (CHROMagar^{TM} Orientation) for 24 hours (*Enterobacter* A47) and 48 hours (marine bacteria) at 30 °C, to obtain isolated colonies. Afterwards, inocula were prepared by placing an isolated colony from the plate into 200 mL of Schatz medium, supplemented with yeast extract and peptone, and using glucose as carbon source. Incubation occurred for 24 hours (*Enterobacter* A47) and 32 hours (marine bacteria) in an orbital shaker (New Brunswick Scientific), at 30 °C and 200 rpm.

2.1.2. Cultivation media

Schatz medium (Fondi et al., 2015) was used for all experiments with the following composition (per liter): KH_2PO_4, 1 g; NH_4NO_3, 1 g; NaCl, 30 g; $MgSO_4.7\ H_2O$, 0.2 g; $FeSO_4.7\ H_2O$, 0.01 g; $CaCl_2.2\ H_2O$, 0.01 g. After autoclaving (20 min at 121 °C, 1 bar – Uniclave 88, Portugal), the medium was supplemented with yeast extract, peptone and glucose solutions (autoclaved separately) to give concentrations of 4 $g.L^{-1}$, 2 $g.L^{-1}$ and 30 $g.L^{-1}$, respectively. For the bioreactor experiments E2 – E4, a modified Schatz medium was used, in which the concentrations of NaCl, yeast extract and peptone were altered to 8.1 $g.L^{-1}$, 0.8 $g.L^{-1}$ and 0.4 $g.L^{-1}$, respectively, according to the results determined in the design of experiments.

Every step involved in the handling of the bacterial strain was carried out in a laminar flow chamber (Heraeus SB 48, Germany).

2.1.3. Shake flask assays

The experiments were performed in 500 mL baffled shake flasks with 200 mL of Schatz medium, supplemented with yeast extract and peptone, and using glucose as carbon source. The

medium was inoculated by placing an isolated colony from the plate into the culture medium and growth occurred at 30 °C and 200 rpm.

The assays were run for 96 hours and 3 mL samples were periodically taken for measurement of the optical density at 550 nm and 450 nm, for marine bacteria and *Enterobacter* A47, respectively, and pH. At the end of the experiments, 20 mL samples were also collected for quantification of biomass and EPS.

2.1.4. Bioreactor operation

All assays were carried out in a bioreactor (BioStat B-plus, Sartorius, Germany) with an initial working volume of 2 L. Medium composition was the same as for the inocula. The bioreactor was inoculated aseptically with 10% (v/v) inocula and operated in batch mode.

The temperature was maintained at 30.0 ± 0.1 °C and the pH was automatically controlled at 7.0 ± 0.05, in the assays with marine bacteria, by the automatic addition of NaOH (5 M) and HCl (5 M). In order to explore the effect of pH on the EPS production by *Enterobacter* A47, pH was automatically controlled at 6.0, 7.0 and 8.0 ± 0.05, according to each experiment. A silicon-based antifoam (Sigma-Aldrich, Germany) was used in order to prevent the formation of foam throughout the process.

The air flow rate was maintained at 2 SLPM (standard liters per minute) during the cultivation and the dissolved oxygen concentration (DO) was controlled at 30% of the air saturation by automatic adjustment of the stirrer speed (300-800 rpm) provided by two 6-blade impellers.

Culture broth samples (20 mL) taken periodically during the cultivation runs were centrifuged at 8000 x g, for 10 min, (Sigma 4-16 KS, Germany) in order to separate the biomass from the cell-free supernatant. The cell-free supernatant was preserved at -20 °C for the determination of glucose concentration and for the quantification of the EPS produced. The cell pellet was used to determine the cell dry weight (CDW).

2.1.5. Experimental design assays

To evaluate the impact of medium composition on *Enterobacter* A47 cell growth and EPS production, a statistical approach, namely response surface methodology (RSM) (Lundstedt et al., 1998) was applied. RSM was used to evaluate the impact and the interaction between the experimental variables (X_i): NaCl, yeast extract and peptone concentrations ($g.L^{-1}$) and the observed responses (Y_i): cell dry weight (CDW, $g.L^{-1}$) and EPS production (EPS, $g.L^{-1}$). A central composite rotatable design (CCRD), with three independent variables, was used to study the responses, where X_1, X_2 and X_3 are NaCl, yeast extract and peptone concentrations ($g.L^{-1}$), respectively (Table 3.7). This design was composed of seventeen experiments: eight factorial design points at levels $\pm$ 1; six experiments of axial level $\alpha = \pm 1.682$; and a central point with three replicas, to allow estimating the experimental error (Table 3.6). All experiments were carried out on a randomized order to prevent the effect of unexplained variability due to exogenous factors (Lundstedt et al., 1998; Torres et al., 2012, 2015).

The systems behavior was evaluated by fitting the experimental data to the following second order model:

$$Y_p = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2 \qquad \text{Eq. 2.1}$$

Where Y_p corresponds to the predicted responses, X_1, X_2 are the coded values of the independent variables; b_0, b_i, b_j, b_{ij} (i, j = 1,2) are the coefficient estimates, where b_0 is the interception, b_1 and b_2 the linear terms, b_{11} and b_{22} the quadratic terms and b_{12} the interaction term. In order to identify an appropriate reduced quadratic model, the significance of each source of variation was obtained from statistical analysis – analysis of variance (ANOVA) and multiple linear regression (MLR). The statistical analysis was carried out using the software Statistica, version 8.0 (StatSoft Inc., Tulsa, USA). The fitted model (Eq. 2.1) was evaluated for each response variables based upon the correlation coefficients (R^2), regression parameter significance (*p*-value) and tested lack of fit. To be considered a good predictive tool, the model should satisfy the following criteria: a good correlation value ($R^2 > 0.7$, which is acceptable for biological samples, according to Lundstedt et al., 1998), with statistical meaning (p-value < 0.05, for a 95% confidence level) and with no lack of fit (p-value > 0.05, for a 95% confidence level), i.e. the model error was in the same range as the pure error (Lundstedt et al., 1998). The factors and their interaction were also evaluated by *p*-value at 95% confidence level. The effect of NaCl, yeast extract and peptone concentrations on the response was given by statistics and the surface plots analysis.

The experiments were carried out in 500 mL baffled shake flasks containing 200 mL of Schatz medium, supplemented with yeast extract and peptone, and using glucose as carbon source. The amount of NaCl added was based on the design of experiments, as well as the quantity of yeast extract and peptone (Table 3.6). The medium was inoculated with 10% (v/v) of inoculums and growth occurred at 30 °C and 200 rpm.

The assays were run for 32 hours and 5 mL samples were periodically taken for measurement of the optical density at 450 nm and determination of biomass. At the end of the experiments, 20 mL samples were also collected for EPS quantification.

2.2. Analytical techniques

2.2.1. Apparent viscosity

In bioreactor runs, the apparent viscosity of the cultivation broth samples was measured using a rotational viscometer (FungiLab Alpha Series, Spain) during the assay. The viscosity, in centipoise (cP), was measured at different rotational speeds at room temperature (20 °C).

2.2.2. Determination of cell growth

Culture growth was followed by measuring the optical density of the culture broth at 550 and 450 nm, for marine bacteria and *Enterobacter* A47, respectively (with a VWR V-1200 spectrophotometer). The cell dry weight (CDW) was determined by gravimetry, after washing the cell pellet with deionized water (resuspension in water, centrifugation at 8000 x g, for 10 min, and, finally, resuspension in water and filtration through 0.20 µm filters) and dried at 100 °C, for 24 hours. This analysis was performed in duplicate.

2.2.3. Glucose concentration

Glucose concentration in the cell-free supernatant was determined by high performance liquid chromatography (HPLC) using a VARIAN Metacarb column (BioRad) coupled to an infrared (IR) detector. The analysis was performed at 30 °C, with sulphuric acid (H_2SO_4 0.01 N) as eluent, at a flow rate of 0.5 $mL.min^{-1}$. The samples were diluted (1:10 or 1:50, depending on the sample) in H_2SO_4 0.01 N and filtered using Vectra Spin Micro Polysulfone filters (Whatman),

which had a pore diameter of 0.2 μm, at 3000 rpm for 10 min. A standard calibration curve was constructed by preparing solutions with different glucose (99 %, Scharlau) concentrations: 1.0 $g.L^{-1}$, 0.5 $g.L^{-1}$, 0.25 $g.L^{-1}$, 0.125 $g.L^{-1}$ and 0.0625 $g.L^{-1}$ (calibration curves in appendix 6.1).

2.2.4. Exopolysaccharide quantification

For EPS quantification, the cell-free supernatant, which contained the polymer, was dialyzed using a 12000-14000 molecular weight cut-off membrane (Zellutrans Carl Roth – Regenerated Cellulose Tubular Membrane) against deionized water, with constant stirring. The water was changed frequently and the conductivity was measured throughout the dialysis process, until a value bellow 10 $\mu S.m^{-1}$ was reached. Sodium azide, at a concentration of 10 $mg.L^{-1}$, was added to avoid possible biological degradation of the polysaccharide during the dialysis process.

Afterwards, the purified polymer was freeze dried (Scanvac, CoolSafe) for 48 hours, and weighed, allowing the determination of the polysaccharide content.

2.3. Calculus

2.3.1. Volumetric productivity

The volumetric productivity (r_p, $g.\ L^{-1}.\ d^{-1}$) of the EPS production process was determined as following:

$$r_P = \frac{\Delta P}{\Delta t}$$

Eq. 2.2

where ΔP corresponds to the variation of concentration of product (EPS, $g.L^{-1}$) in a Δt interval (days).

2.4. Biopolymer characterization

2.4.1. Sugar composition

For the compositional analysis, polymer samples (~5 mg) were dissolved in deionized water (5 mL) and hydrolyzed with trifluoroacetic acid (TFA) (0.1 mL TFA 99%) at 120 °C, for 2 hours. The hydrolysate was used for the identification and quantification of the constituent monosaccharides by HPLC using a CarboPac PA10 column (Thermo Dionex), equipped with pulsed amperometric detector (Dionex ICS3000, ThermoFisher Scientific Inc.). The analysis was performed at 30 °C with sodium hydroxide (NaOH 4 mM) as eluent, at a flow rate of 0.9 $mL.min^{-1}$. D-(+)-galactose (99%, Fluka), D-(+)-glucose anidra (99%, Scharlau), D-(+)-fucose (98%, Sigma), D-(+)-xylose (99%, Merck), L-rhamnose monohydrate (99%, Fluka), D-(+)-mannose (99%, Fluka), D-glucuronic acid (98%, Alfa Aesar) and D-(+)-galacturonic acid monohydrate (97%, Fluka) were used as standards (1-50 ppm).

3. Results and discussion

3.1. Screening assay

3.1.1. Shake flask assays

The screening assay was performed to see which bacteria, *Pseudoalteromonas atlantica* MD12-331 A, *Pseudoalteromonas shioyasakiensis* MD12-375, *Pseudoalteromonas mariniglutinosa* MD12-501, *Psychrobacter submarinus* MD12-530 b and *Enterobacter* A47, would grow better by using a Schatz medium (with 30 g.L^{-1} of NaCl), supplemented with yeast extract (4 g.L^{-1}) and peptone (2 g.L^{-1}), and glucose (30 g.L^{-1}) as carbon source (standard conditions). Due to the frequent variation of the sea water composition (Sharqawy et al., 2010), in this study, a Schatz medium was used as culture medium. This medium was chosen because it is commonly used in studies with marine bacteria (Fondi et al., 2015; Wilmes et al., 2011).

The assays were performed in 500 mL baffled shake flasks with 200 mL of culture medium and were run for 96 hours. The ability of each strain to produce EPS was also evaluated.

Enterobacter A47 is a well-known EPS-producer able to grown on glycerol (Alves et al., 2010; Torres et al., 2011, 2012, 2014), glucose, xylose (Freitas et al., 2014) and lactose (Antunes et al., 2015). Several strains of *Pseudoalteromonas atlantica* and *Pseudoalteromonas shioyasakiensis* have also been described to synthesize EPS (Matsuyama et al., 2014; Perkins-Balding et al., 1999). On the other hand, little is known about *Pseudoalteromonas mariniglutinosa* and *Psychrobacter submarinus* and their ability to produce EPS.

Table 3.1 – Cell dry weight ($g.L^{-1}$) and EPS production ($g.L^{-1}$) obtained in the screening assay, after 96 hours shake flask cultivations on Schatz medium, supplemented with yeast extract and peptone, and using glucose as carbon source.

Bacteria strain	CDW ($g.L^{-1}$)	EPS ($g.L^{-1}$)
Pseudoalteromonas atlantica MD12-331 A	1.70	0.26
Pseudoalteromonas shioyasakiensis MD12-375	1.02	0.24
Pseudoalteromonas mariniglutinosa MD12-501	0.98	0.18
Psychrobacter submarinus MD12-530 b	0.84	0.14
Enterobacter A47	2.26	0.67

The screening assay showed that all cultures were able to grow in Schatz medium, supplemented with yeast extract and peptone, and using glucose as carbon source (Table 3.1). Nevertheless, the marine bacteria with the highest cell growth was *Pseudoalteromonas atlantica* MD12-331 A that reached a CDW of 1.70 $g.L^{-1}$, within 96 hours of cultivation (Table 3.1). Among the remaining marine bacteria tested, *Pseudoalteromonas shioyasakiensis* MD12-375 and *Pseudoalteromonas mariniglutinosa* MD12-501 had very similar cell growth (CDW values of 1.02 $g.L^{-1}$ and 0.98 $g.L^{-1}$, respectively) (Table 3.1), but inferior to the one achieved by *Pseudoalteromonas atlantica* MD12-331 A.

Concerning EPS production, by marine bacteria, the maximum value was attained by *Pseudoalteromonas atlantica* MD12-331 A that reached a production of 0.26 $g.L^{-1}$ of EPS (Table 3.1). The other tested *Pseudoalteromonas* and *Psychrobacter* strains synthesized EPS as well, but in less quantity (0.14-0.24 $g.L^{-1}$) (Table 3.1). According with the literature, *Pseudoalteromonas atlantica* MD12-331 A, *Pseudoalteromonas mariniglutinosa* MD12-501 and *Psychrobacter submarinus* MD12-530 b produced 1.46 $g.L^{-1}$, 1.39 $g.L^{-1}$ and 0.78 $g.L^{-1}$ of EPS. It has also been reported that *Pseudoalteromonas shioyasakiensis* MD12-375 achieved an EPS production of 4.75 $g.L^{-1}$ (Roca et al., 2016). These values are higher than those attained in the screening assay (0.14-0.26 $g.L^{-1}$) (Table 3.1), which may have been due to differences in the culture medium (sea water was used instead of the Schatz medium) and in the duration of the assay (120 hours instead of 96 hours).

Even though EPS production is a common trait within *Pseudoalteromonas* strains as a way of enhancing survival in extreme marine environments (Finore et al., 2014) very few have been studied in detail. So, in order to study and learn more about the strains selected and their

ability to produce EPS, by using a Schatz medium, supplemented with yeast extract and peptone, and glucose as carbon source, these strains were chosen to proceed with the bioreactor assays.

However, of all bacteria studied, *Enterobacter* A47 was the one with the highest cell growth, with a CDW of 2.26 $g.L^{-1}$, and an EPS production of 0.67 $g.L^{-1}$ (Table 3.1). Although, this bacterium is a well-known EPS producer, using glycerol byproduct from the biodiesel industry (Alves et al., 2010; Torres et al., 2011, 2012, 2014), as well as other carbon sources, including glucose (Freitas et al., 2014) , this culture had never been tested in a Schatz medium, nor in any other saline medium. Thus, in order for the results obtained to be explored, this bacterium was also chosen to proceed with the bioreactor assays.

3.1.2. Bioreactor assays

Bioreactor experiments were conducted with the marine bacteria, *Pseudoalteromonas atlantica* MD12-331 A, *Pseudoalteromonas shioyasakiensis* MD12-375, *Pseudoalteromonas mariniglutinosa* MD12-501 and *Psychrobacter submarinus* MD12-530 b, and *Enterobacter* A47, in order to evaluate their performance under controlled conditions of pH (7.0), temperature (30 °C) and dissolved oxygen concentration (30%).

Batch cultivations were performed in 2 L bioreactors using the standard Schatz medium (NaCl, 30 $g.L^{-1}$), supplemented with yeast extract (4 $g.L^{-1}$) and peptone (2 $g.L^{-1}$), and glucose (30 $g.L^{-1}$) as carbon source.

3.1.2.1. Marine bacteria cultivation assays

Figure 3.1 presents the cultivation profiles of *Pseudoalteromonas atlantica* MD12-331 A (P1 – Fig. 3.1 a.), *Pseudoalteromonas shioyasakiensis* MD12-375 (P2 – Fig. 3.1 b.), *Pseudoalteromonas mariniglutinosa* MD12-501 (P3 – Fig. 3.1 c.) and *Psychrobacter submarinus* MD12-530 b (P4 – Fig. 3.1 d.).

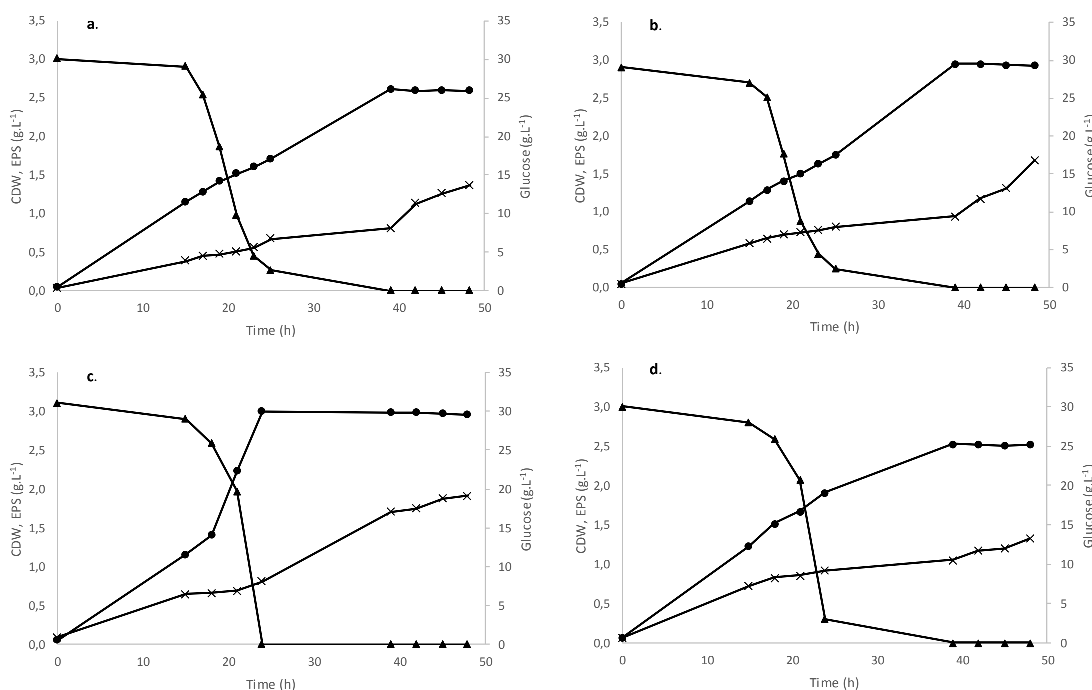

Figure 3.1 – Cultivation profile of *Pseudoalteromonas atlantica* MD12-331 A (P1 – a.), *Pseudoalteromonas shioyasakiensis* MD12-375 (P2 – b.), *Pseudoalteromonas mariniglutinosa* MD12-501 (P3 – c.) and *Psychrobacter submarinus* MD12-530 b (P4 – d.), wherein experimental results of CDW (●), EPS (×) and glucose (▲) are represented throughout the cultivation run.

The strain that presented the greatest growth was *Pseudoalteromonas mariniglutinosa* MD12-501 (run P3) reaching a maximum CDW of 3.00 $g.L^{-1}$, around 24 hours of cultivation (Fig. 3.1 c.). *Pseudoalteromonas shioyasakiensis* MD12-375 (run P2) attained a similar CDW (2.95 $g.L^{-1}$) but it took a longer time (39 hours) (Fig. 3.1 b.). The other strains, *Pseudoalteromonas atlantica* MD12-331 A (run P1) and *Psychrobacter submarinus* MD12-530 b (run P4), reached slightly lower CDW values (2.61 and 2.53 $g.L^{-1}$, respectively) within 39 hours (Fig. 3.1 a. and d.).

In all runs, cell growth was observed in the first 15 hours of cultivation, but glucose consumption was very reduced during that period of time. Hence, in that period cell growth probably occurred due to yeast extract and/or peptone present in the medium which can be used as carbon sources.

In terms of glucose consumption, glucose concentration decreased in the first 24 hours (from 31.09 $g.L^{-1}$ to 0 $g.L^{-1}$) for run P3, limiting the growth of the bacteria (Fig. 3.1 c.). In runs P1, P2 and P4 glucose was also all consumed, but it took around 39 hours (Fig. 3.1 a., b., and d.).

Regarding EPS production, 1.37 $g.L^{-1}$, 1.68 $g.L^{-1}$, 1.91 $g.L^{-1}$ and 1.33 $g.L^{-1}$ of EPS were produced in runs P1, P2, P3 and P4, respectively (Table 3.2). For all assays, EPS production was

improved during the stationary phase. The EPS production observed after glucose depletion might have been due to the consumption of other medium components, such as yeast extract and/or peptone, which may have functioned as substrate for the synthesis of EPS.

Table 3.2 – Marine bacteria exopolysaccharide production studies comparison.

Strain	Location	Carbon and nitrogen source	Culture mode	EPS ($g.L^{-1}$)	r_p ($g. L^{-1}. d^{-1}$)	References
Pseudoalteromonas atlantica MD12-331 A	Ocean sediments from Madeira Archipelago	Glucose/ NH_4NO_3, yeast extract and peptone	Batch Bioreactor 30 °C	1.37	0.69	This study
Pseudoalteromonas shioyasakiensis MD12-375	Ocean sediments from Madeira Archipelago	Glucose/ NH_4NO_3, yeast extract and peptone	Batch Bioreactor 30 °C	1.68	0.84	This study
Pseudoalteromonas mariniglutinosa MD12-501	Ocean sediments from Madeira Archipelago	Glucose/ NH_4NO_3, yeast extract and peptone	Batch Bioreactor 30 °C	1.91	0.96	This study
Psychrobacter submarinus MD12-530 b	Ocean sediments from Madeira Archipelago	Glucose/ NH_4NO_3, yeast extract and peptone	Batch Bioreactor 30 °C	1.33	0.67	This study
Pseudoalteromonas SM20310	Arctic sea ice of the Canada Basin	Glucose/ Yeast extract and peptone	Shake flasks 15 °C	0.57	0.19	Liu et al., 2013
Alteromonas sp. JL2810	Sea water from South China Sea	Glucose/ NH_4Cl	Bioreactor 25 °C	0.77	0.13	Zhang et al., 2015
Halomonas sp.TG39	N.A.	Glucose/ Yeast extract and peptone	Shake flasks 28 °C	0.66	0.22	Gutierrez et al., 2009
Saccharophagus degradans	Salt marsh cord grass in the Chesapeake bay watershed	Glucose/ NH_4Cl and yeast extract	Pulse-fed Bioreactor 30 °C	4.12	1.37	González-García et al., 2015

N.A.: data not available.

Considering 48 hours the time frame of the cultivation assay, a volumetric productivity of 0.69 $g_{EPS}.L^{-1}.d^{-1}$, 0.84 $g_{EPS}.L^{-1}.d^{-1}$, 0.96 $g_{EPS}.L^{-1}.d^{-1}$ and 0.67 $g_{EPS}.L^{-1}.d^{-1}$ was achieved in runs P1, P2, P3 and P4, respectively (Table 3.2). These values are higher than those reported in the literature for other marine *Pseudoalteromonas*, *Alteromonas* or *Halomonas* EPS-producing strains (Table 3.2). For instance, *Pseudoalteromonas* SM20310, isolated from Arctic sea ice of the Canada basin, produced 0.57 $g.L^{-1}$ of EPS after 3 days, corresponding to a productivity of 0.19 $g_{EPS}.L^{-1}.d^{-1}$. Also, a productivity of 0.13 $g_{EPS}.L^{-1}.d^{-1}$ was achieved in a 6 days cultivation of *Alteromonas* sp. JL2810 and a productivity of 0.22 $g_{EPS}.L^{-1}.d^{-1}$ was achieved in a 3 days cultivation of *Halomonas* sp. TG39, when grown on a cultivation medium with glucose as carbon source (Table 3.2).

On the other hand, there are marine bacteria that have shown higher performances, such as *Saccharophagus degradans*, which produced 4.12 $g.L^{-1}$ of EPS within 3 days of cultivation on glucose, corresponding to a productivity of 1.37 $g_{EPS}.L^{-1}.d^{-1}$ (Table 3.2). However, *Pantoea* sp. BM39 is the highest EPS producer reported so far, with an EPS production of 21.3 $g.L^{-1}$ within 18 hours cultivation on glucose, corresponding to a volumetric productivity of 28.4 $g_{EPS}.L^{-1}.d^{-1}$ (González-García et al., 2015).

Concomitant with EPS production, in all the assays, there was a small increase of the apparent viscosity of the broth (from 1 mPa.s at the beginning of the run, to 2 mPa.s within 48 h of cultivation, measured at 1.7 s^{-1}). This increase in the broth's viscosity may be related with the presence of EPS in the broth. For instance, *Halomonas almeriensis*, a species of moderately halophilic bacterium, excretes a low viscosity (3.5 mPa.s, measured at 12 s^{-1}) exopolysaccharide with pseudoplastic behavior and high cation binding potential that can act as a bio-detoxifier and emulsifier (Llamas et al., 2012). Also, levan (a well-known exopolysaccharide produced by a large group of bacteria, such as *Halomonas* sp.) is a polymer with low viscosity (10 mPa.s, measured at 10 s^{-1}) (Küçükasik et al., 2011).

In summary, EPS production was improved during bioreactor cultivation for all the marine bacteria tested. In shake flasks, *Pseudoalteromonas atlantica* MD12-331 A, *Pseudoalteromonas shioyasakiensis* MD12-375, *Pseudoalteromonas mariniglutinosa* MD12-501 and *Psychrobacter submarinus* MD12-530 b produced 0.26 $g.L^{-1}$, 0.24 $g.L^{-1}$, 0.18 $g.L^{-1}$ and 0.14 $g.L^{-1}$, after a 96 hours cultivation, respectively, while in the bioreactor assays, EPS production reached 1.37 $g.L^{-1}$, 1.68 $g.L^{-1}$, 1.91 $g.L^{-1}$ and 1.33 $g.L^{-1}$, after a 48 hours cultivation. This corresponds to over 10-fold improvements of the polymer production for all strains.

Pseudoalteromonas mariniglutinosa MD12-501, which had not been the highest EPS producer in the shake flasks cultivation, reached the highest EPS production (1.91 g.L^{-1}) during bioreactor cultivation.

The EPS obtained in runs P1 – P4 were characterized in terms of sugar composition (Table 3.3).

Table 3.3 – Sugar composition content of the exopolysaccharides produced by *Pseudoalteromonas atlantica* MD12-331 A (P1), *Pseudoalteromonas shioyasakiensis* MD12-375 (P2), *Pseudoalteromonas mariniglutinosa* MD12-501 (P3), *Psychrobacter submarinus* MD12-530 b (P4), *Pseudoalteromonas* sp. SM20310 and *Pseudoalteromonas* MD12-642. Fuc, fucose; Glc, glucose; Gal, galactose; Rha, rhamnose; Man, mannose; Xyl, xylose; GlcN, glucosamine; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; GlcA, glucuronic acid; GalA, galacturonic acid.

Bacteria strain	Sugar composition (%mol)											Refs.
	Fuc	**Glc**	**Gal**	**Rha**	**Man**	**Xyl**	**GlcN**	**GlcNAc**	**GalNAc**	**GlcA**	**GalA**	
P1	–	96	–	4	–	–	–	–	–	–	–	This study
P2	–	79	–	21	–	–	–	–	–	–	–	This study
P3	12	44	18	14	–	–	–	–	–	3	9	This study
P4	12	46	18	13	–	–	–	–	–	2	9	This study
P. sp. SM20310	–	11	9	2	72	1	–	4	2	–	–	Liu et al., 2013
P. MD12-642	–	–	–	19	–	–	14	–	–	26	42	Roca et al., 2016

The results obtained in this study indicate that the polymer produced by the bacterium *Pseudoalteromonas atlantica* MD12-331 A (run P1) was mostly composed of glucose (96 %mol) and presented a small content in rhamnose (4 %mol). The EPS produced in run P2, by *Pseudoalteromonas shioyasakiensis* MD12-375, was identical to that obtained in run P1. However, it had a lower glucose content (79 %mol), while the content in rhamnose was higher (21 %mol) (Table 3.3).

The sugar monomer composition of the EPS produced by *Pseudoalteromonas mariniglutinosa* MD12-501 (run P3) was evaluated and the presence of glucose (44 %mol), galactose (18 %mol), rhamnose (14 %mol), fucose (12 %mol), galacturonic acid (9 %mol) and glucuronic acid (3 %mol) was detected (Table 3.3).

The EPS produced by the bacterium *Psychrobacter submarinus* MD12-530 b (run P4) had a sugar composition similar to that attained in run P3, presenting small differences in the contents in glucose (46 %mol), rhamnose (13 %mol) and glucuronic acid (2 %mol) (Table 3.3).

The production of EPS mainly rich in glucose, by *Pseudoalteromonas* strains sp. AM and sp. SM9913, has been reported by several authors (Al-Nahas et al., 2011; Qin et al., 2007). Liu et al. (2013) reported the production of an EPS composed of mannose (72 %mol) , glucose (11 %mol), galactose (9 %mol), N-acetylglucosamine (4 %mol), rhamnose (2 %mol), N-acetylgalactosamine (2 %mol) and xylose (1 %mol) by *Pseudoalteromonas* sp. SM20310 (Table 3.3). *Pseudoalteromonas* MD12-642 isolated from a marine sediment sample collected at Desertas Islands, as well as the strains used in runs P1 – P4, produced a polymer composed of galacturonic acid (42 %mol), glucuronic acid (26 %mol), rhamnose (19%mol) and glucosamine (14 %mol) (Table 3.3).

3.1.2.2. *Enterobacter* A47 cultivation assay

Figure 3.2 presents the concentration profiles of biomass, EPS and glucose for the cultivation of *Enterobacter* A47 in Schatz medium (run E1).

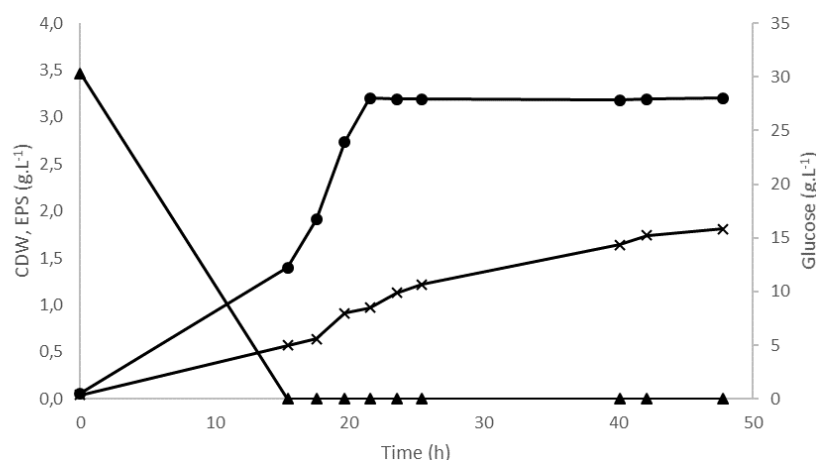

Figure 3.2 – Cultivation profile of *Enterobacter* A47 (run E1), wherein experimental results of CDW (●), EPS (×) and glucose (▲) are represented throughout the cultivation run.

In this assay, *Enterobacter* A47 reached a maximum CDW of 3.20 $g.L^{-1}$, within 22 hours of cultivation (Fig. 3.2). This value is much lower than those reported in literature (5.80-13.58 $g.L^{-1}$) for cultivation in Medium E* supplemented with different carbon sources (Table 3.4).

The fact that the culture has grown less is not related with the carbon source used, once glucose has already been reported as one of the preferred substrates for both growth and EPS production by *Enterobacter* A47. In previous studies, a maximum CDW of 8.14 $g.L^{-1}$ was attained at the end of the batch phase (22 hours of cultivation) (Freitas et al., 2014). However, in run E1 the glucose concentration used (30 $g.L^{-1}$) was half the concentration reported in the literature (60 $g.L^{-1}$) (Freitas et al., 2014), hence the growth was lower.

It has also been reported that an initial nitrogen concentration of 0.7 $g.L^{-1}$ led to a high CDW value (8.14 $g.L^{-1}$) (Freitas et al., 2014). In run E1, the nitrogen concentration used was 1.02 $g.L^{-1}$ and, as already observed, the increase in the initial concentration of nitrogen (1.20 and 1.27 $g.L^{-1}$) leads to higher growth by *Enterobacter* A47 (10.37 and 9.33 $g.L^{-1}$, respectively) (Torres et al., 2014). Therefore, the lower CDW obtained in run E1 (3.20 $g.L^{-1}$) may be the result of a different metabolic capacity by *Enterobacter* A47 towards the different nitrogen sources used. *Enterobacter* A47 did not show preferential growth with organic nitrogen (yeast extract and peptone – run E1) compared to inorganic nitrogen source ($(NH_4)_2HPO_4$ – Freitas et al., 2014; Torres et al., 2014).

As so, at run E1 the carbon to nitrogen ratio (C:N) (12:1, w/w) was lower than that reported in the literature (34:1, w/w) (Freitas et al., 2014). Nevertheless, this ratio is an important parameter for normal bacterial cellular growth and a high value of this ratio (10:1-20:1, w/w) was known to promote EPS synthesis (Torres et al., 2014). Thus, although the C:N ratio used in run E1 is within the mentioned range, this was not suitable for the production of EPS by *Enterobacter* A47.

In addition, since the effect of the salt concentration has never been evaluated for *Enterobacter* A47, the NaCl concentration used (30 $g.L^{-1}$) may also have influenced the growth of the culture. For instance, Neysens et al. (2003) reported that for salt concentrations above 5 $g.L^{-1}$, *Lactobacillus amylovorus* DCE 471 grew more slowly and biomass production became less efficient.

Glucose was totally consumed in less than 15 hours and the culture grew up to 1.40 $g.L^{-1}$ (Fig. 3.2). Then, the culture continued to grow, even without glucose, which shows that it must have used yeast extract and/or peptone as substrate for cell growth.

Concerning EPS production, 0.57 $g.L^{-1}$ of EPS were produced in the first 15 hours, while glucose was available (Fig. 3.2). Then the culture continued to produce, even after growth had

ceased. Indeed, after 25 hours, 1.22 g. L^{-1} of exopolysaccharide were already produced. At the end of the cultivation run, *Enterobacter* A47 had produced 1.81 g.L^{-1} of biopolymer. The EPS production observed after glucose depletion might have been due to the consumption of other medium components, such as yeast extract and/or peptone, which may have functioned as substrates for the culture.

Concomitant with EPS production, there was a slight increase of the apparent viscosity of the broth from 1 to 9 mPa.s (measured at 1.7 s^{-1}) throughout the cultivation run. This alteration in the rheology of the culture broth is probably due to the accumulation of the EPS in the cultivation broth (Alves et al., 2010).

Regarding the assays previously carried out with the bacterium *Enterobacter* A47, the amount of EPS produced in run E1 was similar to that achieved using tomato paste as sole substrate under pH-stat mode (1.65 g.L^{-1}) (Antunes et al., 2017). However, the value obtained was much lower than that reported (13.40 g.L^{-1}) when glucose was used as carbon source (Freitas et al., 2014).

Moreover, the amount of EPS produced was lower than all the others reported in previous studies (Table 3.4), which may have been due to differences in the C:N ratio and to the use of different nitrogen and carbon sources. As already mentioned, at run E1 the C:N ratio was lower than that used in previous studies and, as previously reported, EPS producing bacteria need a high C:N ratio to promote EPS synthesis (Torres et al., 2014). The type and concentration of the carbon and nitrogen sources has probably influenced EPS synthesis.

In addition, as observed by Torres et al. (2014), increasing the initial nitrogen concentration led to higher *Enterobacter* A47 growth, but it was prejudicial for EPS synthesis. Although nitrogen source is necessary for cell growth and the synthesis of the enzymes necessary for polysaccharide formation, an excess of nitrogen generally reduces the conversion of the carbohydrate substrate to the polysaccharide (Torres et al., 2014).

Besides that, the amount of EPS produced may have been lower due to differences in the cultivation mode, cultivation conditions and carbon source composition. In this work, several parameters were different, namely, the culture medium used was different, the mode of cultivation used was a batch mode, the air flow rate used was 2.0 SLPM instead of 0.4 SLPM, and the dissolved oxygen level was controlled at 30% instead of 10% (Antunes et al., 2017; Freitas et al., 2014; Torres et al., 2011, 2012, 2014).

Table 3.4 – Parameters obtained in the bioreactor cultivations of *Enterobacter* A47 using different substrates and cultivation modes.

Substrate	Cultivation mode	CDW (g.L^{-1})	EPS (g.L^{-1})	r_P (g. L^{-1}. d^{-1})	References
Glucose	Batch mode E1	3.20	1.81	0.91	This study
Glycerol	Continuous feeding	5.80 – 7.68	7.23 – 7.97	1.89 – 2.04	Freitas et al., 2014 Torres et al., 2011, 2012, 2014
Glucose	DO-stat	8.14	13.40	3.78	Freitas et al., 2014
Tomato paste	pH-stat	10.22	1.65	0.57	Antunes et al., 2017
	DO-stat	9.81	3.43	1.16	Antunes et al., 2017
	Continuous feeding 4 g.L^{-1}.h^{-1}	10.14	3.99	1.34	Antunes et al., 2017
	6 g.L^{-1}.h^{-1}	10.74	4.54	1.56	Antunes et al., 2017
	11 g.L^{-1}.h^{-1}	13.58	8.77	2.92	Antunes et al., 2017

Considering 48 hours the time frame of the cultivation assay, a volumetric productivity of 0.91 g_{EPS}.L^{-1}.d^{-1} was achieved in run E1 (Table 3.4). Comparing this value with the ones achieved in previous studies with the bacterium *Enterobacter* A47, it was slightly higher than that attained using tomato paste as sole substrate, under pH-stat mode (0.57 g_{EPS}.L^{-1}.d^{-1}). However, the value obtained was lower than that reported in the literature (3.78 g_{EPS}.L^{-1}.d^{-1}), when glucose was used as carbon source (Table 3.4). Furthermore, the value obtained was lower than all the others reported in literature, 1.16-2.92 g_{EPS}.L^{-1}.d^{-1}, when glycerol and tomato paste, under DO-stat or at continuous feeding, were used as sole substrate (Table 3.4).

Concerning the assays carried out with marine bacteria, the value attained in run E1 was higher than that obtained for the *Pseudoalteromonas* and *Psychrobacter* strains tested in this study (runs P1, P2 and P4), except for *Pseudoalteromonas mariniglutinosa* MD12-501 (run P3), whose volumetric productivity was slightly higher (0.96 g_{EPS}.L^{-1}.d^{-1}) (Table 3.2).

The composition of the polymer synthesized can change depending on the type of carbon source used. Hence, the EPS obtained in this assay was characterized in terms of sugar composition (Table 3.5).

Table 3.5 – Sugar composition content of the exopolysaccharides produced by *Enterobacter* A47 from various substrates under different cultivation modes.

Substrate	Cultivation mode	Sugar composition (%mol)				References
		Fucose	Galactose	Glucose	Glucuronic acid	
Glucose	Batch mode E1	37	23	33	6	This study
Glycerol	Continuous feeding	30 – 36	22 – 29	25 – 34	9 – 10	Freitas et al., 2014 Torres et al., 2011, 2012, 2014
Glucose	DO-stat	29	29	26	16	Freitas et al., 2014
Tomato paste	pH-stat	20	45	24	11	Antunes et al., 2017
	DO-stat	28	35	25	12	Antunes et al., 2017
	Continuous feeding 4 $g.L^{-1}.h^{-1}$	27	31	32	10	Antunes et al., 2017
	6 $g.L^{-1}.h^{-1}$	33	27	29	11	Antunes et al., 2017
	11 $g.L^{-1}.h^{-1}$	37	27	23	12	Antunes et al., 2017

The extracellular polysaccharide obtained was composed of fucose (37 %mol), galactose (23 %mol), glucose (33 %mol) and glucuronic acid (6 %mol) (Table 3.5). This sugar monomer profile is within the range of the monosaccharide profile reported for FucoPol, except for a slightly lower content in glucuronic acid (Table 3.5).

According to the results, the cultivation conditions used in run E1 had a significant impact on EPS production and productivity, however the polymer´s composition was similar to that obtained in previous assays.

3.1.2.3. Conclusions

From the bioreactor assays carried out with marine bacteria and *Enterobacter* A47, using the standard Schatz medium, the ones with the best results were *Pseudoalteromonas*

mariniglutinosa MD12-501 and *Enterobacter* A47, with an EPS production of 1.91 and 1.81 $g.L^{-1}$, and volumetric productivity values of 0.96 and 0.91 $g_{EPS}.L^{-1}.d^{-1}$, respectively.

Although *Pseudoalteromonas mariniglutinosa* MD12-501 presented slightly higher EPS production and productivity values, the polymer produced by *Enterobacter* A47 presented a higher fucose content, which is very interesting since fucose is one of the rare sugars difficult to obtain in Nature. Thus, *Enterobacter* A47 was selected to proceed with the studies.

3.2. Influence of NaCl, yeast extract and peptone concentrations on cell dry weight and EPS production by *Enterobacter* A47

To improve bioreactor operational conditions, several optimizing strategies have been developed. Response surface methodology (RSM) is an efficient statistically strategy for designing experiments, building models, searching optimum conditions of factors for desirable responses and evaluating the relative significance of several affecting factors even in the presence of complex interactions (Song et al., 2007; Zhou et al., 2010).

Tables 3.6 and 3.7 show the matrix of the central composite rotatable design (CCRD) and the observed responses studied CDW ($g.L^{-1}$, Y_1) and EPS ($g.L^{-1}$, Y_2).

Table 3.6 – Matrix of the central composite rotatable design (CCRD), composed of seventeen experiments: eight factorial design points at levels ± 1; six experiments of axial level α = ± 1.682; and a central point with three replicas.

		X_1 (NaCl)		X_2 Yeast extract)		X_3 (Peptone)	
	Run number	Coded level	Real value ($g.L^{-1}$)	Coded level	Real value ($g.L^{-1}$)	Coded level	Real value ($g.L^{-1}$)
Factorial design	1	-1	8.1	-1	0.8	-1	0.4
	2	1	31.9	-1	0.8	-1	0.4
	3	-1	8.1	1	3.2	-1	0.4
	4	1	31.9	1	3.2	-1	0.4
	5	-1	8.1	-1	0.8	1	1.6
	6	1	31.9	-1	0.8	1	1.6
	7	-1	8.1	1	3.2	1	1.6
	8	1	31.9	1	3.2	1	1.6
Central point	9	0	20.0	0	2.0	0	1.0
	10	0	20.0	0	2.0	0	1.0
	11	0	20.0	0	2.0	0	1.0
Axial points	12	-1.682	0.0	0	2.0	0	1.0
	13	1.682	40.0	0	2.0	0	1.0
	14	0	20.0	-1.682	0.0	0	1.0
	15	0	20.0	1.682	4.0	0	1.0
	16	0	20.0	0	2.0	-1.682	0.0
	17	0	20.0	0	2.0	1.682	2.0

Table 3.7 – Central composite rotatable design (CCRD) with three independent variables X_1 (NaCl, $g.L^{-1}$), X_2 (yeast extract, $g.L^{-1}$) and X_3 (peptone, $g.L^{-1}$), and the observed responses studied Y_1 (CDW, $g.L^{-1}$) and Y_2 (EPS, $g.L^{-1}$).

Run number	NaCl ($g.L^{-1}$) X_1	Yeast extract ($g.L^{-1}$) X_2	Peptone ($g.L^{-1}$) X_3	CDW ($g.L^{-1}$) Y_1	EPS ($g.L^{-1}$) Y_2
1	8.1	0.8	0.4	1.40	0.48
2	31.9	0.8	0.4	1.16	0.42
3	8.1	3.2	0.4	0.88	0.28
4	31.9	3.2	0.4	1.24	0.47
5	8.1	0.8	1.6	1.50	0.50
6	31.9	0.8	1.6	1.08	0.47
7	8.1	3.2	1.6	0.96	0.30
8	31.9	3.2	1.6	1.28	0.51
9	20.0	2.0	1.0	1.04	0.40
10	20.0	2.0	1.0	1.02	0.40
11	20.0	2.0	1.0	1.08	0.43
12	0.0	2.0	1.0	0.87	0.30
13	40.0	2.0	1.0	0.00	0.00
14	20.0	0.0	1.0	0.00	0.00
15	20.0	4.0	1.0	1.28	0.55
16	20.0	2.0	0.0	1.02	0.36
17	20.0	2.0	2.0	1.06	0.45

3.2.1. Influence of NaCl, yeast extract and peptone concentrations on cell dry weight

The results obtained under the different NaCl, yeast extract and peptone concentrations tested are presented in Table 3.7. The highest CDW values (1.40-1.50 $g.L^{-1}$) were achieved at low concentrations of NaCl and yeast extract (8.1 $g.L^{-1}$ and 0.8 $g.L^{-1}$, respectively).

The results show that high concentrations of NaCl, yeast extract and peptone (run 8 – 31.9 $g.L^{-1}$, 3.2 $g.L^{-1}$ and 1.6 $g.L^{-1}$, respectively) and high concentration of yeast extract (run 15 – 4.0 $g.L^{-1}$) also resulted in a similar CDW (1.28 $g.L^{-1}$).

The central point conditions (runs 9, 10 and 11) resulted in intermediate CDW values (1.02-1.08 $g.L^{-1}$). Under the remaining conditions, the CDW was lower, except for runs 16 and 17 (peptone concentration of 0.0 and 2.0 $g.L^{-1}$, respectively), wherein similar CDW values (1.02 and 1.06 $g.L^{-1}$, respectively) were obtained.

Within the concentration extremes tested (0.0 and 40.0 $g.L^{-1}$ of NaCl, 0.0 and 4.0 $g.L^{-1}$ of yeast extract, 0.0 and 2.0 $g.L^{-1}$ of peptone), for the highest concentration of NaCl (run 13) and lower concentration of yeast extract (run 14) no cell growth was observed. In run 12, where no

NaCl was added to the medium, CDW was very low (0.87 $g.L^{-1}$). On the other hand, the CDW was not significantly affected by peptone within the range 0.0-2.0 $g.L^{-1}$.

The effect of NaCl or yeast extract or peptone concentration on the different responses evaluated by the one-to-one factor did not allow the identification of the interaction between the three variables, which was only possible using the response surface methodology (RSM).

RSM – ANOVA (analysis of variance) and MLR (multiple linear regression) – analysis used for CDW is summarized in Tables 3.8 and 3.10.

Table 3.8 – Analysis of variance (ANOVA) of the second order model for parameter CDW.

Source of variation	CDW				
	Sum of squares	Degrees of freedom	Mean squared	F-value	*p*-value
Model					0.002
NaCl (L)	0.068	1	0.068	72.750	0.013[a]
NaCl (Q)	0.011	1	0.011	11.556	0.077
YE (L)	0.022	1	0.022	23.694	0.040[a]
YE (Q)	0.173	1	0.173	185.183	0.005[a]
Peptone (L)	0.008	1	0.008	8.684	0.098
Peptone (Q)	0.0003	1	0.0003	0.407	0.588
NaCl.YE	0.224	1	0.224	240.482	0.004[a]
NaCl.Peptone	0.006	1	0.006	6.482	0.126
YE.Peptone	0.001	1	0.001	1.339	0.367
Lack of fit	0.010	3	0.003	3.455	0.233
Pure error	0.002	2	0.001		
Total SS	0.469	14			
R^2	0.975				

Adj. R^2 (0.931); CV=16.3%; [a] Model terms are significant.

The model *p*-value = 0.002 means that the model is significant. An appropriate analysis of variance (ANOVA) of the second order model showed a good fit ($R^2 = 0.975$), indicating that 97.5% of the variability in the response could be explained by the model. The adjusted R^2 is a corrected value for R^2 after the elimination of unnecessary model terms. The value of the adjusted R^2 (0.931) is also high to advocate for a high significance of the model. The *p*-value of the lack of fit was 0.233, indicating that the lack of fit was not significant relative to the pure error (Table 3.8). The coefficient of variation (CV) is the ratio of the standard error of estimate to the mean value of the observed response, and as a general rule a model can be considered reasonably

reproducible if the CV is not greater than 10%. CV of 16.3% showed that the statistical quality of the experimental results was acceptable (Mustafa et al., 2016).

3.2.2. Influence of NaCl, yeast extract and peptone concentrations on EPS production

The results obtained for EPS production under different NaCl, yeast extract and peptone concentrations tested are presented in Table 3.7. Maximum EPS concentrations (0.51-0.55 $g.L^{-1}$) were achieved for the highest yeast extract concentration tested (4.0 $g.L^{-1}$ – run 15), and for high concentrations of NaCl, yeast extract and peptone (run 8 – 31.9 $g.L^{-1}$, 3.2 $g.L^{-1}$ and 1.6 $g.L^{-1}$, respectively).

The results show that low concentrations of NaCl, yeast extract and peptone (run 1 – 8.1 $g.L^{-1}$, 0.8 $g.L^{-1}$ and 0.4 $g.L^{-1}$, respectively) and low concentrations of NaCl and yeast extract (run 5 – 8.1 $g.L^{-1}$ and 0.8 $g.L^{-1}$, respectively) and high concentration of peptone (1.6 $g.L^{-1}$), also resulted in a high EPS concentration (0.48-0.50 $g.L^{-1}$).

The central point conditions resulted in intermedium EPS concentrations, as well as for run 2 (0.40-0.43 $g.L^{-1}$). Under the remaining conditions, EPS concentrations were lower, except for runs 4, 6 and 17, wherein a high EPS concentration was obtained (0.45-0.47 $g.L^{-1}$). At runs 13 and 14, no EPS production was observed since there was no growth. In the absence of salt (run 12) the EPS production was lower (0.30 $g.L^{-1}$), however in this run the CDW was also lower.

RSM (ANOVA and MLR) analysis used for EPS production is summarized in Tables 3.9 and 3.10.

Table 3.9– Analysis of variance (ANOVA) of the second order model for parameter EPS production.

Source of variation	EPS				
	Sum of squares	Degrees of freedom	Mean squared	F-value	*p*-value
Model					0.005
NaCl (L)	0.041	1	0.041	136.048	0.007[a]
NaCl (Q)	0.005	1	0.005	17.526	0.053
YE (L)	0.001	1	0.001	4.793	0.160
YE (Q)	0.024	1	0.024	79.793	0.012[a]
Peptone (L)	0.003	1	0.003	10.706	0.082
Peptone (Q)	$5x10^{-4}$	1	0.0005	1.630	0.330
NaCl.YE	0.030	1	0.030	100.042	0.010[a]
NaCl.Peptone	$3x10^{-4}$	1	$3x10^{-4}$	1.042	0.415
YE.Peptone	$1x10^{-5}$	1	$1x10^{-5}$	0.042	0.857
Lack of fit	0.003	3	$9,9x10^{-4}$	3.307	0.241
Pure error	$6x10^{-4}$	2	$3x10^{-4}$		
Total SS	0.094	14			
R^2	0.962				

Adj. R^2 (0.893); CV=19.4%; [a] Model terms are significant.

Statistical analysis (RSM) was also used to evaluate the impact of NaCl, yeast extract and peptone concentrations on the quadratic model for describing EPS production. The model *p*-value = 0.005 means that the model is significant. The correlation coefficient (R^2) and the adjusted correlation coefficient (adj. R^2) were 0.962 and 0.893, respectively, which illustrated that there are excellent correlation between the independent variables and the fitted model can describe the independent variables well. The *p*-value of the lack of fit was 0.241, indicating that the lack of fit was not significant relative to the pure error (Table 3.9). The insignificant *p*-value thus indicates that the model was good and fitted well to the experimental data. CV of 19.4% showed that the statistical quality of the experimental results was acceptable (Mustafa et al., 2016).

3.2.3. Multiple linear regression (MLR)

Multiple linear regression (MLR) gave information about linear (L), quadratic (Q) and interaction effect of NaCl, yeast extract and peptone concentrations on CDW and EPS production (Table 3.10). The CDW was affected mostly by the linear NaCl and yeast extract, the quadratic terms of yeast extract and the interaction between NaCl and yeast extract, for a significance level of 5% ($p < 0.05$). Linear NaCl, the quadratic terms of yeast extract and the interaction between NaCl and yeast extract are the factors which had a significant effect on EPS production, for a significance level of 5% ($p < 0.05$) (Table 3.10).

Table 3.10 – Multiple linear regression (MLR) analysis of the polynomial model: constants and *p*-values for linear, quadratic and interaction effects of NaCl, yeast extract and peptone concentrations for the responses CDW and EPS production.

Effect		CDW (Y_1)	*p*-Value	EPS (Y_2)	*p*-Value
Constant		0.999	$3x10^{-4}$	0.386	$6x10^{-4}$
Linear	NaCl (X_1)	0.127	0.013[a]	0.098	0.007[a]
	YE (X_2)	- 0.072	0.040[a]	- 0.018	0.160
	Pept. (X_3)	0.048	0.098	0.018	0.082
Quadratic	NaCl * NaCl (X_1^2)	- 0.073	0.077	- 0.051	0.053
	YE * YE (X_2^2)	0.291	0.005[a]	0.108	0.012[a]
	Pept. * Pept. (X_3^2)	0.017	0.589	- 0.007	0.330
Interaction	NaCl * YE (X_1X_2)	0.299	0.004[a]	0.109	0.010[a]
	NaCl * Pept. (X_1X_3)	- 0.061	0.126	0.008	0.415
	YE * Pept.(X_2X_3)	0.028	0.367	- 0.002	0.857

[a] Model terms are significant.

3.2.4. Influence of NaCl, yeast extract and peptone concentrations on cell dry weight and EPS production – 3D response graphs

The influence of NaCl, yeast extract and peptone concentrations and their interaction effects can be analyzed by using 3D response graphs. Figure 3.3 (1-3) show the 3D response graphs for CDW. The response surface graphs are drawn by varying two parameters and keeping the other parameter at zero level.

Figure 3.3 (1) shows the response graph for two varying parameters, NaCl and yeast extract concentrations, by keeping the third parameter (peptone concentration) at zero level, which indicates that at minimal (8.1 and 0.8 $g.L^{-1}$, respectively) and at maximal (31.9 and 3.2 $g.L^{-1}$, respectively) of interaction parameters, the CDW is maximum (1.40-1.50 $g.L^{-1}$). The relation between NaCl concentration with respect to peptone concentration is presented in Figure 3.3 (2). The results show that the increase of NaCl concentration until 20.0 $g.L^{-1}$ and a peptone concentration up to 1.6 $g.L^{-1}$ increase the CDW, remaining stable from these values on. Figure 3.3 (3) shows the response graph for two varying parameters, yeast extract and peptone concentration, by keeping the third parameter (NaCl concentration) at zero level. At extremes of interaction parameters, the CDW is maximum. The results suggest that CDW was not significantly affected by peptone concentration, but rather by yeast extract concentration.

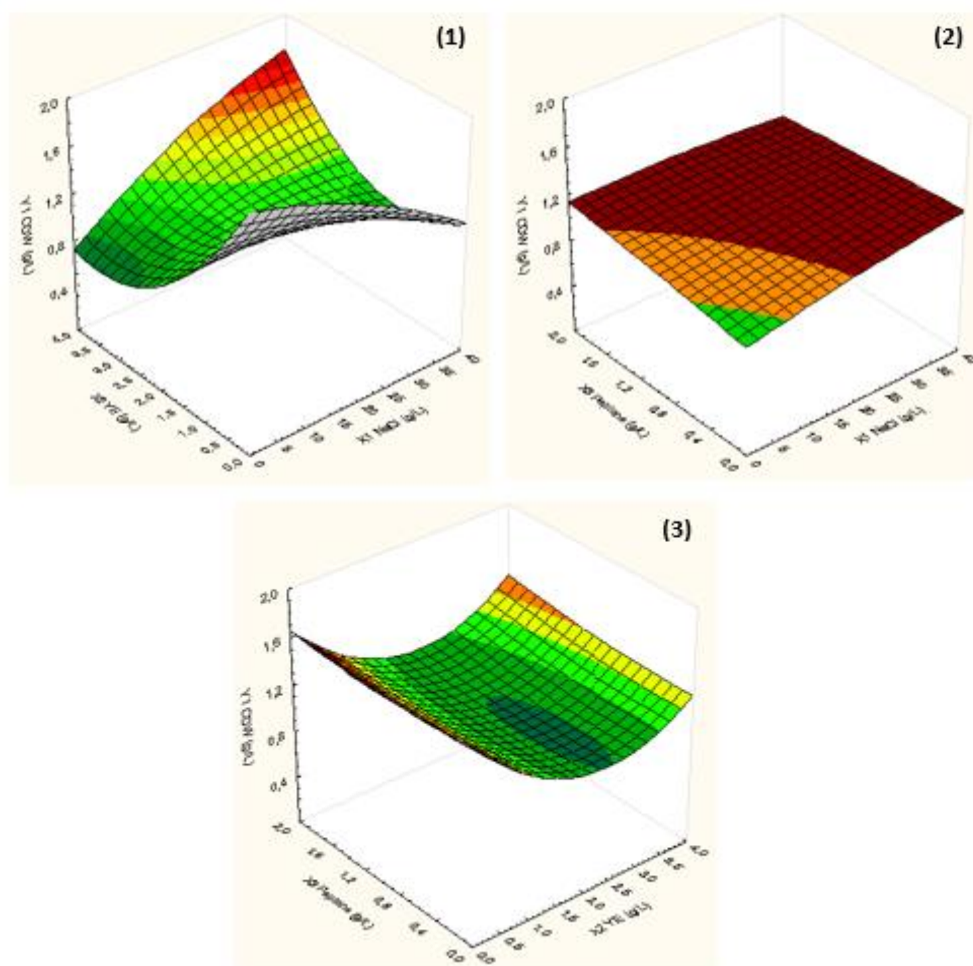

Figure 3.3 – (1) Response surface plot of CDW as a function of NaCl and yeast extract concentrations; (2) Response surface plot of CDW as a function of NaCl and peptone concentrations; (3) Response surface plot of CDW as a function of yeast extract and peptone concentrations.

Figure 3.4 (1-3) show the 3D response graphs for EPS production. The relation between NaCl concentration with respect to yeast extract concentration is presented in Figure 3.4 (1). The results show that at minimal (8.1 and 0.8 $g.L^{-1}$, respectively) and maximal values (31.9 and 3.2 $g.L^{-1}$, respectively) of interaction parameters, the EPS production is maximum (0.51-0.55 $g.L^{-1}$). Figure 3.4 (2) shows the response graph for two varying parameters, NaCl and peptone concentration, by keeping the third parameter (yeast extract concentration) at zero level, which indicates that the increase of NaCl and peptone concentrations until 20.0 $g.L^{-1}$ and 1.6 $g.L^{-1}$, respectively, increase the EPS production.

According with literature, Khalifa et al. (2016) reported that *Enterobacter cloacae* MSR1 exhibited the ability to grow on a medium with 40 $g.L^{-1}$ of NaCl. Sheng et al. (2006) observed that the amount of EPS produced by *Rhodopseudomonas acidophila* increased at a NaCl concentration of 8 $g.L^{-1}$. Also, Chen et al. (2006) reported that, in desert soil bacterium

Microcoleus vaginatus, exposure to NaCl (29.2 g.L^{-1}) resulted in a nearly 50% increase in extracellular carbohydrate. On the other hand, the halotolerant strain *Rhizobium meliloti* EFB1 produced 40% less exopolysaccharides in the presence of 17.5 g.L^{-1} of NaCl (Lloret et al., 1998).

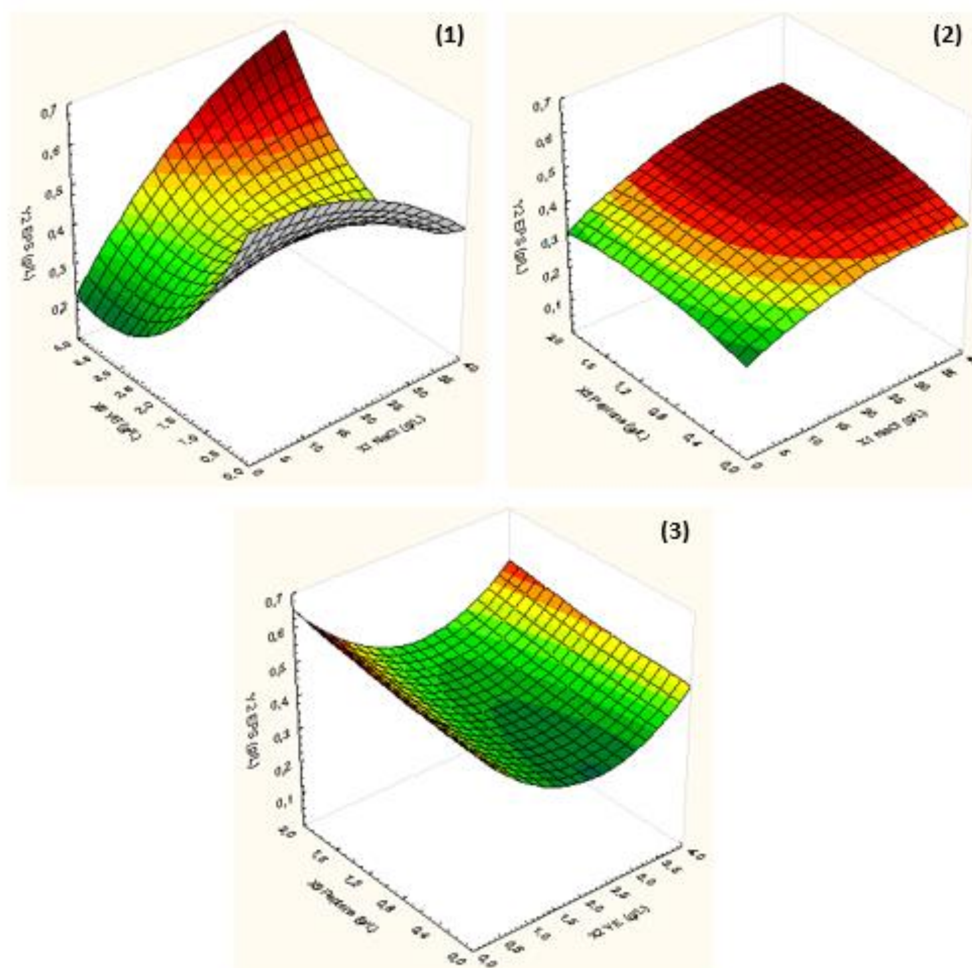

Figure 3.4 – (1) Response surface plot of EPS production as a function of NaCl and yeast extract concentrations; (2) Response surface plot of EPS production as a function of NaCl and peptone concentrations; (3) Response surface plot of EPS production as a function of yeast extract and peptone concentrations.

The relation between yeast extract and peptone concentrations is presented in Figure 3.4 (3). The results show that at extremes of interaction parameters, the EPS production is maximum. However, low concentration of yeast extract (0.8 g.L^{-1}) and high concentration of peptone (1.6 g.L^{-1}) also resulted in a high EPS production (0.47-0.50 g.L^{-1}).

Concerning yeast extract and peptone concentrations, Meade et al. (1994) reported that, for *Enterobacter cloacae*, the use of yeast extract and peptone decreased EPS yields. However,

Hamedi et al. (2007) observed that 4.0 g.L^{-1} of yeast extract gave the best production of EPS for *Agaricus blazei* Murill DPPh 131. Furthermore, the results obtained, in the experimental design, are similar to those attained for *Pseudoalteromonas rubra* QD1-2, by Ding et al. (2014) who found that for this strain the optimum concentrations of yeast extract and peptone were 3.125 g.L^{-1} and 2.21 g.L^{-1}, respectively.

3.2.5. Conclusions

Enterobacter A47 was able to grow and synthesize EPS under most of the experimental conditions assessed.

It was found that maximum CDW values were achieved at low concentration of NaCl (8.1 g.L^{-1}). However, intermediate and high concentrations of NaCl (20.0 and 31.9 g.L^{-1}) also resulted in a high CDW. Concerning EPS synthesis, it was observed that the optimum NaCl concentration range was 20-31.9 g.L^{-1}. However, low concentrations of NaCl (8.1 g.L^{-1}) also resulted in a high EPS production.

Regarding yeast extract and peptone concentrations, it was found that maximum CDW values were achieved at low concentration of yeast extract (0.8 g.L^{-1}). Nevertheless, high concentrations of yeast extract and peptone (3.2 and 1.6 g.L^{-1}, respectively) and high concentration of yeast extract and intermediate concentration of peptone (4.0 and 1.0 g.L^{-1}, respectively) also resulted in a high CDW. Concerning EPS production, it was verified that the optimum yeast extract and peptone concentrations range was 3.2-4.0 g.L^{-1} and 1.0-1.6 g.L^{-1}, respectively. However, low concentrations of yeast extract and peptone (0.8 g.L^{-1} and 0.4 g.L^{-1}, respectively) and low concentration of yeast extract (0.8 g.L^{-1}) and high concentration of peptone (1.6 g.L^{-1}), also resulted in a high EPS production.

Thus, since the supplements used are expensive and low concentrations of NaCl, yeast extract and peptone (run 1 – 8.1 g.L^{-1}, 0.8 g.L^{-1} and 0.4 g.L^{-1}, respectively) also resulted in a high EPS production (0.48 g.L^{-1}), these conditions, namely the use of the modified Schatz medium, were chosen to proceed with the *Enterobacter* A47 bioreactor assays.

3.3. Effect of pH on EPS production by *Enterobacter* A47

This study aimed to assess the impact of pH on EPS production by *Enterobacter* A47, as well as on the polymer´s composition, using the modified Schatz medium described in the previous section.

In order to evaluate the effect of the pH value on EPS production by *Enterobacter* A47, using the modified medium, batch fermentations were performed in 2 L bioreactors and pH values of 6.0, 7.0 and 8.0 were tested, and compared with the results obtained in run E1, in which the pH was 7.0 and the standard Schatz medium was used.

Figure 3.5 shows the cultivation profiles of *Enterobacter* A47 at the three different pH conditions tested: (pH 6.0: E2 – Fig. 3.5 a.), (pH 7.0: E3 – Fig. 3.5 b.) and (pH 8.0: E4 – Fig. 3.5 c.).

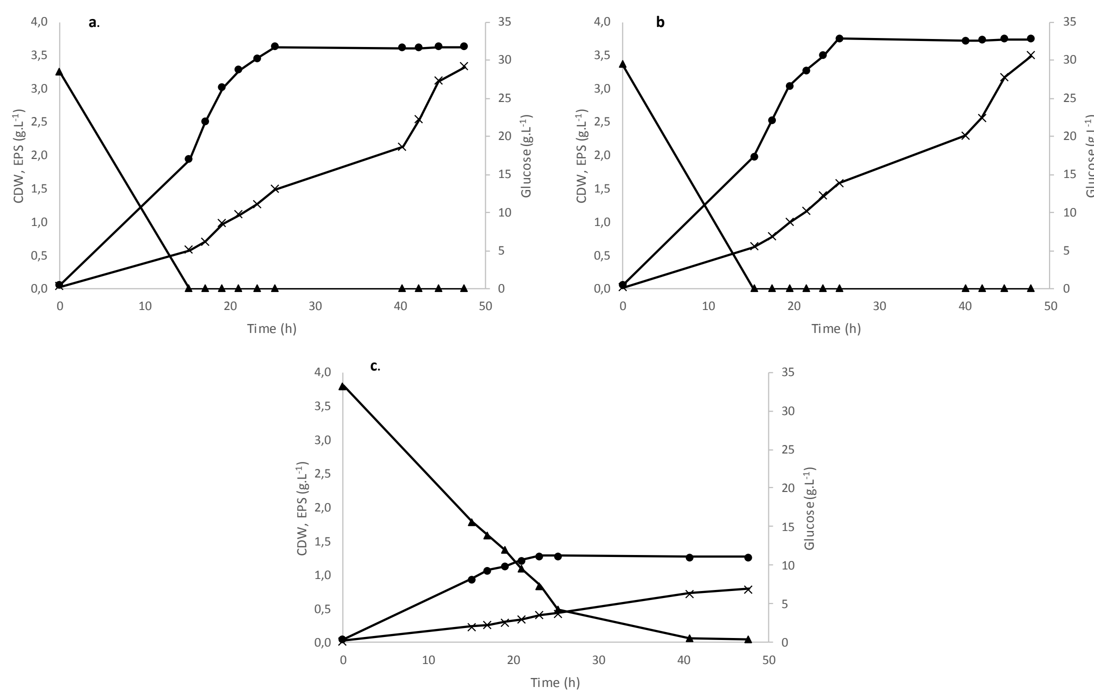

Figure 3.5 – Cultivation profile of *Enterobacter* A47 at different pH values (pH 6.0: E2 – a.; pH 7.0: E3 – b.; pH 8.0: E4 – c.), wherein experimental results of CDW (●), EPS (×) and glucose (▲) are represented throughout the cultivation run.

In runs E2 (pH 6.0) and E3 (pH 7.0), *Enterobacter* A47 reached maximum CDW values of 3.63 $g.L^{-1}$ and 3.75 $g.L^{-1}$, respectively, within 25 hours of cultivation (Fig. 3.5 a. and b.).

In run E4 (pH 8.0), *Enterobacter* A47 showed much lower growth, with a maximum CDW of 1.28 $g.L^{-1}$ within 25 hours of cultivation (Fig. 3.5 c.). Also, in run E4 cell growth appeared to cease when glucose was depleted, unlike other assays where there was significant growth after glucose had been consumed.

These values are much lower than those reported in literature (5.80-13.58 $g.L^{-1}$) for the cultivation in Medium E* supplemented with different carbons sources (Table 3.11). Although the concentration of nitrogen used (0.48 $g.L^{-1}$) was lower than the concentration used in run E1 (1.02 $g.L^{-1}$), the carbon source concentration remained unchanged (30 $g.L^{-1}$). As such, the C:N ratio (25:1, w/w) remained lower than that reported in literature (34:1, w/w), when glucose was used as carbon source (Freitas et al., 2014). Thus, the CDW achieved was lower. However, the CDW obtained in runs E2 and E3 (3.63 $g.L^{-1}$ and 3.75 $g.L^{-1}$, respectively) was slightly higher than that of run E1 (3.20 $g.L^{-1}$) (Table 3.11).

In terms of glucose consumption, in runs E2 and E3, glucose was totally consumed in less than 15 hours and the culture grew to 1.94 $g.L^{-1}$ and 1.98 $g.L^{-1}$, respectively (Fig. 3.5 a. and b.), limiting the growth of the bacteria. In run E4 glucose was also completely consumed, but it took around 41 hours (Fig. 3.5 c.).

Concerning EPS production, compared with the Schatz medium at pH 7.0 (run E1), with the modified Schatz medium (run E3) there was a significant increase in EPS production (3.49 $g.L^{-1}$) (Table 3.11). Thus, it was found that the concentrations of NaCl, yeast extract and peptone in the culture medium had a significant effect on cell growth and EPS production by the bacterium *Enterobacte*r A47, as had already been verified in the experimental design.

It was observed that EPS production was also influenced by the pH value. In the assay at pH 6.0 (run E2) the EPS production (3.33 $g.L^{-1}$) was similar to the production obtained in the assay at pH 7.0 (run E3), whereas at pH 8.0 (run E4) the production of EPS was much lower (0.79 $g.L^{-1}$) (Table 3.11).

In runs E2 and E3, in the first 15 hours were produced 0.58 $g.L^{-1}$ and 0.63 $g.L^{-1}$ of EPS, respectively, while glucose was available (Fig. 3.5 a. and b.). Then the cultures continued to produce, even after growth had ceased. Indeed, after 40 hours, 2.13 $g.L^{-1}$ and 2.29 $g.L^{-1}$ of EPS were already produced in runs E2 and E3, respectively. At the end of the assay, *Enterobacter* A47 had produced 3.33 $g.L^{-1}$ (run E2) and 3.49 $g.L^{-1}$ (run E3) of biopolymer. The EPS production observed after glucose depletion might have been due to the consumption of other medium components, such as yeast extract and/or peptone, which may have functioned as carbon sources for the culture.

Concomitant with EPS production, in run E2, there was an increase of the apparent viscosity of the broth from 1 to 40 mPa.s (measured at 1.7 s^{-1} and 0.025 s^{-1}, respectively). Also, in run E3, there was an increase of the apparent viscosity of the broth from 1 to 50 mPa.s (measured at 1.7 s^{-1} and 0.025 s^{-1}, respectively) throughout the cultivation run.

In run E4, the EPS production was much lower (0.79 $g.L^{-1}$) and occurred while glucose was available. Regarding the apparent viscosity of the broth, there was only a small increase in the apparent viscosity from 1 to 3 mPa.s (measured at 1.7 s^{-1}) throughout the cultivation run.

Table 3.11 – Parameters obtained in the bioreactor cultivation of *Enterobacter* A47 using different substrates, pH values and cultivation modes.

Substrate	Cultivation mode	pH	CDW ($g.L^{-1}$)	EPS ($g.L^{-1}$)	r_p ($g. L^{-1}. d^{-1}$)	References
	Batch mode					
Glucose	E1	7.0	3.20	1.81	0.91	This study
	E2	6.0	3.63	3.33	1.67	This study
	E3	7.0	3.75	3.49	1.75	This study
	E4	8.0	1.28	0.79	0.40	This study
Glycerol	Continuous feeding	5.6	N.A.	7.50	N.A.	Torres et al., 2012
		7.0	N.A.	7.79	N.A.	
		8.4	N.A.	2.67	N.A.	
Glycerol	Continuous feeding	7.0	5.80 – 7.68	7.23 – 7.97	1.89 – 2.04	Freitas et al., 2014 Torres et al., 2011, 2014
Glucose	DO-stat	7.0	8.14	13.40	3.78	Freitas et al., 2014
Tomato paste	pH-stat, DO-stat or Continuous feeding	7.0	9.81 – 13.58	1.65 – 8.77	0.57 – 2.92	Antunes et al., 2017

N.A.: data not available.

Regarding the results reported in the literature, the amount of EPS produced in runs E2 and E3 was higher than that achieved under pH-stat mode (1.65 $g.L^{-1}$) and similar to that obtained under DO-stat mode (3.43 $g.L^{-1}$), using tomato paste as sole substrate (Antunes et al., 2017) (Tables 3.4 and 3.11). However, the values obtained were lower than that reported in the literature (13.40 $g.L^{-1}$) (Freitas et al., 2014), when glucose was used as carbon source (Table 3.11).

The quantity of EPS produced in run E4 was lower than all results previously reported (Table 3.11). At pH 8.0, there may have been changes that led to the cells becoming less viable,

which in turn may have led to a lower production of sugar nucleotide precursors involved in EPS synthesis. Therefore the control of pH at 8.0 was not an adequate strategy to obtain a high EPS production.

The fact that EPS production was lower than that reported in previous studies may be related to differences in the carbon and nitrogen sources concentration and composition, cultivation mode and cultivation conditions. In this work, as was referred for run E1, several parameters were different, namely, the culture medium, the cultivation mode (batch), the air flow rate (2.0 SLPM instead of 0.4 SLPM), and the dissolved oxygen level was controlled at 30% instead of 10% (Antunes et al., 2017; Freitas et al., 2014; Torres et al., 2011, 2012, 2014).

The conclusions obtained are similar to those attained by Torres et al. (2012) who found that *Enterobacter* A47 produced the higher EPS concentration (7.79 $g.L^{-1}$) at pH 7.0 (Table 3.11). Also, an optimal pH of 7.0 was reported for growth and EPS synthesis by *E. agglomerans* WD50 (Prasertsan et al., 2006) and *E. cloacae* WD7 (Prasertsan et al., 2008). Similarly, in other genera it has been seen that EPS production depends on pH. For instance, Kiliç and Dönmez (2008) observed that the suitable pH level to obtain a maximum EPS yield by *P. aeruginosa* is 7.0. Also, according to Quintelas et al. (2011) the highest EPS production by the *Arthrobacter viscosus* was recorded at pH 7.0.

Torres et al. (2012) also reported that, for *Enterobacter* A47, at pH 5.6 the EPS production (7.50 $g.L^{-1}$) was similar to the production obtained at pH 7.0 (7.79 $g.L^{-1}$), whereas at pH 8.4 the production of EPS was much lower (2.67 $g.L^{-1}$) (Table 3.11).

Considering 48 hours the time frame of the cultivation assay, a similar volumetric productivity was achieved in runs E2 and E3 (1.67 $g_{EPS}.L^{-1}.d^{-1}$ and 1.75 $g_{EPS}.L^{-1}.d^{-1}$, respectively), while that of run E4 was considerably lower (0.40 $g_{EPS}.L^{-1}.d^{-1}$) (Table 3.11).

The values attained in runs E2 and E3 are higher than those obtained in previous studies (0.57-1.56 $g_{EPS}.L^{-1}.d^{-1}$), using tomato paste as sole substrate (Antunes et al., 2017), under pH-stat mode, DO-stat mode, and under continuous feeding at 4 $g.L^{-1}.h^{-1}$ and 6 $g.L^{-1}.h^{-1}$ (Tables 3.4 and 3.11). Moreover, the productivity values achieved are higher than the ones obtained for bacterial alginate (0.43-1.53 $g_{EPS}.L^{-1}.d^{-1}$) (Peña et al., 2000) and are in the range of the ones presented for the EPS produced by *Enterobacter cloacae* WD7 (1.68 $g_{EPS}.L^{-1}.d^{-1}$) (Prasertsan et al., 2008), using glucose or sucrose as substrates. However, the values obtained were lower than that reported in the literature (3.78 $g_{EPS}.L^{-1}.d^{-1}$), when glucose was used as carbon source (Table 3.11). Furthermore, the values attained were lower than all the others reported in literature, 1.89-2.92 $g_{EPS}.L^{-1}.d^{-1}$ (Table 3.11).

The sugar composition of the EPS obtained under the different pH values tested is provided in Table 3.12.

Table 3.12 – Sugar composition content of the exopolysaccharides produced by *Enterobacter* A47 from various substrates under different pH values and cultivation modes. Fuc, fucose; Gal, galactose; Glc, glucose; GlcA, glucuronic acid; Rha, rhamnose; GlcN, glucosamine.

Substrate	Cultivation mode	pH	Sugar composition (%mol)						References
			Fuc	Gal	Glc	GlcA	Rha	GlcN	
Glucose	Batch mode E1	7.0	37	23	33	6	–	–	This study
	E2	6.0	47	27	18	7	–	–	This study
	E3	7.0	41	24	29	6	–	–	This study
	E4	8.0	49	29	16	6	–	–	This study
Glycerol	Continuous feeding	5.6	13	13	58	8	6	2	Torres et al., 2012
		7.0	36	26	28	10	–	–	
		8.4	–	12	37	11	29	11	
Glycerol	Continuous feeding	7.0	30 – 36	22 – 29	25 – 34	9 – 10	–	–	Freitas et al., 2014 Torres et al., 2011, 2014
Glucose	DO-stat	7.0	29	29	26	16	–	–	Freitas et al., 2014
Tomato paste	pH-stat, DO-stat or Continuous feeding	7.0	20 – 37	27 – 45	23 – 32	10 – 12	–	–	Antunes et al., 2017

The glycosyl composition analysis of the exopolysaccharides revealed some differences when *Enterobacter* A47 was grown in the modified Schatz medium and in media with different pH. The results obtained in this study indicate that lower concentrations of NaCl, yeast extract and peptone (run E3) led to an increase in fucose (41 %mol), when compared to run E1, where the fucose content was 37 %mol. Concomitantly, there was a decrease in glucose (from 33 %mol to 29 %mol), while the content in glucuronic acid remained the same (6 %mol) (Table 3.12). This sugar monomer profile is different from typical FucoPol, namely, the polymer had a considerably higher fucose content, while the glucuronic acid content was lower.

Lowering the pH from 7.0 to 6.0 (run E2) had a much more noticeable effect on the composition of the EPS since fucose and galactose contents were increased to 47 %mol and 27 %mol, respectively, while glucose content was reduced to 18 %mol. Additionally, there was also a slight increase in the glucuronic acid content to 7 %mol (Table 3.12). However, the sugar monomer profile is different from that obtained by Torres et al. (2012) at pH 5.6, namely, the polymer had higher fucose and galactose contents, while the glucose content was lower. In addition, the polymer obtained did not show rhamnose and glucosamine (Table 3.12).

The pH increase from 7.0 to 8.0 also had large effects on the EPS composition, since the content in fucose and galactose also increased (49 %mol and 29 %mol, respectively), while the content in glucose decreased (16 %mol). The content in glucuronic acid remained the same when compared to the assay at pH 7.0 (run E3) (Table 3.12). Nevertheless, the sugar monomer profile is different from that obtained by Torres et al. (2012) at pH 8.4, namely, the polymer had a higher galactose content, while the glucose and glucuronic acid contents were lower. Moreover, the polymer obtained had fucose, but did not show rhamnose and glucosamine (Table 3.12).

3.3.1. Conclusions

According to the results, the pH values tested in these assays seemed not only to have an impact in EPS production and productivity, but also in the polymer´s composition. Although the growth and EPS production have been lower than those obtained in previous studies, the fact that the polymer has a higher fucose content makes it very interesting, since fucose is a rare sugar that is difficult to obtain. In addition, it was observed that the highest EPS production (3.49 $g.L^{-1}$) and productivity (1.75 $g_{EPS}.L^{-1}.d^{-1}$), by *Enterobacter* A47, were reached at pH 7.0.

4. Conclusions and future work

In this thesis, the production of bacterial exopolysaccharides was studied, using a Schatz medium, supplemented with yeast extract and peptone, and glucose as carbon source.

In the shake flask assays, all the bacteria studied, *Pseudoalteromonas atlantica* MD12-331 A, *Pseudoalteromonas shioyasakiensis* MD12-375, *Pseudoalteromonas mariniglutinosa* MD12-501 and *Psychrobacter submarinus* MD12-530 b and *Enterobacter* A47, were able to grow and produce EPS under the culture conditions tested. The cultures achieved EPS productions of 0.14-0.67 $g.L^{-1}$, within 96 hours of cultivation, corresponding to volumetric productivities of 0.04-0.17 $g_{EPS}.L^{-1}.d^{-1}$.

In the bioreactor assays, the marine bacteria were able to produce 1.33-1.91 $g.L^{-1}$ of EPS, within 48 hours of cultivation, corresponding to volumetric productivities of 0.67-0.96 $g_{EPS}.L^{-1}.d^{-1}$, with the highest EPS production being achieved by *Pseudoalteromonas mariniglutinosa* MD12-501. The polymers produced by *Pseudoalteromonas atlantica* MD12-331 A and *Pseudoalteromonas shioyasakiensis* MD12-375 were composed of glucose (96 %mol and 79 %mol, respectively) and rhamnose (4 %mol and 21 %mol, respectively). The polymers produced by *Pseudoalteromonas mariniglutinosa* MD12-501 and *Psychrobacter submarinus* MD12-530 b were composed of glucose (44 and 46 %mol, respectively), galactose (18 %mol), rhamnose (14 and 13 %mol, respectively), fucose (12 %mol), galacturonic acid (9 %mol) and glucuronic acid (3 and 2 %mol, respectively).

Furthermore, *Enterobacter* A47 was able to grow and synthesize 1.81 $g.L^{-1}$ of EPS, within 48 hours of cultivation, corresponding to a volumetric productivity of 0.91 $g_{EPS}.L^{-1}.d^{-1}$. The extracellular polysaccharide obtained was composed of fucose (37 %mol), galactose (23 %mol), glucose (33 %mol) and glucuronic acid (6 %mol). Although *Pseudoalteromonas mariniglutinosa* MD12-501 presented slightly higher EPS production and productivity values, the polymer produced by *Enterobacter* A47 presented a higher fucose content, which is very interesting since fucose is one of the rare sugars difficult to obtain in Nature. Thus, *Enterobacter* A47 was selected for further optimization studies.

However, in future studies, the culture conditions of marine bacteria can still be optimized.

The influence of NaCl, yeast extract and peptone concentrations on cellular growth and EPS production by *Enterobacter* A47 was also evaluated. The optimal NaCl, yeast extract and peptone concentrations were determined to be 8.1 $g.L^{-1}$, 0.8 $g.L^{-1}$ and 0.4 $g.L^{-1}$, respectively, due to the high EPS production achieved. Moreover, the reduction in the concentration of the supplements used implied a considerable diminution of the overall EPS cost production, since yeast extract and peptone are expensive.

In future studies, exopolysaccharide production by *Enterobacter* A47 can be further optimized in terms of carbon source concentration. Moreover, operational parameters, such as dissolved oxygen concentration and aeration, can also affect cell growth and polymer synthesis and, hence, their effect should be studied. Additionally, other bioreactor operation modes (e.g. fed-batch, continuous) may also be tested, as well as feeding strategies (e.g. pulses).

In this work, the impact of pH on EPS production, as well as on the polymer´s composition, using the modified Schatz medium by *Enterobacter* A47 was also investigated. It was found that the use of the modified Schatz medium and pH control at 7.0 led to an increase in EPS production, when compared to the standard conditions. In fact, an EPS production of 3.49 $g.L^{-1}$ was achieved, within 48 hours of cultivation, corresponding to a volumetric productivity of 1.75 $g_{EPS}.L^{-1}.d^{-1}$. It was also observed that the decrease from pH 7.0 to pH 6.0 had little effect on EPS production (3.33 $g.L^{-1}$), whereas at pH 8.0 a significant reduction in EPS synthesis (0.79 $g.L^{-1}$) was observed.

The extracellular polysaccharides obtained at pH 6.0, 7.0 and 8.0 were composed of fucose (47, 41 and 49 %mol, respectively), galactose (27, 24 and 29 %mol, respectively), glucose (18, 29 and 16 %mol, respectively) and glucuronic acid (7, 6 and 6 %mol, respectively). As a function of pH, *Enterobacter* A47 revealed the ability to synthesize different exopolysaccharides, a feature that can be further exploited to obtain tailored polymer compositions. In addition, the polymers obtained had a high fucose content, which, as previously mentioned, is very important. Thus, future studies can be done to increase the productivity of this fucose-enriched polymer.

Moreover, in the future, the total nitrogen must be determined, and NH_4^+ and NO_3^- must be quantified, since they can be used as nitrogen source.

Also, in future studies, the functional properties (e.g. rheology, film-forming capacity, emulsion forming and stabilizing capacity, flocculation) of the distinct exopolysaccharides, synthesized by *Enterobacter* A47, should be further explored in order to determine if their different physical-chemical characteristics conferred them different interesting properties.

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6. Appendixes

6.1. Glucose calibration curves

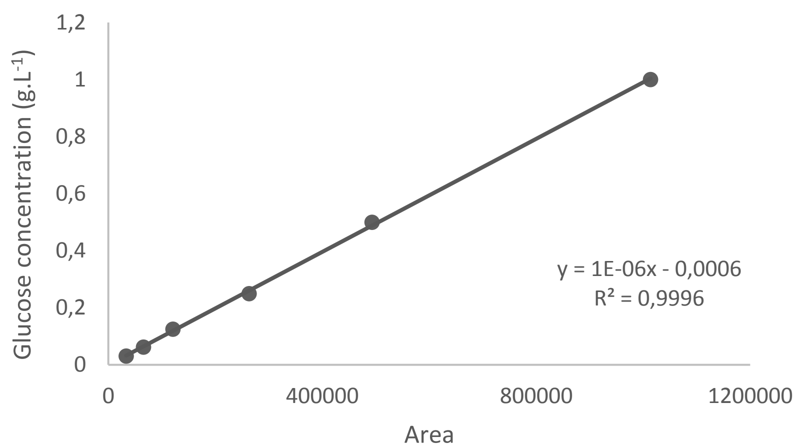

Figure 6.1 – Glucose calibration curve used in runs P1 – P4.

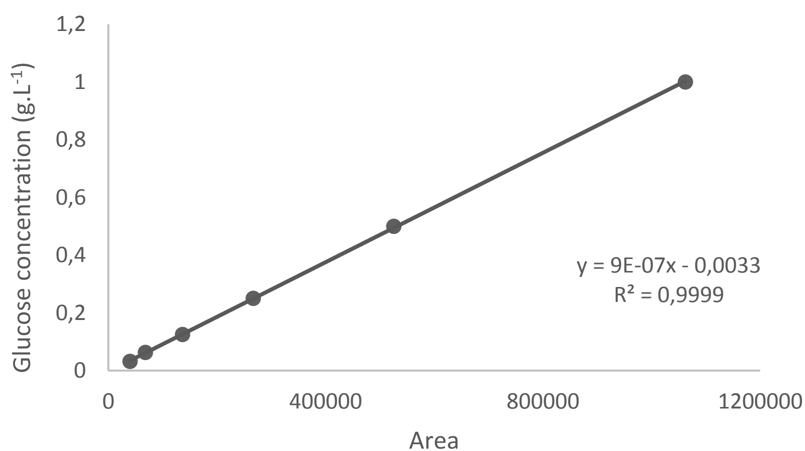

Figure 6.2 – Glucose calibration curve used in runs E1 – E3.

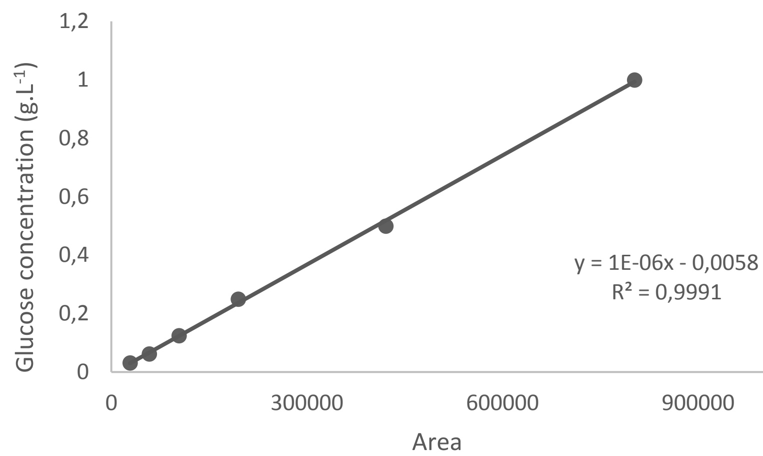

Figure 6.3 – Glucose calibration curve used in run E4.