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Exopolysaccharide production by *Enterobacter* A47: optimization of cultivation conditions and study of polymer functional properties

Dissertação para obtenção do Grau de Mestre em Biotecnologia

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Resumo

O FucoPol é um biopolímero de elevado conteúdo em fucose, produzido pela bactéria *Enterobacter* A47 DSM 23139. Trata-se de um exopolissacárido microbiano (EPS) cuja composição engloba açúcares neutros (fucose, galactose, glucose), açúcar acídico (ácido glucurónico), e ainda grupos acilo substituintes (acetato, piruvato e succinato).

No âmbito deste trabalho foram considerados dois objectivos principais: a optimização da produção de EPS e o estudo das propriedades funcionais de diversos polímeros produzidos pela *Enterobacter* A47. No estudo da optimização do FucoPol, foi avaliado tanto o efeito do oxigénio dissolvido em diferentes concentrações (10, 30 e 60%), como o impacto da redução de concentração de fosfato no meio de cultura. Desta forma, não só foi possível observar o efeito de ambos os parâmetros no crescimento celular e na capacidade produtiva bacteriana, como também, a sua influência na composição química do EPS. A produção máxima de EPS (6.11 g L^{-1}) foi obtida a 10% de oxigénio dissolvido, e, embora a redução de fosfato no meio de cultivo não tenha influenciado a capacidade produtiva bacteriana, esta originou uma diminuição do conteúdo em fucose até 24%mol.

Posteriormente, foram avaliadas as propriedades funcionais de exopolissacáridos produzidos pela *Enterobacter* A47, a diferentes condições de cultivo. A maioria dos polímeros evidenciou um comportamento reofluidificante, atingindo maior viscosidade aparente (0.2 - 0.3 Pa.s) a menores taxas de escoamento (0.3 a 1 s^{-1}) para os polímeros EPS-g, EPS-s e GNEX. Também na sua maioria, os polímeros apresentaram capacidade para formar e estabilizar emulsões a diferentes temperaturas. Relativamente à capacidade filmogénica, EPS-s, EPS-g e EPS-x demonstraram boas propriedades mecânicas juntamente com elevada permeabilidade ao vapor de água. Todos os polímeros demonstraram actividade floculante à concentração de 0.01%, sendo esta substancialmente diminuída a uma menor concentração de EPS (0.001%).

Palavras-chave: *exopolissacárido*, *oxigénio dissolvido*, *fosfato*, *propriedades funcionais*.

Abstract

FucoPol is a fucose-containing biopolymer produced by the bacterium Gram-negative, *Enterobacter* A47 DSM 23139. It is an exopolysaccharide (EPS) composed of neutral sugars (fucose, galactose, glucose), an acidic sugar (glucuronic acid), and also non-saccharide substituents (acetate, pyruvate and succinate).

In this work, two primary objectives were considered: the optimization of EPS production by varying two different parameters, and the functional properties' assessment of different EPS produced by the bacterium. In the optimization study, the influence of different dissolved oxygen concentrations (controlled at 10, 30 and 60% of air saturation), and the impact of phosphate concentration reduction in the culture medium (5.25 and 3.81 g L^{-1}) were assessed. The objective was to evaluate not only the effect of both parameters on cellular growth and exopolysaccharide-synthesis, but also to determine their influence in EPS chemical composition. The best results were obtained with DO at 10%, with the highest EPS production (6.11 g L^{-1}), and although a reduction of phosphate concentration didn't affect the EPS production, it reduced the exopolysaccharide's fucose content to 24%mol.

Secondly, the functional properties of nine distinct exopolysaccharides synthesized by *Enterobacter* A47 under different cultivation conditions were evaluated. Rheologically, most of the EPS polymer solutions showed shear-thinning behavior, wherein EPS-s, EPS-g and GNEX achieved the highest apparent viscosity (0.2 - 0.3 Pa.s) at lower shear rates (0.3 to 1 s^{-1}). Also, most of the polymers presented emulsifying capacity at different temperatures. Concerning the film-forming capacity, EPS-s, EPS-g and EPS-x films demonstrated to have good mechanical properties and high water vapour permeability. And, all polymers described flocculating activity at 0.01% of EPS, which was significantly diminished at lower concentration, 0.001%.

Keywords: *exopolysaccharide*, *dissolved oxygen*, *phosphate*, *functional properties*.

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Nomenclature

Acronyms

CDW ($g\ L^{-1}$)	Cell dry weight
DO (%)	Dissolved oxygen
EPS ($g\ L^{-1}$)	Exopolysaccharide
GRP	Glycerol-rich product
vvm	Volume of gas per volume of liquid per minute

Kinectic parameters

μ (h^{-1})	Specific growth rate
$Y_{X/S}$ ($g_{cell}\ g_{glycerol}^{-1}$)	Yield of biomass on glycerol
$Y_{P/S}$ ($g_{EPS}\ g_{glycerol}^{-1}$)	Yield of exopolysaccharide on glycerol
r_P ($g\ L^{-1} h^{-1}$)	Volumetric productivity

1. Introduction

Bacterial cells possess a cellular membrane that separates them from the exterior and whose sole function is to select the movement of ions, nutrients, metabolic products, among others. The cellular membrane can present different external structures: flagella and fimbriae, for locomotion and adhesion purposes, respectively, a cell wall and a capsule (Lopes et al. 2005) (Figure 1.1).

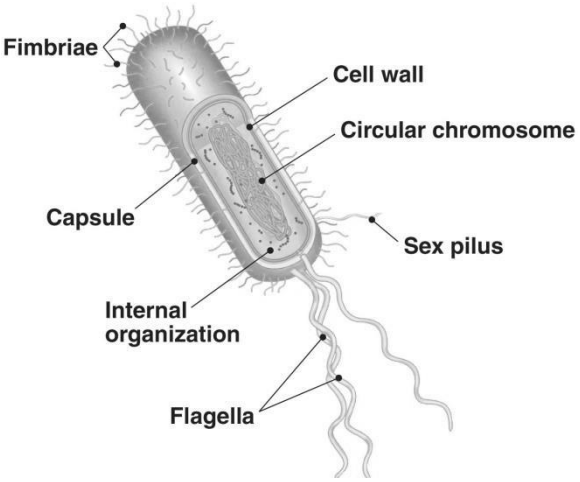

Fig. 1.1 – Bacterial external structure representation and its circular DNA molecule identified as circular chromosome. (http://science.kennesaw.edu/~jdirnber/Bio2108/Lecture/LecBiodiversity/BioDivProkaryotes.html) assessed at 11.09.2013

On the other hand, the capsule, mostly constituted by polysaccharides, is often associated to the cell's protective response towards external factors (Lopes et al. 2005). Its physiological roles can vary from desiccation protection due to its high water content, protection against predatory microorganisms or even blockage of other microorganisms' interactions. The capsule can be highly structured, being associated with the cell surface membrane and may be covalently bound to it, or highly disorganised, resulting into a slime layer of extracellular polymer, loosely bound to the cell surface (Vanhooren et al. 1998). The later, are known as extracellular polysaccharides or exopolysaccharides (EPS) and their different structural organization is dependent on the specific bacterial secretion systems (Whitney et al. 2013).

The EPS are hydrophilic and high molecular weight polymers, mainly composed of carbohydrates, with glucose, galactose and mannose being the most common monomers, wherein neutral sugars (e.g. rhamnose and fucose), some uronic acids (mainly glucuronic and galacturonic acids) and aminosugars (N-acetylamino sugars) are also frequently present. Bacterial EPS might also contain non-sugar functional groups, like ester-linked and ketal-linked pyruvate as organic substituents (Sutherland 1994).

Polysaccharides can be derived from many natural sources. They may be plant based (e.g. guar gum, arabic 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, gelan, pullulan and bacterial alginate) (Kaur et al. 2012).

Microorganisms are found to be more advantageous than plants, crustacean and algae for exopolysaccharide production, due to their intrinsic characteristics. Bacteria usually have

higher specific growth rates and allow manipulation of the growth conditions for improving fermentation yields, productivity and biopolymer's properties (Freitas et al. 2009a).

Many Gram-negative EPS-producing bacterial species have been reported in the literature over the last decades. For example, *Azotobacter vinelandii* (Sabra et al. 1999), *Agrobacterium* sp. (Lee et al. 1999), *Sphingomonas paucimobilis* (Ashtaputre et al. 1995) and *Xanthomonas campestris* (Liakopoulou-Kyriakides et al. 1999) and their products have gainned considerable commercial interest in the past few years.

These biopolymers possess numerous properties depending on their chemical composition, molecular structure, average molecular weight, and distribution. The main properties of polysaccharides that are relevant for their industrial development include rheology modifier of aqueous solutions, gel-forming ability, and emulsifying and/or flocculating capacity, among others. As such, they can be valorized into food, pharmaceuticals, cosmetics and agriculture applications, just to name a few (Freitas et al. 2011a).

1.1 Substrate

The major limitation for EPS production at large scale is their inherent production costs. For this reason expensive substrates (e.g. refined sugars as glucose, sucrose, etc.) usually used in microbial EPS production are being replaced by some manufactures by cheaper and more accessible substrates, like waste residues or agro-industrial by-products, in order to have more cost-effective processes (Du et al. 2011).

Biodiesel is a well-known biofuel that has been contributing to the worldwide bioenergy production scenario, with a significant share of the renewable sources. It is originated by the transesterification of vegetable oils and animal fats in the presence of a catalyst, through a primary alcohol (usually methanol or ethanol), leading to the product formation of fatty acid methyl esters, FAMES (Rywinska et al. 2013), along with a major by-product formation, crude glycerol.

The worldwide biodiesel production has been increasing exponentially, raising from 4 million m^3 in 2005 to 19.21 million m^3 in 2010. Approximately 1 kg of crude glycerol is generated for every 10 kg of biodiesel produced (Delgado et al. 2013). Consequentially, the crude glycerol production, that initiated with almost 0.5 million m^3 in 2005 rose to 2.0 million m^3 in 2010 (Almeida et al. 2012). This parallel increase of crude glycerol originated an enormous amount of energy stocked (turning it into a waste residue), as opposed to the purified glycerol that is utilized as a raw material in several industrial sectors.

The main disadvantages of crude glycerol for use in several of the glycerol traditional applications rely on its variable composition and lower purity degree associated to contaminants. Crude glycerol contains several impurities, such as fat and protein contents ranging from 1 to 13 % and 0.06 to 0.44 %, respectively, whereas metals (Ca^{2+}, K^{+}, Mg^{2+}) and other macroelements (P and S) are commonly present in smaller concentrations, from 4 to 163 mg L^{-1}, in exception for Na^{2+}, which averages barely over 1% (Rywinska et al. 2013). However, these industrial wastes' inherent characteristics can be overcome by refining their composition, or using microbes tolerant to inhibitors present in this case in crude glycerol, converting it into various biotechnological products.

On the other hand, industrial waste prices can also be decisive for the approval of crude glycerol as a primary carbon resource. As such, crude and pure glycerol's prices in Europe were assessed at 200-260 and 480-530 euros per ton, respectively, in September of 2011 (Almeida et al. 2012), constituting an attractive and crucial factor to its growing preference towards biotechnological applications.

1.2 Exopolysaccharide composition

Bacterial EPS of Gram-negative bacteria can be homopolysaccharides, usually with D-glucose as the repeating unit, or heteropolysaccharides (Figure 1.2 and 1.3), whose repeating units differ not only along its structure but also in its size distribution, ranging from disaccharides to octasaccharides.

In heteropolysaccharides, sugar monomers can be of up to four different types and classified into neutral, uronic or aminated sugars. In addition to its sugar backbone, heteropolysaccharides may also contain non-carbohydrate substituents, such as amino acids, organic acids (e.g. acetate, succinate, pyruvate, glycerate, proprionate and hydroxybutanoate) and inorganic acids (phosphate and sulphate) (Sutherland 2001).

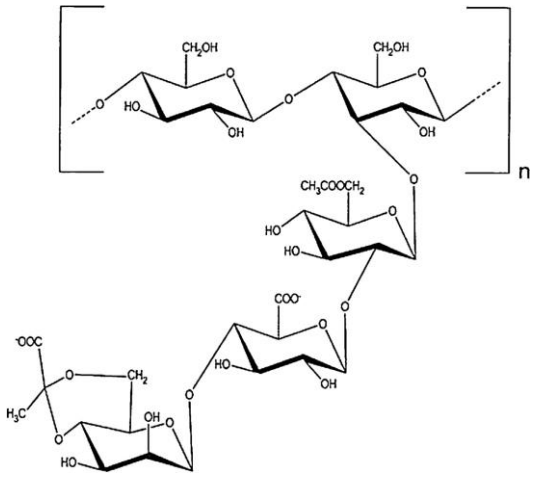

Fig. 1.2 - The ideal repeating unit of xanthan as reported by Jansson et al. 1975.

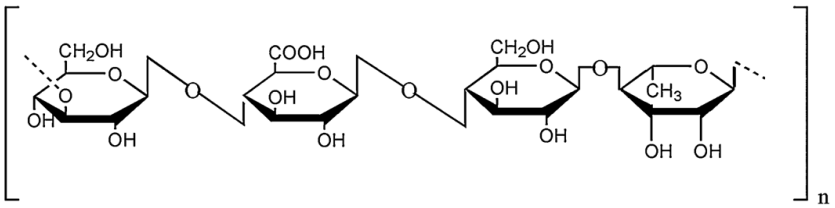

Fig. 1.3 - Gellan structure: repeating units of D-glucose, D-glucuronic acid, D-glucose, and L-rhamnose (Prajapati et al. 2013).

The acyl groups play an important role in the exopolysaccharide properties since the charged residues, found on the exterior of the extended molecules, may promote interaction with ions and other macromolecules (Chandrasekaran 1997). That is originated for instance by pyruvate, glycerate and succinate, which confer an anionic character to the polysaccharides (Freitas et al. 2009a).

1.3 Exopolysaccharide structure

A polysaccharide is constituted by long chains of monosaccharide units linked together by glycosidic linkage. The glycosidic linkages can be either *alfa* or *beta*, and vary between 1→3; 1→4; 1→6 or 1→2 to form either linear or ramified structures (Kumar et al. 2007).

For instance, the bacterial EPS curdlan is composed entirely of 1→3 β-linked D-glucose residues, while fungal scleroglucan has the same backbone structure as curdlan but has attached side-chains of 1→6 β-linked D-glucose (Sutherland 1994).

The EPS structure has direct influence on its function once the presence of side-chains on linear polysaccharide macromolecules promote conformational disorder and inhibit ordered assembly, therefore resulting in solubility in aqueous solutions (Rinaudo 2004).

1.4 Bacterial biosynthetic pathways

Bacterial EPS are synthesised by either Gram-positive or Gram-negative bacteria, by two very distinct mechanisms. In Gram-positive bacteria, EPS (e.g. levans, alternans and dextrans) are synthesized by an extracellular process (Vanhooren et al. 1998) but in Gram-negative bacteria they are synthesized intracellularly (e.g. xanthan, gellan, cellulose, succinoglycan) (Sutherland 2001).

The intracellular biosynthesis is regulated by enzymes that are located in various regions of the cell. In the citoplasm, glucose-1-phosphate (G-1P) is converted (Figure 1.4) to the key molecule in exopolysaccharide synthesis, uridine diphosphate glucose (UDP-Glc) (Figure 1.4). Afterwards, in the cell periplasmic membrane, glycosyltransferases transfer the nucleosides diphosphates sugars (NDPs) to form the repeating unit attached to a glycosyl carrier lipid. Finally, the macromolecules are polymerized and secreted (Kumar et al. 2007).

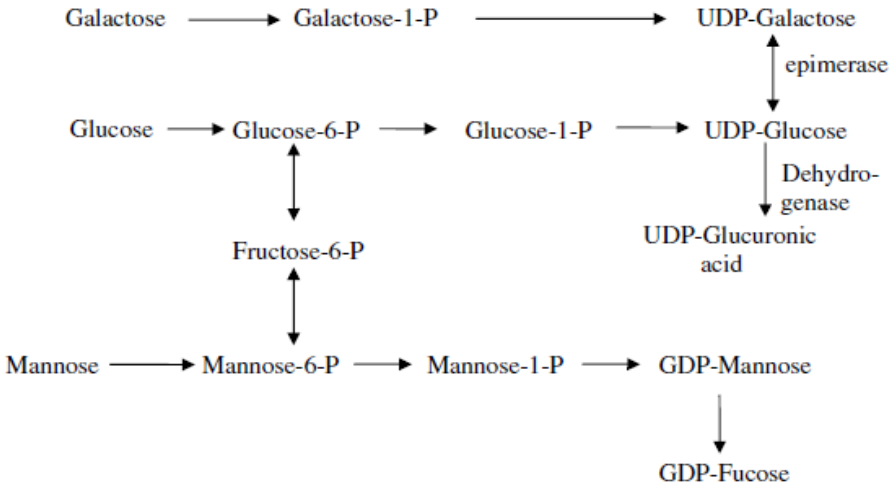

Fig. 1.4 - Catabolic mechanism representing the sugar nucleotide synthesis and the interconversion of various monosaccharides through epimerization, dehydrogenation and decarboxylation, occurred in the cell cytoplasm. (Kumar et al. 2007).

1.5 Exopolysaccharides properties

Microbial polysaccharides stand out from traditional plant polysaccharides due to their unique or superior physical properties (Sutherland 1998). Aside from their intrinsic biodegradability, non-toxicity and biocompability characteristics, this type of natural polysaccharides can possess many properties that could be broadly used in industrial applications: as emulsion stabilizing and gelling agents in food products; as foam stabilizing agents in the beverage industry and to fire-fighting fluids; as inhibitors of crystal formation in

frozen food, pastilles and sugar syrups; as film-forming agents that have special interest in food coatings; as flocculant agents, applied into water clarification; as hydrating agents in cosmetics and pharmaceutical products, among others (Sutherland 1998). Most of these applications have been developed and improved, for xanthan, gellan and hyaluronan (Mishra et al. 2013), and their potential has also been introduced into bioplastic industry and designed to meet therapeutic trends, as drug-delivery composites, for example (Halley et al. 2011).

1.5.1 Rheological properties

Various microbial polysaccharides show high water solubility, producing aqueous solutions with interesting rheological properties that can be used as viscosifying, thickening, stabilizing and/or gelling agents in several applications (Freitas et al. 2009a).

Aqueous solutions of polysaccharides can be characterized as Newtonian or non-Newtonian fluids (Verbeeten 2010).

For a Newtonian fluid, there is a direct relationship between the shear stress (τ) and its shear rate ($\dot{\gamma}$) (Figure 1.5). A non-Newtonian fluid is a fluid whose flow properties aren´t proportional to the shear rate, being non-linear and even time-dependent (Figure 1.5).

Without any applied force, the EPS in the aqueous media are randomly arranged and present no resistance. But, when a unidirectional shear stress is applied, an initial resistance can be observed, before the fluid starts to flow. Hence, when the biomolecules are moving, they tend to entangle themselves with each other, resulting in an enhanced resistance to flow (McNeil et al. 1993; Gibbs et al. 2000). Thus the relationship between shear stress and shear rate is not constant, depending on the imposed degree of shearing (Seviuor et al. 2011).

Regarding shear stress, shear-thinning fluids appear to be thinner (exhibiting lower viscosity) at higher shear stresses due to the increased alignment of the molecules, while at low shear stresses, they appear to be thicker (exhibiting higher viscosity) in result of the entanglements between their molecules (Figure 1.5).

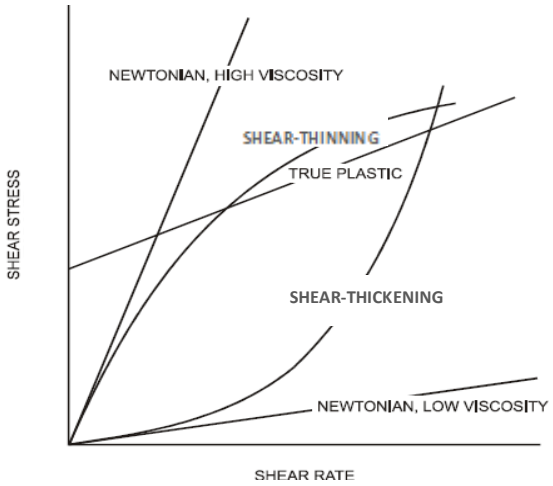

Fig. 1.5 - Flow curves corresponding to different types of liquid behavior: Newtonian fluids and non-Newtonian fluids. (http://www.globalspec.com/reference/10735/179909/chapter-3-physical-properties-of-fluids-vapor-pressure-and-boiling-point-of-liquids) assessed at 11.09.2013

Shear-thinning fluids are the largest and probably most important class of non-Newtonian fluids (Cross 1965).

Many non-Newtonian fluids, like most EPS aqueous solutions, exhibit both viscous and elastic properties when undergoing deformation, behavior that is known as viscoelasticity. Further, non-linear viscoelastic materials exhibit mechanical properties that are dependent upon time and magnitude of the stress that is applied to the material, simultaneously (Vélez-Ruiz et al. 1997). On the contrary, mechanical properties of linear viscoelastic materials only are dependent upon time.

The viscoelastic behavior of EPS aqueous solutions is evaluated by measuring the storage modulus and the loss modulus (Figure 1.6).

The storage modulus (G') or elastic modulus, measures the ability of the material to store energy or its ability to recover while the loss modulus (G'') or viscous modulus, expresses the ability of the material to dissipate energy as heat (per cycle of deformation). In a perfect elastic-solid polymer wherein all energy is stored, G'' is zero, in opposition to a liquid that without any elastic properties, i.e., all the energy is dissipated, G' is in turn, zero (Rao 2007).

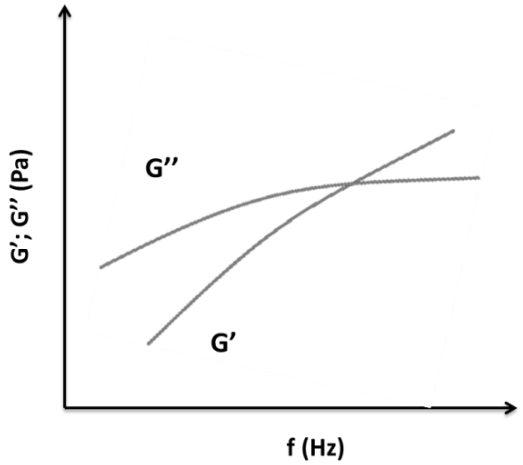

Fig. 1.6 - Frequency sweep graphic, representing both elastic (G') and viscous (G'') modulus at fixed stress and temperature.

1.5.2 Emulsion forming and stabilizing capacity

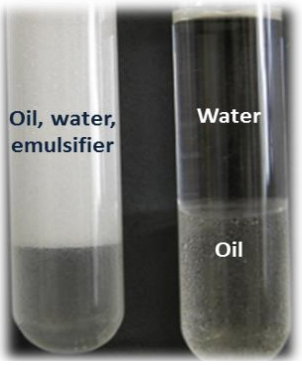

Fig. 1.7 - An emulsion *vs* an unblended oil-water liquid system. (http://www.fiocruz.br/ccs/media/emulsao%20biosurfactante.JPG) assessed at 14.09.2013.

In an emulsion, one liquid (the dispersed phase) is dispersed in other liquid (the continuous phase), presenting a two-phase system (Figure 1.7). This system may exhibit structural changes depending on the characteristics of the phases, and the conditions that they are under (Vianna-Filho 2013).

Emulsifying compounds can be low molecular weight molecules that efficiently lower the surface tension and interfacial tensions or high molecular weight molecules, which bind tightly to surfaces thus being more effective at stabilizing water/oil emulsions (Abbasi et al. 2008). These characteristics are found in some neutral, eg. galactomannans, or in anionic polysaccharides, eg. xanthan gum and fugogel, which possess emulsion forming and stabilizing capacity (Vianna-Filho 2013).

Biobased emulsions can be utilized in engineered multilayered 'smart' delivery systems, for targeted delivery of specialized bioactive agents and functional foods, to combat diseases and to promote and sustain good health. Hence, biobased emulsions can play a fundamental role in many fields, including the pharmaceutical, cosmetics and food industries (Imam et al. 2012).

1.5.3 Flocculation capacity

Flocculation is first and foremost a chemical process wherein particles dispersed in a liquid, and not actually dissolved in solution, come out of suspension in the form of stable flocs or aggregates (Chaiwong et al. 2008) (Figure 1.8). This separation occurs by the addition of a flocculating agent, into the previous heterogeneous mixture.

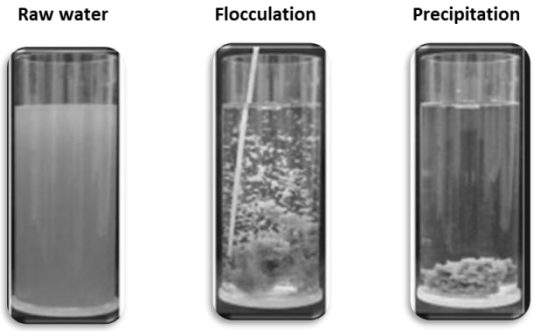

Fig. 1.8 – Representation of the water treatment steps (an example).

Differently to precipitation, in a flocculated system, there's no cake formation, since all the particles behave like flocs or aggregates. Still, and because the particles are not in physical contact it is also possible to reverse the formation of aggregates.

Conventionally, many different flocculants are used in several industrial processes, such as, wastewater treatment, dredging, brewing, downstream processing, fermentation and food processing (Dermlin et al. 1999). However the most commonly used products are synthetic flocculants, organic (eg. polyacrylamide, polyethylenimine) and inorganic (eg. polyaluminium chloride and aluminium sulphate), that have shown to be carcinogenic and neurotoxic (Kumar et al. 2004).

In light of this, the naturally occurring flocculant agents (eg. chitosan, sodium alginate, gelatin and microbial polymers), have been recently preferred due to their biodegradability factor and innocuous degradative intermediates (Mandal et al. 2013). The flocculating substances produced by microorganisms are expected to be useful since they are environmentally safer and also can be produced uniformly and reliably by fermentation (Prasertsan et al. 2006).

1.5.4 Film-forming capacity

As mentioned before, polysaccharides have a huge variety of functional attributes. Their unique macromolecular features turn them into very attractive raw materials for the public consumption, in chemicals, pharmaceuticals, tissue engineering products, nutraceuticals, biomedical devices, building materials and enhanced oil recovery aids (Imam et al. 2012).

Some natural polysaccharides, from soluble soybean (Tajik et. al. 2013), chitosan (Bourbon et al. 2011), levan and alginate (Freitas et al. 2011a), for example, have film-forming capacity (Figure 1.9).

This property can be useful in bioplastics and packaging industries where they are used for tailoring the mechanical and barrier properties of those materials and improving the efficiency of packaged foods conservation (Tajik et. al. 2013).

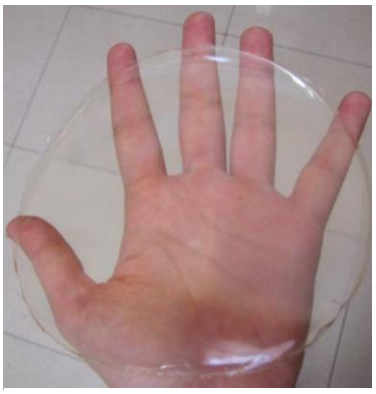

Fig. 1.9 - Soluble soybean polysaccharide based film. (Tajik et. al. 2013)

In order to characterize the exopolysaccharide biofilm's properties several parameters should be studied, including their water vapour permeability and the mechanical properties.

The water vapour permeability measures the diffusion capacity of the water molecules through the polymer matrix (Alves et al. 2010a), while the mechanical properties of the films, namely tensile strength and elongation at break are important to evaluate their ability to perform in different applications (Xu et al. 2005).

1.6 FucoPol

FucoPol is a high molecular fucose-containing exopolysaccharide (EPS) produced by *Enterobacter* A47 (DSM 23139) (Freitas et al. 2011b).

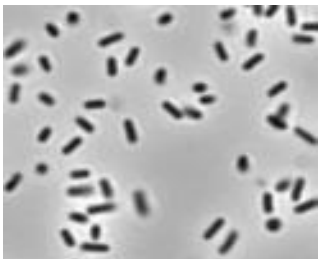

Fig. 1.10 - *Enterobacter* A47 (DSM 23139) rod-shaped morphology.

This bacterial strain belongs to a large family of Gram-negative bacteria, the *Enterobacteriaceae*. The *Enterobacteriaceae* include many genera: *Escherichia, Shigella, Salmonella, Klebsiella, Serratia, Proteus, Enterobacter* and others. This family has rod-shaped morphology with typically 1-5 µm in length (Figure 1.10), it is non-spore-forming, it is facultative anaerobe or aerobe and it can also ferment a wide range of carbohydrates (Murray et al. 2003).

Fig. 1.11 – Freeze-dried biopolymer, FucoPol, produced by *Enterobacter* A47 (DSM 23139).

This biopolymer (Figure 1.11) is composed of fucose (36-37%mol), galactose (25-26%mol), glucose (27-28%mol), glucuronic acid (10-11%mol), pyruvate (9-13%mol), succinate (2-3%mol) and acetate (5-8%mol) (Torres et al. 2012). Fucose is a neutral sugar commonly found in exopolysaccharides

produced by bacteria of the genus *Enterobacter,* such as *E. amnigenus*, *E. sakazakii* and *E. cloacae*.

E. amnigenus originates a heteropolymer containing glucose, galactose, fucose, mannose, glucuronic acid and pyruvil (Cescutti et al. 2005); *Enterobacter* sp. CNCM 1-2744 has fucose, galactose, glucose and glucuronic monomers in a ratio of 2:2:1:1 (Philbe 2002); Enterobacter sp. SSYL (KCTC 0687BP) produces a exopolysaccharide with a 40-70% and 8-10% of glucuronic acid and fucose content, respectively (Yang 2002); *Enterobacter sakazakii* strains ATCC 53017, ATCC 29004 and ATCC 12868 form an exopolysaccharide with 13-22% of fucose content (Harris et al. 1989) and *Enterobacter cloacae* secretes a biopolymer with glucose, galactose, glucuronic acid, fucose and acetyl groups in a molar ratio of 5:4:4:11:1 (Meade et al. 1994).

The monosaccharide fucose adds an increased market-value to the biopolymer since L-fucose and fucose-rich oligo- and polysaccharides (FROP-s) have proven to promote acceleration of wound healing and scavenge free radicals. As a starting point, this rare sugar has anti-inflammatory and anti-aging properties, enhancing its biological properties that can be incorporated into pharmaceutical and cosmetic products (Péterszegi et al. 2003a; Péterszegi et al. 2003b). However, for this particular exopolysaccharide (FucoPol), its biological activity related to the monomer fucose is yet to be determined.

FucoPol production is a patented process (Reis et al. 2011) which consists on the use of glycerol byproduct from the biodiesel industry, as the carbon source. Its standard operation results in a specific growth rate of 0.30-0.32 h^{-1}, a volumetric productivity of 0.53-0.56 $g_{EPS}g_{CDW}^{-1}d^{-1}$ and EPS production of 7.23-7.79 g L^{-1} (Torres et al. 2012). Its main known properties are related to viscous shear-thinning solutions in aqueous media, film-forming, emulsifying and flocculating capacity, as well as biological activity due to fucose content (Freitas et al., 2011b).

Enterobacter A47 can also originate EPS with different compositions when cultivated in different conditions. The distinct EPS produced by *Enterobacter* A47 possess different properties that can be useful in different applications. This feature can be useful to obtain tailored EPS production by simply altering the cultivation conditions (Torres et al. 2012).

2 Motivation

The microbial polysaccharides have been raising commercial interest against synthetic polymers, over the past years. This increasing attention has been associated with their natural sources and renewable resources based production. Microbial polysaccharides are able to provide biodegradable and biocompatible polymers, as opposed to the synthetic polymers, and utilize industrial wastes/byproducts for polymer production, which consequently heighten their value-added characteristics.

The glycerol byproduct of biodiesel industry was presented as the sole carbon source of the novel patented bacterial fucose-rich exopolysaccharide, FucoPol (Reis et al., 2011). In addition to previous studies on the effect of the environmental conditions, pH and temperature (Torres et al. 2012), and nitrogen/glycerol ratio (Torres et al. 2013), it was also necessary to determine the oxygen dissolved requirements of such production. Furthermore, an evaluation on the reduction of the phosphate concentration into FucoPol production was needed, in order to decrease its associated high costs.

The present work's third goal was to assess the functional properties of the exopolysaccharides produced by *Enterobacter* A47, using different cultivation conditions. These EPS which are described in Table 2 (page 25), were previously produced by other investigators prior to this thesis. It was important to examine the impact of the different chemical characteristics into the functional properties of those polymers, namely, in rheology, film-forming capacity, emulsion formation and stabilizing capacity and flocculating activity.

3 Materials and Methods

3.1 EPS Production

3.1.1 Microorganism

Enterobacter A47 (DSMZ 23139) was preserved in 20% (v/v) glycerol as a cryoprotectant agent, at -80 °C. To perform the reactivation of the microorganism, it was grown in a chromagar (CHROMagar^{TM} Orientation) plate, during 24 h at 30 °C, to obtain isolated colonies.

3.1.2 Cultivation Media

Luria Broth (LB) medium, used for preparation of the pre-inocula, had the following composition (per liter): yeast extract (Cultimed), 5.0 g; bacto-tryptone (LaborSpirit) 10.0 g and NaCl (Panreac) 10.0 g.

Medium E*, which was used for preparation of the inocula for bioreactor and shake flasks experiments, had the following composition (per liter): $(NH_4)_2HPO_4$ (Scharlau), 3.3 g; K_2HPO_4 (Panreac), 5.8 g; KH_2PO_4 (Panreac), 3.7 g; 10 mL of a 100 mM $MgSO_4$ solution (Cmd Chemicals) and 10 mL of a micronutrients solution. This micronutrients solution had the following composition (per liter of 1N HCl (Scharlau)): $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich), 2.78 g; $MnCl_2 \cdot 4H_2O$ (Acros-Organics), 1.98 g; $CoSO_4 \cdot 7H_2O$ (Merck), 2.81 g; $CaCl_2 \cdot 2H_2O$ (Scharlau), 1.67 g; $CuCl_2 \cdot 2H_2O$ Merck), 0.17 g; $ZnSO_4 \cdot 7H_2O$ (Merck), 0.29 g.

Every step involved in the handling of the bacterial strain was carried out in a laminar flow chamber (Heraeus SB 48, Germany). For every flask or bottle containing solutions required to the bioreactors' experiments, sterilization (Uniclave 77, Portugal) was achieved at 120 °C, during at least 20 min.

3.1.3 Inocula preparation

Pre-inocula for the experiments were prepared by inoculating a single *Enterobacter* A47 colony grown on a chromagar plate into 40 mL LB Medium (pH 6.8-7.0) and incubating it in an orbital shaker (IKA ® KS 260 basic orbital shaker, Germany) at 30 °C, 200 rpm during 24 h.

Inocula were prepared in Medium E*, supplemented with ≈40 g L^{-1} of glycerol-rich product, (SGC, SGPS, Portugal) or glycerol 99% (Sigma-Aldrich) as the carbon source. 20 mL of the pre-

inocula prepared as described above were transferred into 200 mL Medium E*, in 500 mL shake flasks, and incubated at 30 °C, 200 rpm during 48-72h.

3.1.4 Shake Flasks Assays

For the study of the effect of phosphate on the bioprocess, six shake flasks assays were performed (in duplicate experiments) by varying the concentration of this nutrient's sources in Medium E*(K_2HPO_4 and KH_2PO_4). Hence the phosphate concentrations tested were:

Table 1 - Phosphate concentrations of the different experiments in the shake flasks assays.

Experiment	A	B	C	D	E	F
PO_4^{3-} (g L^{-1})	8.12	5.25	3.81	3.09	2.95	2.37

3.1.5 Bioreactor cultivation assays

All the experiments were executed in 2 L bioreactors (BioStat B-plus, Sartorius, Germany) with the following operation mode: a batch phase characterized by the exponential microbial growth phase, followed by a fed-batch phase during which a feeding solution was supplied to the culture at a constant rate of 2.5 mL h^{-1}. This solution was identical to Medium E* (described above), but had a higher carbon source concentration, 200 g L^{-1}. The bioreactor was operated with a constant aeration rate of 0.125 vvm, with controlled temperature and pH, 30 °C and 6.8-7.0, respectively. The pH control was achieved with 2M NaOH (eKa) and 2M HCl (Scharlau). Silicone based anti-foam (VWR) was used to control foam formation during the process.

The dissolved oxygen concentration (DO) was controlled by the automatic variation of the stirrer speed (300–800 rpm). In the study of the effect of phosphate on the bioprocess, the DO was controlled at 10% of the air saturation, while in the study of the effect of DO, it was maintained at different air saturation values: 10, 30 or 60% during the batch and fed-batch phases.

To measure the broth viscosity and to quantify the kinetic parameters, ≈25 mL samples were periodically taken during the cultivation assays.

3.2 Exopolysaccharide Extraction

For extraction of the biopolymer from the cultivation broth, it was diluted with deionized water. This dilution depended on the broth's viscosity: 1-50 mPa.s (no dil.); 50-300 mPa.s (dil. 1:2); 300-1000 mPa.s (dil. 1:3), which was measured at shear rates from 0.3 to 55.8 s^{-1}. The diluted broth was then centrifuged (Sartorius 4K 15, Germany) at 11 627 g for 15 min to separate the biomass from the cell-free supernatant, which contained the biopolymer. After this separation step, the cell-free supernatant was subjected to a thermal treatment at 70 °C for 1 h followed by centrifugation at 11 627 g for 15 min, to remove any remaining cell debris and denatured proteins. The treated supernantant was subjected to dialysis with a 10,000 MWCO membrane (SnakeSkin™ Pleated Dialysis Tubing, Thermo Scientific) against deionized water, with constant stirring at 4 °C. The dialysis was monitored through measurements of the ionic content in the solution for an average of 48 h, so that the conductivity reached below 25 µS m^{-1}, and contaminants adsorbed by the biopolymer were reduced.

For the study of the functional properties the polymers were extracted with an alternative procedure, to guaranty a higher purity degree. A similar procedure was performed, but the thermal treatment of the cell-free supernatant was replaced by protein precipitation with trichloroacetic acid (TCA). 4 mL TCA 99% (Sigma-Aldrich) at a concentration of 100% (w/v) was added to the cell-free supernatant (40 mL) and the mixture was kept at 4 °C for 15 min. After centrifugation at 11 627 g for 15 min to remove the denatured proteins, the treated supernatant was dialyzed, as described above.

In both procedures, the dialyzed supernatants were frozen in liquid nitrogen and freeze-dried (Telstar Cryodos-50, Spain) at - 40 °C and 340 mbar for 48 h.

3.3 Analytical Techniques

3.3.1 Apparent Viscosity

The apparent viscosity of the broth samples taken from the bioreactor was measured using a digital viscometer (Brookfield, EUA).

3.3.2 Cell Dry Weight

The broth samples were centrifuged (16 743 g during 15 min). The cell-free supernatant was kept at -20 °C for the quantification of ammonium, glycerol and exopolysaccharide, while the cell pellet was used for the gravimetric quantification of the cell dry weight (CDW). The pellet was washed twice in deionized water, filtered in a vacuum system through a cellulose acetate membrane 0.2 µm (GVS), and, finally, dried at 100 °C (Memmert U15, Germany) for 24 h.

3.3.3 Ammonium concentration

To determine the ammonium concentration, 1 mL of the cell-free supernatant sample was mixed with 20 µL ISA (Ionic Strength Adjuster) which is a reagent composed of NaOH, 200 g; EDTA, 8.6107 g; and thymolphthalein, 0.0158g per liter of deionized water. After 5 min, the conductivity was measured using a potentiometric sensor (Thermo Electron Corporation Orion 9512). The ammonium concentration was determined using a NH_4Cl (Sigma-Aldrich) standards (0.0056 – 1.8 g L^{-1}).

3.3.4 Glycerol concentration

Glycerol concentration in the cell-free supernatant was determined by high performance liquid chromatography (HPLC) with a Varian 87H column (Metacarb), coupled to a refractometer (Merck). This analysis was performed at 50 ◦C, using 0.01 N H_2SO_4 (Fischer Chemical) as eluent at a flow rate of 0.6 mL min^{-1}. A standard calibration curve (glycerol 99% - Sigma-Aldrich) with concentrations ranging from 0.15 to 5 g L^{-1} was used.

3.4 Calculations

3.4.1 Specific Growth Rate

The maximum specific growth rate (μ_{max}, h^{-1}) was determined using the following equation:

$$\mu_{max} = \frac{Ln\ (CDW/CDW_0)}{t} \qquad equation\ (1)\ ,$$

Where CDW_0 is the initial cellular density.

3.4.2 Volumetric Productivity

The EPS volumetric productivity (r_P, g L^{-1} h^{-1}) was determined by the following equation:

$$r_P(gL^{-1}h^{-1}) = \frac{dP}{dt} \qquad equation\ (2)\ ,$$

Where *dP* (g L^{-1}) is the variation of product formation during the experiment and *dt* (h) is the time period of the experiment delay.

3.4.3 Product and Biomass Yield

The biomass yield on substrate ($Y_{x/s}$, g_{cell} $g_{glycerol}^{-1}$) was determined considering the biomass produced (ΔX, g_{cell}) per substrate consumed (ΔX, $g_{glycerol}$) during the assay:

$$Y_{X/S}\left(g_{cell}g_{glyc}^{-1}\right) = \frac{\Delta X}{\Delta S} \qquad equation\ (3)\ ,$$

The product yield on substrate ($Y_{p/s}$, g_{EPS} $g_{glycerol}^{-1}$) is based on the product formed (ΔP, g_{EPS}) per substrate consumed (ΔX, $g_{glycerol}$):

$$Y_{P/S}\left(g_{EPS}g_{glyc}^{-1}\right) = \frac{\Delta P}{\Delta S} \qquad equation\ (4)\ .$$

3.5 Chemical Characterization of the Polymers

3.5.1 Elemental Analysis

The measurement of the biopolymers' composition in C, N, O, H and S, was executed at 60 kPa pressure with a carrier gas flow of 150 mL min^{-1} and column temperature of 1000 °C (Manufacturer Eurovector, Model EuroEA, Italy).

3.5.2 Exopolysaccharide Composition

3.5.2.1 Sugar Monomers

The exopolyssacharide's sugar composition was performed by hydrolyzing 20 µL of 1 % (w/v) of EPS solution with 20 µL of 0.04 % (v/v) of trifluoroacetic acid, TCA 99% (Scharlau). The

reaction was done at 120 °C for 2 h. After hydrolysis, 92 µL of 1.6 % NH_4OH was added to the hydrolyzed sample.

Prior to analysis, all samples were prepared according to the following 96-well PMP-derivatization method: 75 µL of derivatization reagent were added to 25 µL of sample in a 96-well-PCR micro titer plate. The plate was sealed with a silicone cap mat, mixed well and centrifuged at 2.000 g for 2 min at 20 °C. After incubation (100 min at 70 °C) in a PCR-cycler and a following automated cool down to 20 °C, an aliquot of 20 µL was transferred to a fresh 96-well micro titer plate and mixed with 130 µL acetic acid. The samples were then transferred into a 96-well filter plate and centrifuged at 2.500 g for 5 min at 20 °C. Finally, the plate was sealed with a 96-well silicon cap mat.

The analysis was performed by a HPLC analysis of monosaccharide-PMP-derivatives. The HPLC system (Ultimate 3000RS, Dionex) was composed of a degasser (SRD 3400), a pump module (HPG 3400RS), an autosampler (WPS 3000TRS), a column compartment (TCC 3000RS), a diode array detector (DAD 3000RS) and an ESI-ion-trap unit (HCT, Bruker).

Data was collected and analyzed with Bruker Hystar, QuantAnalysis and Dionex Chromelion software. The column (Gravity C18, 100 mm length, 2 mm i.d.; 1.8 µm particle size; Macherey-Nagel) was tempered to 50 °C. Mobile phase A consisted of 5 mM ammonium acetate buffer with 15 % acetonitrile and a chromatographic flow rate of 0.6 mL/min. A switch valve behind the UV-detector (245 nm) refused the first 3 min of chromatographic flow. Before entering ESI-MS the flow was splitted 1:20 (Accurate-Post-Column-Splitter, Dionex).The autosampler temperature was set to 20 °C and an injection volume of 10 µL was used. Calibration 200 - 2mg/L: Man, D-mannose; GlcUA, D-glucuronic acid; GlcN, D-glucosamine; GalUA, D-galacturonic acid; Rib, D-ribose; Rha, L-rhamnose; Gen, D-gentiobiose; GalN, D-galactosamine; GlcNAc, N-acetyl-D-glucosamine; Lac, D-lactose; Cel, D-cellobiose; Glc, D-glucose; GalNAc, N-acetyl-D-galactosamine; Gal, D-galactose; Ara, L-arabinose; Xyl, D-xylose; Fuc,L-Fucose; 2-d-Glc, 2-deoxy-D-glucose; 2-d-Rib, 2-deoxy-D-ribose.

The ion-trap was operated in the ultra-scan mode (26.000 m/z/sec) from 50 to 1.000 m/z. The ICC target was set to 200.000 with a maximum accumulation time of 50 ms and four averages. The ion source parameters were set as follows: capillary voltage 4 kV, dry temperature 325 °C, nebulizer pressure 40 psi and dry gas flow 6 L/min. Auto MS mode with the smart target mass of 600 m/z and a MS/MS fragmentation amplitude of 0.5 V was used.

3.5.2.2 Volatile Fatty Acids

The analysis of the acyl groups' composition was performed by hydrolyzing 5mg of EPS dissolved in 5mL of deionized water, with 100 µL of trifluoroacetic acid, TCA 99% (Scharlau). The reaction was done at 120 °C in 2 h (Hach Lange LT 200, Germany).

A standard solution composed by a mixture of acetic acid (Fischer Chemicals), succinic acid (Merck) and pyruvic acid (Sigma-Aldrich), each in a 1 g L^{-1} concentration, with 0.001 M H_2SO_4 as solvent, was hydrolyzed under the same conditions as the samples. This standard solution was successively diluted to: 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625 and 0.0078125 g L^{-1}. All the reagents used in the standard solution had a purity degree ranging from 98 to 100%.

The analysis was performed by HPLC (Merck) with an Aminex 87H column (Biorad), coupled to a UV-Visible detector (Merck), at 50 ◦C, 210 nm and a flow rate of 0.6 mL min^{-1} with 0.001 M H_2SO_4 as eluent.

3.5.2.3 Protein, Water and Inorganic Content

For determination of the protein content, 5.5 mL samples of aqueous EPS solutions (4.5 g L^{-1}) were mixed with 1 mL 20% NaOH and hydrolyzed at 100 °C for 5 min. After cooling on ice, 170 µL of 25% (v/v) $CuSO_4 \cdot 5H_2O$ (Riedel de Haen) were added. After mixing, the samples were centrifuged (VWR MicroStar 17, EUA) at 10000 rpm during 5 min and the optical density was measured at 560 nm (Spectrophotometer Helios Alpha, Thermo Spectronic, UK). The protein standard solutions contained BSA 98% (Sigma-Aldrich) in 3.0 to 0.015625 g L^{-1} concentrations.

In relation to the water content quantification, 10 mg of EPS was placed in a glass fiber 1.2 µm (VWR), folded and placed at 100 °C overnight. Afterwards the same sample was placed at 550 °C (Muffle Nabertherm B150, Germany) during 20 h.

The water content was determined as the amount of water that was evaporated from the EPS sample, after 24 h at 100 °C, as suggested by the following formula:

$$Water\ content\ (\%) = \frac{(m_{EPS+filter}) - (m_{EPS+filter})_{100\,^{\circ}C}}{m_{EPS}} \times 100 \qquad equation\ (5)\ .$$

The inorganic content was determined as the inorganic salts content of the EPS sample after pyrolysis, in which all organic matter is decomposed.

3.6 Functional Properties

EPS with distinct composition produced by the bacterium *Enterobacter* A47 under different cultivation conditions were characterized in terms of their functional properties. Such polymers were obtained in previous studies and their physical-chemical characteristics are present in Table 2.

3.6.1 Rheological Properties

The rheology of 1% (w/w) biopolymers' aqueous solutions in 0.1 M NaCl (Panreac) were studied using a controlled stress rheometers (Haake Mars III, Thermo Scientific, Germany), equipped with a cone and plate geometry: diameter 3.5 cm and a 2° angle. During the experiments, the shearing geometry was covered with paraffin oil in order to prevent water loss. Flow curves were determined at 25 °C using a steady state flow ramp in the shear range of 1–700 s^{-1}. The shear rate was measured point by point with consecutive 30 s steps of constant shear rate. The viscosity was recorded for each point to obtain the flow curves. Frequency sweeps were carried out at a controlled stress of 1 Pa (shown by stress sweeps to give values within the linear viscoelastic region) in order to measure the dynamic moduli G' and G''.

3.6.2 Film-forming capacity

A preliminary test was made for all biopolymers to test their capacity to form films by solvent casting: 5 mL of 1% (w/w) EPS aqueous solutions were put in plastic Petri dishes (*d*=90 mm) and placed at 40 °C, for 24 h, for solvent evaporation. For the biopolymers which had film-forming capacity, their properties were studied, including the mechanical properties and water vapor permeabilities. For this purpose, films were prepared using 19.5 g of 1.25% (w/w) EPS aqueous solutions with 30% (v/v) ($g_{glycerol}\ g_{dry\ polymer}^{-1}$) glycerol 99% content as a plasticizer. The solutions were put in plastic Petri dishes and placed at 40 °C for approximately 48 h, for solvent evaporation. The films were peeled from the dishes' surfaces and kept in a desiccator with a controlled relative humidity atmosphere of 45 % until testing. Their thickness was measured at three different points using a digital micrometer (Mitutoyo, UK).

Mechanical properties were tested by performing tensile tests of the different films, which were carried out by attaching 20 mm × 60 mm film strips on tensile grips and stretching them at 1 mm s^{-1} in tension mode. To this assessment was used a TA-Xtplus texture analyser (Stable

Micro Systems, Surrey, England). The stress at break was calculated as the ratio of the maximum force to the films' initial cross-sectional area. The elongation at break was determined as the ratio of the extension of the sample upon rupture by the initial gage length. The slope of stress as a function of strain, within the elastic deformation of the stress–strain curve, was taken as the Young's modulus, which was calculated according to the following equation:

$$Y = \frac{\tau}{\varepsilon} = \frac{\frac{F}{A}}{\frac{\Delta l}{l}} \qquad equation\ (6)\ ,$$

Where, Y (Pa) is the Young's modulus (or elastic modulus).

Water vapour permeability's evaluation was performed gravimetrically at 25.0 ± 1.0 °C. The films samples were sealed with silicone on the top of the vials (*d*=2.5 cm), placed in a desiccator containing a saturated $Mg(NO_3)_2.6H_2O$ solution (a_w = 0.534) and equipped with a fan to promote air circulation. Room temperature and relative humidity inside the desiccator were monitored over time using a thermohygrometer (Vaisala, Finland). To impose the selected driving force, a saturated KBr solution was placed inside the vial (a_w = 0.809) and water vapour flux was determined by weighing the vial in regular time periods for 47 h. Three independent runs were performed for each EPS film. The water vapour permeability was determined by the following equation:

$$WVP = \frac{N_W \times \delta}{\Delta P_{W,eff}} \qquad equation\ (7),$$

Where, N_w is the water vapour molar flux, δ is the film thickness and $\Delta P_{w,eff}$ is the effective driving force, expressed as the water vapour pressure difference between both sides of the film, calculated taking into account the mass transfer resistance of the stagnant film of air below the test film.

3.6.3 Emulsion forming and stabilizing capacity

The capacity of the EPS to stabilize emulsions was measured using 0.5% (w/v) EPS aqueous solutions that were mixed with sunflower oil (Continente) in a 2:3 (v/v) ratio and stirred in the vortex for 2 min. Each EPS-oil mixture was subjected to various tests: emulsion stability at

different temperatures, 4, 23 and 37 °C, independently, for several days and temperature fluctuations, such as, freezing at -20 °C and sequential thawing at 20 °C for a time period of 24 h, and increasing temperature from 40 to 100 °C. Each test was carried out once for each EPS-oil mixture.

The emulsification index (E_{Index}, %), was determined by the following equation:

$$E_{Index} = \frac{h_e}{h_t} \times 100 \qquad equation\ (8)\ ,$$

Where h_e (cm) is the height of the emulsion layer and h_t (cm) is the total height of the mixture.

3.6.4 Flocculation capacity

To test the flocculating activity of the different polymers, a 5 g L^{-1} Kaolin clay (Fluka) suspension was prepared along with a 1% (w/v) $CaCl_2$ (Kemira) solution. EPS aqueous solutions at two different concentrations, 0.01 and 0.001% (w/v) were tested. With a final ratio volume of 40:1:0.1 (Kaolin:$CaCl_2$:EPS), the samples were stirred in the vortex at 2400 rpm, for 1 min. After 5 min the optical density was measured at 550 nm.

A blank sample containing deionized water instead of the EPS solution was used. All measurements were performed in duplicate.

Flocculation (%) was determined using the following equation:

$$Flocculation\ (\%) = \left(\frac{OD_{H_2O} - OD_{EPS}}{OD_{H_2O}}\right) \times 100 \qquad equation\ (9)\ ,$$

Where OD_{H20} is the optical density for the suspension containing deionized water and OD_{EPS} is the optical density for the suspension containing exopolysaccharide.

Table 2 - Biopolymers description including cultivation conditions and physical characterization. Biopolymers produced by *Enterobacter* A47

EPS	Cultivation conditions				Average Molecular Weight	Sugar composition (%mol)						Acyl groups' composition (% (w/v))		
	Substrate	T (°C)	pH	Fed-batch		fucose	galactose	glucose	rhamnose	glucuronic acid	glucosamine	pyruvate	acetate	succinate
EPS-x (*)	Xylose	30	7.0	pO_2 control with 200g/L xylose	1.7×10^6	38	18	27	0	17	0	15	0	2
EPS-g (*)	Glucose	30	7.0	pO_2 control with 200g/L glucose	4.2×10^6	29	29	26	0	16	0	15	5	2
EPS-m (*)	GRP + Methanol	30	7.0	pO_2 control with pure methanol	4.5×10^6	22	23	30	3	19	2	2	0	2
S4 (*[1])	GRP	15.9	7.0	Constant feeding with 200g/L GRP	6.3×10^6	26	21	36	2	12	2	3	3	2
S7 (*[1])		30	5.6		9.8×10^6	13	13	58	6	8	2	3	4	0
S8 (*[1])		40	8.0		1.3×10^7	0	24	54	10	8	4	0	1	0
S10 (*[1])		30	8.4		8.4×10^6	0	12	37	29	11	11	1	3	0
EPS-s (*[1])		30	7.0		4.3×10^6	36	26	28	0	10	0	9	7	2
GNEX (*[2])		30	7.0	Constant feeding with 400g/L GRP	7.2×10^5	26	34	30	0	10	0	4	5	4

(*) Freitas et al. 2013; (*[1]) Torres et al. 2012; (*[2]) Torres et al. 2013

4 EPS Production by *Enterobacter* A47

One of the aims of this thesis was to optimize the production of biopolymer FucoPol, otherwise known as a fucose-containing biopolymer.

In order to improve productivity many factors have to be taken into account, such as, the type of bioreactor used, the mode of operation, the medium composition, and the culture conditions: temperature, pH, dissolved oxygen concentration, aeration rate and stirring speed.

In previous studies, the mode of operation (Torres et al. 2013) and the effect of temperature and pH (Torres et al. 2012) on exopolysaccharide production by Enterobacter A47, were established. Hence, in this thesis, two other factors were chosen to be studied, the effect of dissolved oxygen concentration and the effect of phosphate concentration, on EPS production.

4.1 Effect of Dissolved Oxygen Concentration

4.1.1 Cultivation Assays

For this particular study, dissolved oxygen concentration (DO, %) was selected because it is extremely important in aerobic bioprocesses. The DO corresponds to the relative amount of oxygen gas in the media, depending intrinsically on the oxygen gas partial pressure (Atkins, P. et al. 2008). A suitable DO allows a sufficient accessibility from bacterial cells towards the oxygen gas, which in turn determines their growth.

To begin with, experiments were performed at a constant aeration rate of 1 vvm varying the DO concentration (10, 30 and 60%), as shown in Figure 4.1.

Figure 4.1 (A) shows the profile of cell growth and production of the exopolysaccharide under the standard conditions, with DO controlled at 10%, similar to previous assays (Alves et al. 2010b; Torres et al. 2010). Following inoculation of the culture into the bioreactor, bacterial growth started without presenting any lag phase, leading to an immediate decrease of the DO (from 100 to 9.2% within 5 h) due to bacterial activity. The maximum specific growth rate was 0.33 h^{-1} (Table 3), and the biomass concentration rose from 1.48 g L^{-1} to approximately 6.00 g L^{-1} at the end of the batch phase (Figure 4.1 (A)).

The nitrogen source (ammonium) was exhausted in the first 10 h of cultivation. Being the nitrogen source a limitating factor towards cell growth, as soon as ammonium was completely consumed, bacterial growth was restricted. Along with ammonium depletion, a substrate consumption of approximately 15 g L^{-1} (from the 55 g L^{-1} initially available) was also observed.

At this point, the fed-batch phase was implemented (Figure 4.1), in which a feeding solution was fed to the bioreactor to provide more nutrients, namely glycerol.

EPS production slowly started after 1 h of the inoculation, reaching only 0.18 g L^{-1} by the end of the batch phase. In the next 20 h, EPS production was still relatively low, rising to 0.81 g L^{-1}, but immediately after, it started to increase significantly for the remaining cultivation time. An EPS production of 6.11 g L^{-1}, was attained at the end of the run. As shown in Table 3, cultivation with DO controlled at 10% resulted in a volumetric productivity of 0.080 g $L^{-1}h^{-1}$ and an yield on glycerol of 0.11 g_{EPS} $g_{glycerol}^{-1}$.

Biomass concentration declined 18% since its maximum (7.32 g L^{-1}) at ≈20 h. This outcome was probably due to dilution of biomass caused by the volume withdrawn from the bioreactor for sampling and the addition of feeding solution and pH control solutions during the fed-batch phase.

Comparatively with other experiments previously performed with the standard conditions (Alves et al. 2010b; Torres et al. 2010), it is verified a very similar cultivation profile.

Concerning the kinetics parameters, this assay also showed similar growth rate, 0.33 h^{-1}, with the one referred in (Torres et al. 2012), 0.36 h^{-1}, although the biomass yield and produced biomass concentration (0.12 g_{cell} $g_{glycerol}^{-1}$ and 6.60 g L^{-1}), were lower than in previous work (0.49 g_{cell} $g_{glycerol}^{-1}$ and 7.68 g L^{-1}) (Torres et al. 2010). Moreover, the overall product yield on glycerol was also similar, 0.11 g_{EPS} $g_{glycerol}^{-1}$, even when EPS production was lower, 6.11 g L^{-1}, and the productivity much lower, 0.080 g $L^{-1}h^{-1}$, than in (Torres et al. 2010), 7.50 g L^{-1} and 0.28 g $L^{-1}h^{-1}$, respectively.

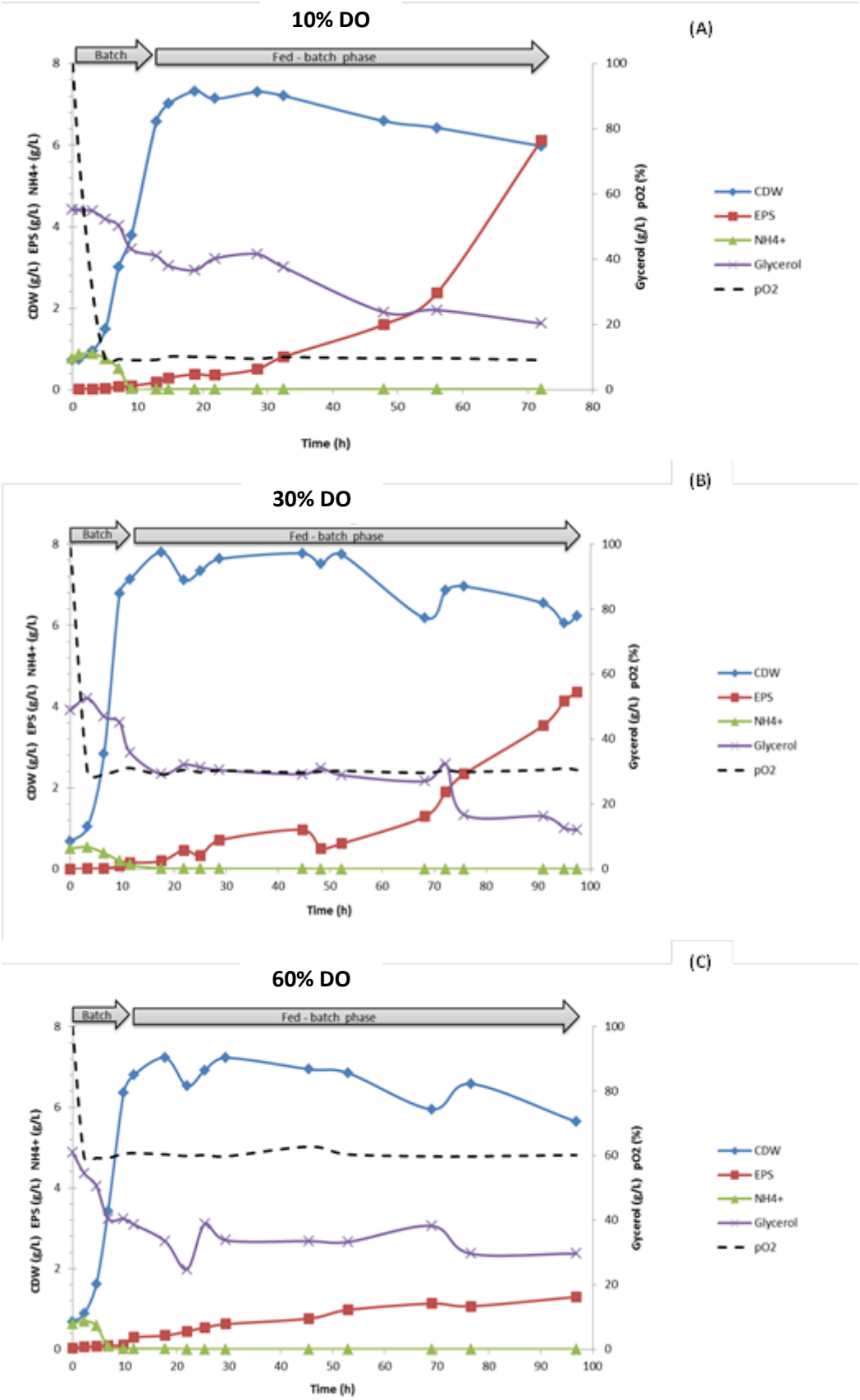

Fig. 4.1 - Cultivation profiles for *Enterobacter* A47 with dissolved oxygen concentration controlled at 10% (A), 30% (B) and 60% (C) air saturation.

Finally, during the cultivation assay, a change in the broth viscosity was observed (Figure 4.2). At the beginning, when the EPS concentration in the broth was low, the broth's viscosity was 1.45 mPa.s (at 11.2 s^{-1}). In the first 30 h, the broth's viscosity was slightly increased to 2.86 mPa.s (at 11.2 s^{-1}). After that, the broth's viscosity increased proportionally to EPS production, till the end of the run, reaching a maximum of 671 mPa.s (at 0.3 s^{-1}) along with the maximum EPS production.

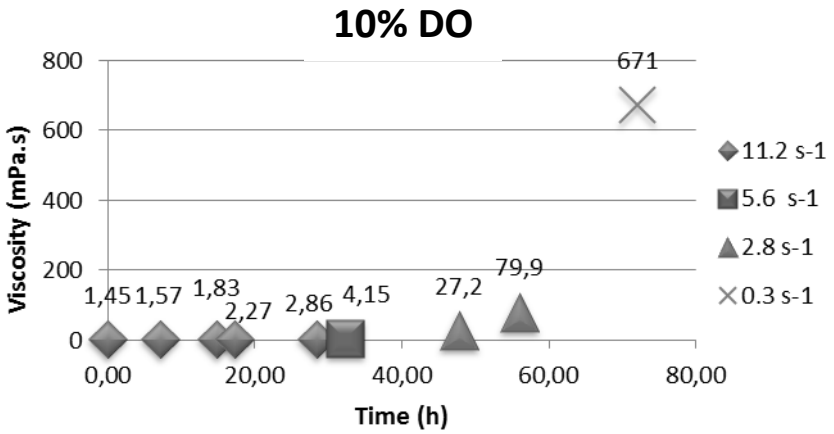

Fig. 4.2 - Apparent viscosity of the culture broth during cultivation of *Enterobacter* A47 with DO controlled at 10%, measured at shear rates of 0.3 – 11.2 s^{-1}.

In the cultivation assay wherein DO was controlled at 30%, a similar cultivation profile was verified, since DO quickly stabilized at 30% within 3.5 h and was kept at this value till the end of the run. Also without lag phase, bacteria grew exponentially for 7 h with a specific growth rate of 0.33 h^{-1}, rising from an initial CDW of 1.04 to 6.78 g L^{-1} in the end of batch phase. During this batch phase, ammonium was consumed and reached a limiting concentration (<0.1 g L^{-1}). 10 g L^{-1} of carbon source were also consumed, from the initially 50 g L^{-1} available (Figure 4.1 (B)). Then, the fed-batch phase was initiated by the addition of the feeding solution to the bioreactor.

Exopolysaccharide production begun, at 10 h and proceeded until the end of the run. After approximately 98 h of cultivation, 4.35 g L^{-1} of EPS were produced, corresponding to an overall volumetric productivity of 0.045 g L^{-1} h^{-1}. These values are lower than the ones obtained under the standard conditions (6.11 g L^{-1} and 0.080 g L^{-1} h^{-1}, respectively), which is probably related to the non-limiting DO concentration (30%). Also, the lower EPS productivity resulted in a lower yield of EPS on glycerol, 0.07 g_{EPS} $g_{glycerol}^{-1}$ in comparison to 0.11 g_{EPS} $g_{glycerol}^{-1}$, with DO at 10% (Table 3). Furthmore, glycerol consumption was higher (64.36 g L^{-1}) than in standard conditions (55.99 g L^{-1}), which indicates that with 30% of air saturation, less EPS production is obtained

with superior substrate consumption. This superior substrate consumption was probably due to its deviation towards other metabolic pathways.

Taking into consideration the maximum value of CDW at standard conditions, 7.32 g L^{-1}, at 30%, CDW reached to 7.80 g L^{-1}, which describes a similar behavior in the biomass concentration even if DO its three times higher.

So, in conclusion, the biomass concentration was not affected with DO at 30% but the EPS productivity ability of the bacteria, decreased considerably.

Relatively to the culture broth viscosity (Figure 4.3), that was initially 1.05 mPa.s (at 11.2 s^{-1}), it increased slowly to 50.1 mPa.s (at 0.6 s^{-1}) and it took another 20 h to reach to 391 mPa.s (at 0.3 s^{-1}), as opposed to the 671 mPa.s (at 0.3 s^{-1}), in 98 h of cultivation with DO controlled at 10% (Figure 4.2). The inferior increase of broth's viscosity was corroborated by the direct relation to the lower EPS production, with DO controlled at 30%.

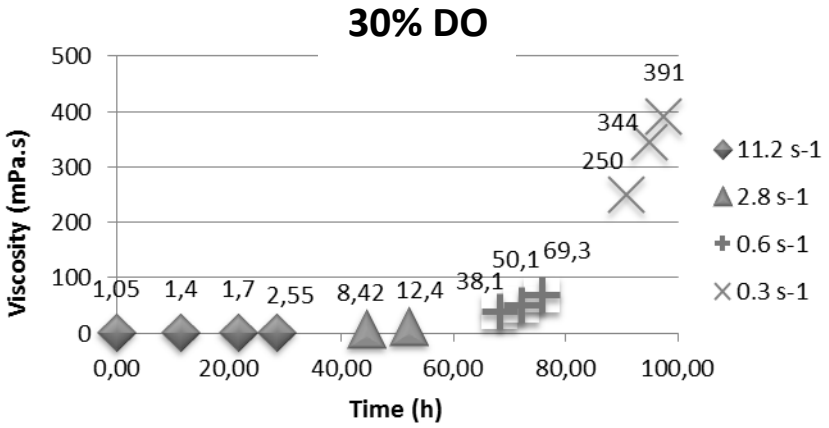

Fig. 4.3 - Apparent viscosity of the culture broth during cultivation of *Enterobacter* A47 with DO controlled at 30%, measured at shear rates of 0.3 – 11.2 s^{-1}.

In the cultivation assay Figure 4.1 (C), wherein the DO concentration was controlled at 60%, identical bacterial growth profile was observed.

The cell growth started without lag phase, increasing exponentially for 9 h, from 0.06 to 6.21 g L^{-1} with a specific growth rate of 0.33 h^{-1}, till the end of the batch phase. This occurred simultaneously with the exhaustion of ammonium, which reached the limiting condition, <0.1 g L^{-1}, and also with the consumption of 20 g $L^{-1}$of glycerol (from the initial 60 g L^{-1} initially available).

After that, the fed-batch phase was implemented and it was continuously fed with a solution containing 200 g L^{-1} of glycerol, equally to both cultivation assays presented previously (DO at 10 and 30%). At the fed-batch phase, DO and ammonium levels were kept at 60% and <0.1 g L^{-1}, respectively.

Even though the exopolysaccharide was slowly produced during the entire cultivation assay, from 0.12 g L^{-1} to only 1.27 g L^{-1}, after 87 h of fed-batch phase, bacteria continued to consume glycerol till the end. Its total glycerol consumption (61.90 g L^{-1}) was higher than glycerol consumption (55.99 g L^{-1}) at standard conditions, probably due to its deviation for other metabolic routes (e.g. protein production), with high DO concentrations. The EPS production and volumetric productivity, 1.27 g L^{-1} and 0.013 g L^{-1} h^{-1}, respectively, were unequivocally lower than standard conditions.

The only parameter that was not affected with DO controlled at 60%, was the biomass concentration that reached a maximum of 7.20 g L^{-1}, identical to the ones verified with DO at 10 and 30%, 7.32 and 7.80 g L^{-1}, respectively. The EPS production, productivity and yield on glycerol were negatively affected, reaching to the lowest values of the present study. For these last reasons, the cultivation assay with DO at 60% is considered an exopolysaccharide non-productive set of conditions.

Regarding the culture broth viscosity (Figure 4.4), concomitant with the low EPS production, it reached a final value of only 25 mPa.s (at 1.4 s^{-1}).

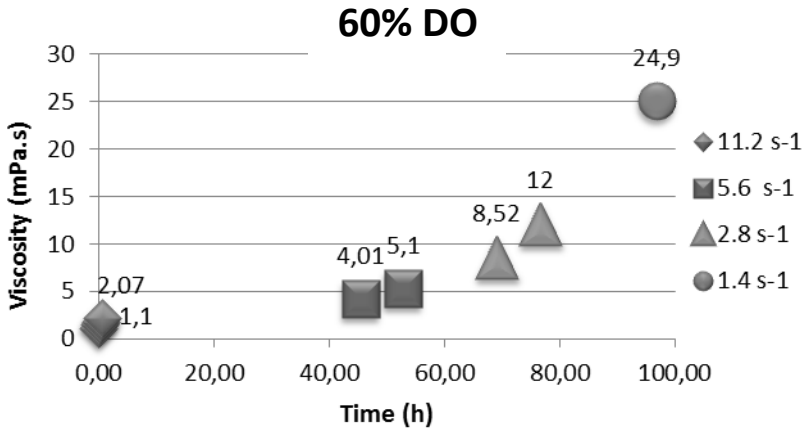

Fig. 4.4 - Apparent viscosity of the culture broth during cultivation of *Enterobacter* A47 with DO controlled at 60%, measured at shear rates of 1.4 – 11.2 s^{-1}.

Resorting to Table 3, all different DO concentration values equally provided optimal bacterial growth, ranging from 6.54 to 7.11 g L^{-1}, with a specific growth rate of 0.33 h^{-1} and biomass yield of 0.12 g_{cell} $g_{glycerol}^{-1}$. Nevertheless, higher DO concentrations, inversely affected the product yield, which was five times lower at 60%, 0.02 g_{EPS} $g_{glycerol}^{-1}$ than it was in standard conditions, 0.11 g_{EPS} $g_{glycerol}^{-1}$.

Table 3 - Kinetic parameters for the cultivation of *Enterobacter* A47 with dissolved oxygen concentration controlled at different air saturation values.

Experiment	A	B	C
DO (%)	10	30	60
μ (h^{-1})	**0.33**	**0.33**	**0.33**
$CDW_{produced}$ ($g\ L^{-1}$)	**6.60**	**7.11**	**6.54**
EPS ($g\ L^{-1}$)	**6.11**	**4.35**	**1.27**
r_p ($g\ L^{-1}h^{-1}$)	**0.080**	**0.045**	**0.013**
glycerol $_{cons.}$ ($g\ L^{-1}$)	**55.99**	**64.36**	**61.90**
$Y_{X/S}$ ($g_{cell}\ g_{glycerol}^{-1}$)	**0.12**	**0.11**	**0.11**
$Y_{P/S}$ ($g_{EPS}\ g_{glycerol}^{-1}$)	**0.11**	**0.07**	**0.02**

According to these data, it's possible to confirm that standard conditions were the most adequate for EPS production by *Enterobacter* A47. A partially-growth associated product was attainable with this specific bacteria caused by two triggering events, carbon source availability and nitrogen limitation. Therefore, similar biomass concentration was originated in all assays by exhaustion of the limiting nutrient and EPS production was reduced for DO concentration above 10% of air saturation.

In comparison to the literature, it is referred that the role of oxygen limitation in polysaccharide biosynthesis could exercise positive or negative influence. For instance, (Peters et al. 1989) and (Amanullah et al. 1998), support the idea that dissolved oxygen above 20%, enhance biopolymer formation and production rate in species such as *Xanthomonas campestris*. Banik et al. 2006, also reported that cultivation of *Sphingomonas paucimobilis* with higher DO levels, from 20 to 100%, increases biomass formation and biopolymer production, as opposed to DO levels below 20%.

However, Rho et al. 1988 support that in some cultures, such as *Aureobasidium pullulans*, oxygen demand is so high, that under conditions at low DO, an optimum biopolymer synthesis is achieved. Moreover, they reinforce that biomass production is not affected by different DO levels. Specifically for bacteria strains, such as *Azotobacter vinelandii*, low DO concentrations (between 2-3%) were the most suitable for alginate production and biomass formation, in the range of 1–10% of air saturation, after which both parameters decreased significantly (according to Sabra et al. 1999).

Therefore, *Enterobacter* A47 apparently falls into the same category that *Aureobasidium pullulans* and *Azotobacter vinelandii*, since it was determined that lower DO promoted high EPS production and that different DO concentrations didn't affect cell growth.

4.1.2 EPS Chemical characterization

Concerning the biopolymers chemical composition, it was examined for its sugar and acyl groups composition (Table 4) and its content in carbon, oxygen, nitrogen, hydrogen and sulphur atoms (by elemental analysis) as well as well as its protein, ashes and water content (Table 5).

Table 4 - Exopolysaccharide sugar and acyl's composition.

Experiment	**Sugar monomers** (%mol)				**Total acyl groups** composition (% (w/v))
	Fucose	**Galactose**	**Glucose**	**Glucuronic acid**	
10%	31	30	31	7	20.67
30%	32	14	45	10	20.52
60%	29	27	37	6	20.27

Regarding the sugar monomers, changes were observed with DO controlled at 30%, more specifically the reduction in galactose and an increase in glucose monomers. In relation to glucuronic acid monomers, their content for all experiments, with exception for 30%, was slightly below of the 10% reported in (Torres et al. 2012), with optimal conditions. However for higher DO concentrations, in gellan production by *Sphingomonas paucimobilis*, Banik et al. 2006 reported that it did not suggested significant variation in glucose and glucuronic acid monomers, in accordance with the results obtained.

Identical acyl groups' contents were obtained for all three biopolymers (20.5 ± 0.2 % (w/v)), which is similar to the value reported for FucoPol produced under the standard conditions (Torres et al. 2010). The non-saccharide total composition of the fucose-containing biopolymer is composed by acetyl, pyruvil and succinyl (Torres et al. 2010). Pyruvil and succinyl confer an anionic character to the exopolysaccharide, which functions as counter ions for the inorganic salts (Freitas et al. 2009a), and also defines its solubility and rheological properties (Rinaudo 2004). Further studies on acyl groups' composition variation throughout the cultivation assays, should be considered in order to speculate the influence of acetyl, pyruvil and succinyl in the exopolysaccharide structure.

Taking into consideration the elemental analysis of all three biopolymers, it is possible to confirm that changes in DO concentration didn't greatly affect the composition of each atom. In fact, carbon, oxygen, nitrogen, hydrogen and sulfur contents were similar for all experiments.

Table 5 – Exopolysaccharide's elemental analysis and its water, protein and inorganic residues content.

Experiment	**Elemental analysis** (%)					Water (% (w/v))	Ashes (% (w/w))	Protein (% (w/v))
	C	**O**	**N**	**H**	**S**			
10%	39.74	35.44	3.61	5.97	0.00	15.73	0.50	3.71
30%	40.17	31.11	3.91	6.08	0.11	12.97	0.70	3.27
60%	40.02	35.07	3.53	5.80	0.06	14.94	0.99	3.60

In addition to this characterization, it was possible to verify an inorganic salt content under 1% (v/v) for all biopolymers produced. Besides that, the protein content also revealed itself a depreciable component into the biopolymers composition, since it wasn't higher than 4% (w/v), as it is referred in (Freitas et al. 2011b).

The inorganic salt compounds' percentage corresponds to all non-aqueous residues that were present in the culture medium and the protein content in turn, was related to the proteins produced by the bacteria, both adsorbed by the polymer. Those low values mentioned above, represent a reduced amount of contaminants in the biopolymer, meaning that the dialysis was successfully achieved ultimately providing a refined biopolymer.

4.2 Effect of Phosphate Concentration

The overall FucoPol production costs are greatly influenced by the phosphate components, K_2HPO_4 and KH_2PO_4, of the culture medium. For this reason, it was studied the impact of the reduction of these two components in the bacterial behavior and performance.

4.2.1 Shake flasks assays

Firstly, preliminary tests in shake flasks were performed, in order to determine the impact of the phosphate reduction in the bacterial growth. Six different phosphate concentrations were tested, as described in Table 1, section 3.1.4.

The shake flasks assays allowed a pre-visualization of the *Enterobacter* A47 behavior for a period of 47 h. All experiments were performed in parallel.

In this time period, bacterial growth in all experiments behaved similarly to each other, and achieved identical optical density values, as shown by the Figure 4.5. For this reason, it was possible to verify that a reduction of the phosphates concentrations, didn´t significantly affect the overall bacterial activity.

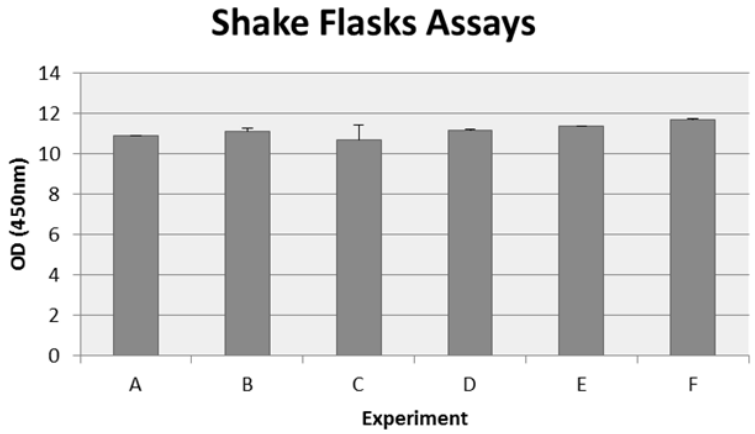

Fig. 4.5 - Optical density of *Enterobacter* A47 grown in the shake flasks assays, for 47h, with different phosphate concentrations: 8.12 g L^{-1} (Experiment A); 5.25 g L^{-1} (Experiment B); 3.81 g L^{-1} (Experiment C); 3.09 g L^{-1} (Experiment D); 2.95 g L^{-1} (Experiment E) and 2.37 g L^{-1} (Experiment F).

It is known that in shake flasks assays, pH and dissolved oxygen concentration control do not exist, only temperature and stirring speed are possible to maintain constant (Gamboa-Suasnavart et al. 2013). Therefore, and since phosphate nutrient acts as a buffering component of the culture media, the reduction of its concentration consequently led to the pH

reduction. The pH initially in 6.90 ± 0.03 was then slowly changed over time reaching to ≤ 4.0 for most experiments.

Once the bacterial growth was not apparently affected by the diminution of phosphate concentration in shake flasks, experiment B and C were firstly chosen to evaluate if the bacteria could adapt to these conditions, throughout the all assay, wherein oxygen levels could only be controlled in bioreactor.

4.2.2 Bioreactor Cultivation assays

Since in a bioreactor experiment, loss of buffering capacity of the culture medium can be overcome (by *online* addition of base and acid in response to pH alterations) and all parameters are simultaneously controlled, two initial cultivation assays were conducted to validate preliminary tests, as shown in Figure 4.6. Those cultivation assays are related to the phosphate concentrations of 5.25 g L^{-1} and 3.81 g L^{-1}, identified as Phalf and Pquarter, respectively. These two conditions were selected to assess, in the first place, if a smaller reduction on the macronutrient phosphate, in the culture medium, did not negatively disturb the EPS production.

It is important to refer that each inoculum was grown in the same phosphate concentration of the correspondent cultivation assay, so that any condition couldn't be favored prior to bioreactor inoculation.

These cultivation assays were also performed with DO at 10%, since it was previously established that it was considered the best DO concentration to promote an optimal bacterial growth and EPS production.

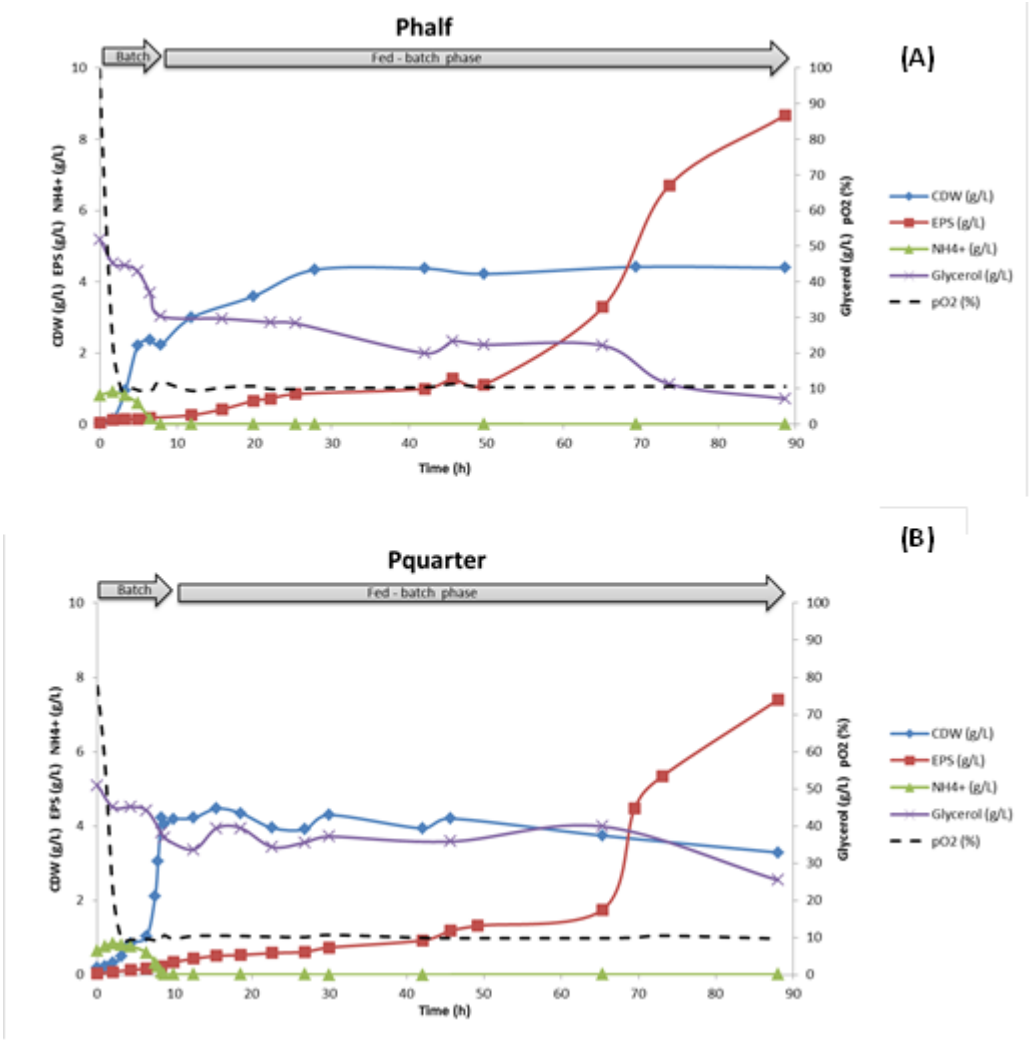

Fig. 4.6 - Cultivation profiles for *Enterobacter* A47 with dissolved oxygen concentration controlled at 10% air saturation and phosphate concentrations: 5.25 g L^{-1} (A) and 3.81 g L^{-1} (B).

The Phalf and Pquarter cultivation assays, in Figure 4.6, showed similar cultivation profiles compared to the standard conditions in Figure 4.1 (A), which contained a total phosphate concentration of 8.12 g L^{-1}.

Following the inoculation of the culture into the bioreactor, it was observed a sharp decrease in DO in the first 5 h of both batch phases, which was kept at 10% till the end of both runs. Ammonium depletion (<0.1 g L^{-1}) was also achieved in the first 9 to 10 h of both batch phases and maintained throughout the entire cultivation assays.

On the other hand, cell growth behaved very differently in these two situations. In the Phalf run, a fast bacterial growth with a specific growth rate of 0.31 h^{-1} was verified, reaching to a biomass concentration of 2.36 g L^{-1} at the end of batch phase. Afterwards, biomass concentration increased in the next 20 h, to a maximum of 4.42 g L^{-1}.

In the Pquarter run, a slow bacterial growth occurred in the first 5 h, probably due to an initial microbial adaptation to the culture conditions. Then, it was followed by an exponential cell growth characterized with a specific growth rate of 0.26 h^{-1}, which led to a biomass concentration of 4.29 g L^{-1}, at the end of batch phase. Biomass concentration did not significantly change by the end of the run.

The carbon source consumption, in both batch phases, presented some differences. From the initially available 52 g L^{-1}, in Phalf, and 51 g L^{-1}, in Pquarter, around 22 and 16 g L^{-1}, respectively, were consumed. Also in both batch phases, as expected, it was verified a very low EPS production, with only 0.18 g L^{-1}, in Phalf, and 0.22 g L^{-1}, in Pquarter.

At this point, for both Phalf and Pquarter experiments, the fed-batch phase started and a feeding solution containing 200 g L^{-1} of carbon source was continuously fed into the bioreactors.

In the fed-batch phase, the glycerol consumption in Phalf, was around 20 g L^{-1}, and it was higher than in Pquarter, with just 10 g L^{-1}. Considering this kinetic parameter it is acknowledged that the glycerol accumulation in the broth may be explained due to the microbial adaptation in these culture conditions.

The biomass concentration was maintained at 4.35 g L^{-1} ± 0.05 for both cultivation assays till the end of the runs. This was supported by the overall biomass yield that revealed to be quite similar, 0.07 g_{cell} $g_{glycerol}^{-1}$ and 0.06 g_{cell} $g_{glycerol}^{-1}$, for Phalf and Pquarter, respectively.

Finally, the EPS production although slow, was enhanced after 50 h and 65 h in Phalf and Pquarter, reaching to 8.64 and 7.38 g L^{-1} maximum values, respectively. However, both conditions promoted similar productivities, 0.10 and 0.08 g L^{-1}, for Phalf and Pquarter, and also equal product yields, 0.13 g_{cell} $g_{glycerol}^{-1}$.

Concomitant with exopolysaccharide synthesis, broth's viscosity increased over time (Figure 4.7). A slight increase on the broth's viscosity was observed in the first 50 and 65 h, for Phalf and Pquarter, respectively. Afterwards, Phalf broth's viscosity started to rapidly increase reaching to 605 mPa.s (at 0.3 s^{-1}), while for Pquarter, a smoother increase of broth's viscosity was promoted, reaching to 274 mPa.s (at 0.6 s^{-1}).

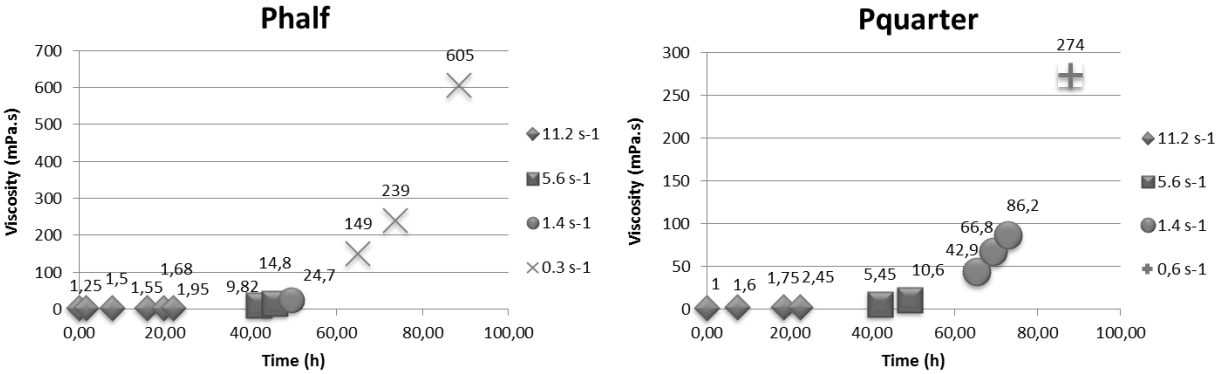

Fig. 4.7 - Apparent viscosity of the culture broth during cultivation of *Enterobacter* A47 with different phosphate concentrations: 5.25 g L^{-1} and 3.81 g L^{-1}, measured at shear rates of 0.3 – 11.2 s^{-1}.

According to Table 6, considering the volumetric productivities and product yield on glycerol, apparently the reduction of phosphate concentration in the medium was favorable for EPS production. So, to conclude, results suggest that an efficient bacterial growth along with high production performance can be reached with a reduction of the phosphate concentration to a quarter of the usually used value in previous work (Torres et al. 2010).

Table 6 - Kinetic parameters for the cultivation of *Enterobacter* A47 in medium supplemented with different phosphate concentrations.

Experiment	Standard(*)	Phalf	Pquarter
PO_4^{3-} (g L^{-1})	8.12 (*)	5.25	3.81
μ (h^{-1})	**0.36**	**0.31**	**0.26**
$CDW_{produced}$ (g L^{-1})	**7.68**	**4,40**	**4.29**
EPS (g L^{-1})	**7.50**	**8.64**	**7.38**
r_p (g $L^{-1}h^{-1}$)	**0.10**	**0.10**	**0.08**
glycerol $_{cons.}$ (g L^{-1})	**66.19**	**64.75**	**55.80**
$Y_{X/S}$ (g_{cell} $g_{glycerol}^{-1}$)	**0.49**	**0.07**	**0.06**
$Y_{P/S}$ (g_{EPS} $g_{glycerol}^{-1}$)	**0.11**	**0.13**	**0.13**

(*) *Torres et al.* 2010

Hereupon it´s possible to affirm that a decrease in the phosphate nutrient concentration didn´t affect the overall production behavior of *Enterobacter* A47, and was in fact proved to be as excellent as in standard conditions.

This conclusion is also beneficial to the environmental and economic cost of the bioprocess once this particular biopolymer can be produced in a sustainable affordable way.

In relation to the literature, it was reported in (Yu et al. 2007), that the highest EPS production, by *Aurebasidium pullulans*, was achieved with a phosphate concentration of 3 g L^{-}

[1]. Although insignificant interference in the EPS production was observed at higher concentrations, untill 9 g L^{-1}, a great decrease in EPS production was verified under 3 g L^{-1}. Furthermore the cell growth was not affected by the different phosphate concentrations.

Umashankar et. al. 1996, reported that *Xanthomonas campestris* had higher EPS production when 4 and 6 g L^{-1} of phosphate were used, while below 4 g L^{-1}, xathan production decreased. In regard to the cell growth, it wasn't affected by phosphate concentrations under 5 g L^{-1}, however with higher concentrations, it slowly decreased.

In this perspective, *Enterobacter* A47 behavior, related to the EPS production, has similar behavior to *Xanthomonas campestris*. This is corroborated with the increase in biopolymer production at phosphate concentrations between 3.81 and 5.25 g L^{-1}.

4.2.3 EPS Chemical characterization

In the phosphate study, the chemical composition of the biopolymers produced was accomplished by the determination of its sugar and acyl contents (Table 7), in first place, and also by the determination of its protein, ashes, water content and elemental analysis (Table 8).

The monosaccharide composition (Table 7) was significantly different from that reported by Torres et al. 2012, for fucose, galactose and glucose. Both exopolysaccharides presented a higher content in glucose, while galactose and fucose were reduced in comparison with the standard FucoPol (Freitas et al., 2011b). Nevertheless, the total acyl groups' content of both biopolymers (21.0 ± 0.5 % (w/v)) matched once again the standard values referred by (Torres et al. 2010).

Table 7 - Exopolysaccharide sugar and acyl composition of the different phosphate concentrations' cultivation assays.

Experiment	**Sugar monomers** (%mol)					**Total acyl groups** composition (% (w/v))
	Fucose	**Galactose**	**Glucose**	**Rhamnose**	**Glucuronic acid**	
Phalf	24	25	45	2	5	20.58
Pquarter	24	25	45	1	5	21.64

According to Table 8, it was verified that carbon, oxygen, nitrogen, hydrogen and sulfur contents were very similar for both Phalf and Pquarter's biopolymers.

Additionally, determination of the inorganic salts' composition revealed that it was less than 4 % (w/w), similarly to the values reported by Freitas et al. 2011b. Regarding the protein

content, it was also determined in a concentration lower than 4 % (w/v). For these last two reasons, it is possible to validate an efficient dialysis, since removal of most of contaminants was achieved.

Table 8 - Exopolysaccharide's elemental analysis and its water, protein and inorganic content of the different phosphate concentrations' cultivation assays.

Experiment	Elemental analysis (%)					Water (% (w/v))	Ashes (% (w/w))	Protein (% (w/v))
	C	**O**	**N**	**H**	**S**			
Phalf	40.91	30.85	4.18	6.20	0.00	17.59	2.80	3.29
Pquarter	41.26	30.99	4.38	6.13	0.10	12.28	1.39	2.99

Finally, considering that fucose render increased value to the EPS, the reduction of its content is not advantageous. Nonetheless, these exopolysaccharides are still industrially desirable due to their content in fucose, as mentioned before in section 1.6, towards pharmaceutical and cosmetic based products. However, the impact of phosphate concentration on EPS composition, especially on fucose content must be studied in greater detail to confirm that saving in production costs justify obtaining an EPS with lower fucose content and, eventually, different functional properties.

5 Functional Properties of the EPS synthesized by *Enterobacter* A47

The results presented in this chapter were published in a peer reviewed paper:

Freitas F., et al. (2013) Controlled production of exopolysaccharides from *Enterobacter* A47 as a function of carbon source with demonstration of their film and emulsifying abilities. Appl Biochem Biotechnol (in press) (DOI: 10.1007/s12010-013-0560-0).

In this chapter, the properties of different exopolysaccharides synthesized by *Enterobacter* A47 were studied.

These sugar and acyl groups composition, as well as the average molecular weight of these exopolysaccharides, are described in Table 2, where it's possible to observe that their physical-chemical characteristics are influenced by the cultivations.

5.1 Rheological properties

Due to their functional properties, microbial polysaccharides enable larger industrial applications. They can be used as thickening, emulsion stabilizer, flocculating and binding agents in various fields, for example, in food, pharmaceutical, cosmetic products, or even in paint manufacture. Nevertheless, these applications rely on the exopolysaccharide behavior in aqueous solutions and for this reason it is crucial a better understanding of the viscosity and viscoelastic properties of such polymers. Hence, the rheological properties of the different EPS synthesized by *Enterobacter* A47 were studied in aqueous solution.

The flow curves obtained for the different EPS produced are presented in Figure 5.1. All EPS solutions had the same concentration 1% (w/w) in 0.1 M NaCl and the flow curves were measured at 25 °C. It is observed that all biopolymers, except S8 and S10, showed a similar apparent viscosity profile as a function of the shear rate (shear-thinning behavior).

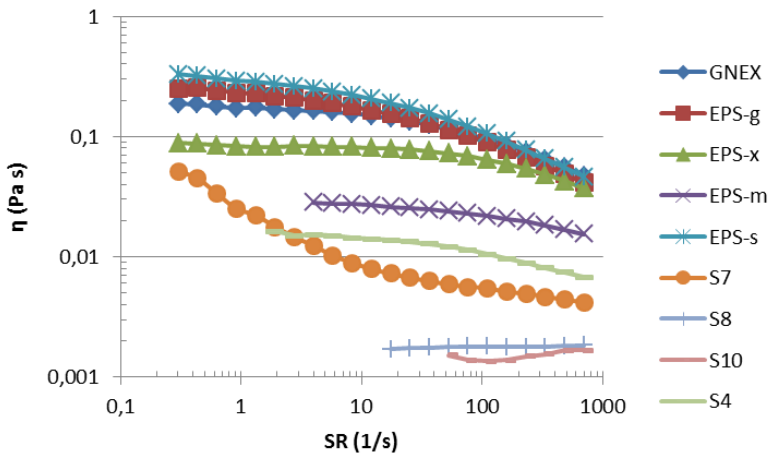

Fig. 5.1 - Comparative flow curves of the different exopolysaccharides produced by *Enterobacter* A47. The measurements were made at 25 °C, with 1% (w/w) of EPS aqueous solutions in 0.1 M NaCl.

The results show that the flow curves of the aqueous solutions of GNEX and EPS-g biopolymers were similar to the EPS obtained under the standard conditions (EPS-s). A Newtonian plateau is observed at 0.2 - 0.3 Pa.s (from 0.3- 17.5 s^{-1}) for EPS-s, GNEX and EPS-g polymers and the apparent viscosity decreases as the shear rate increases. Moreover, a better defined Newtonian plateau was demonstrated with EPS-x at 0.08 Pa.s (from 0.3- 17.5 s^{-1}), with shear-thinning behavior also present as the shear rate was increased.

In turn, for EPS-m and S4 solutions an attenuated shear-thinning behavior was verified. In relation to S7 solution, it showed also a shear-thinning behavior. However, the profile is different of the other solutions (Figure 5.1), since it presents a shear-thinning behavior for lower shear rates and for higher rates it is observed an approaching of the second Newtonian plateau.

Differently, S8 and S10 showed constant apparent viscosity for all measured shear rates, 0.017 and a 0.015 Pa.s, respectively, corresponding to a Newtonian fluid behavior.

The different rheological behavior shown by the polymers is probably related to their composition and average molecular weight. The EPS solutions with the highest apparent viscosity (EPS-s, GNEX and EPS-g) were composed of nearly equimolar amounts of the neutral sugar monomers: 26-36%mol fucose, 26-34%mol galactose and 26-30%mol glucose (Table 2). Moreover, their acyl groups content was higher (13-22 %(w/v)) than that of most of the other tested EPS. The lower apparent viscosity shown by EPS-x solution is probably related to the polymer's lower galactose content (18%mol), since its content in fucose (38%mol) and glucose (27%mol), as well as in acyl groups (17 %(w/v)), was close to those of EPS-s, GNEX and EPS-g solutions. On the other hand, the flow behavior of EPS-m and S4 might be explained by the polymers' considerably lower acyl groups content (4 and 8 %(w/v), respectively) and/or the presence of rhamnose and glucosamine in the polymers' composition (Table 2).

The Newtonian fluid behavior of S8 and S10 solutions might be related to their considerably different composition in comparison with the other tested polymers. The main sugar component of those EPS was glucose (54 and 37%mol, respectively), they had increased rhamnose (10 and 29%mol, respectively) and glucosamine contents (4 and 11%mol, respectively). Additionally, no fucose was detected in the composition of both polymers and their acyl groups content was also lower (1 and 4 %(w/v), respectively) than that of the other tested EPS.

The shear-thinning behavior, in aqueous media, has been observed for others microbial polysaccharides, such as, *Aeromonas nichidenii* 5797 (Xu et. al. 2007) and *Pseudomonas oleovorans* (Freitas et al. 2009b), for example. However, some microbial polysaccharides

display a Newntonian behavior, such as, for instance levan (produced by *Bacillus* sp.) until concentrations up to about 30 % (w/w) (Arvidson et al. 2006).

When the material response to increase stress is monitored at a constant frequency and temperature, it is called a stress sweep. When the material response to increase frequency is monitored at a constant stress and temperature, it is called a frequency sweep. So, a stress sweep evaluates the material resilience while a frequency sweep, evaluates the material viscoelasticity properties (Rao 2007).

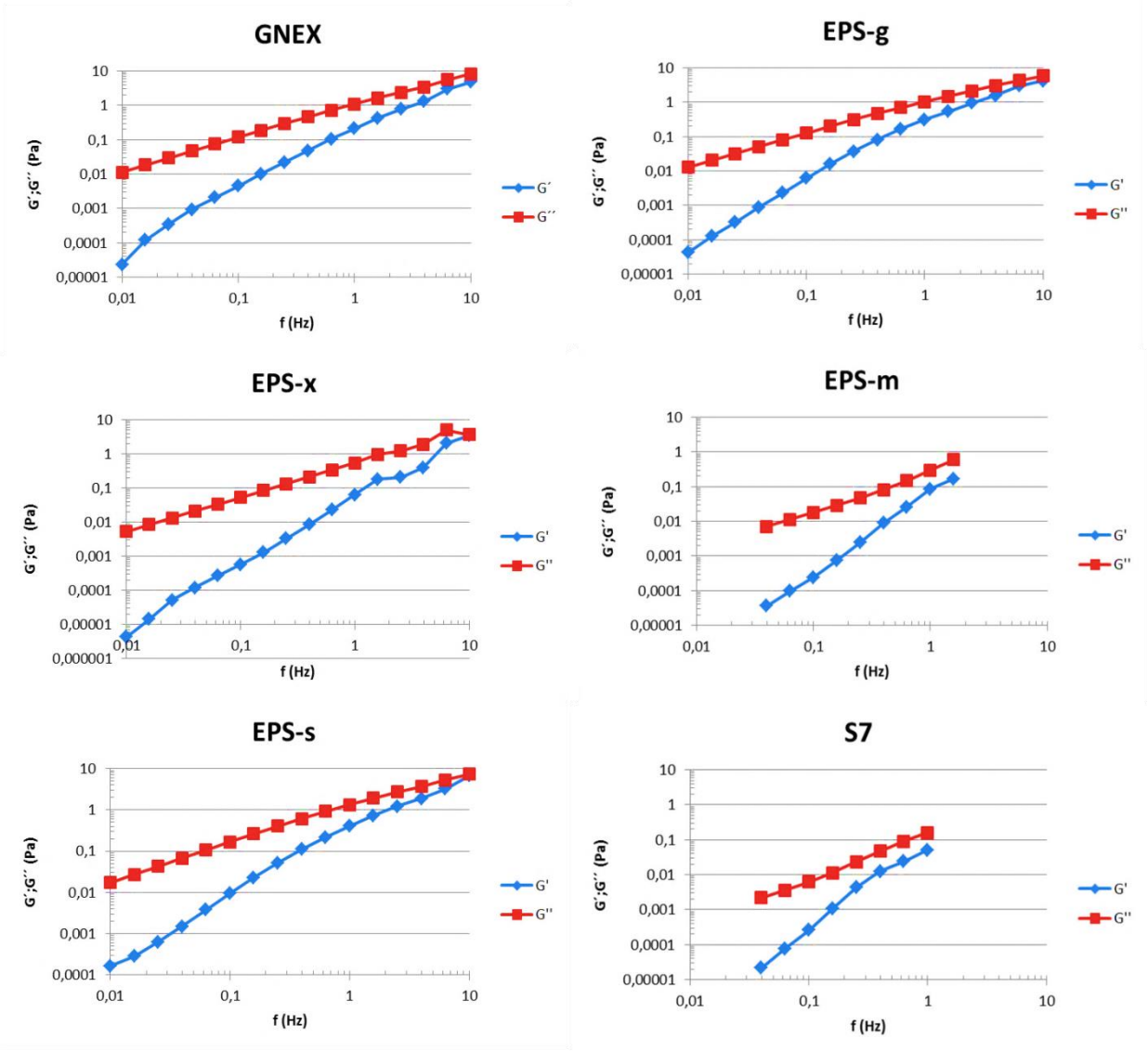

Fig. 5.2 - Mechanical spectra for the different exopolysaccharides, with a 1% (w/w) of EPS solution at frequency values up to 10 Hz, for a fixed tension within the linear viscoelastic region.

In Figure 5.2, the viscous modulus (*G*'') and the elastic modulus (*G*'), of the tested polymers, as a function of the frequency are represented. For all polymers the viscous modulus was higher than the elastic modulus, which is translated into a predominant viscous behaviour.

For GNEX, EPS-g and EPS-s polymers, the same behaviour was observed, wherein the mechanical spectra is characterized by the approaching of a crossover of the dynamic moduli, at the highest frequency, 10 Hz. EPs-x also showed an approaching of a crossover of both modulus at 10 Hz, but its dynamic moduli values were lower than GNEX, EPs-g and EPS-s, particularly in the elastic modulus. This dependence of the dynamic moduli with the frequency indicates the presence of solutions with entangled polymer chains.

For EPs-m and S7 polymers the viscoelastic moduli did not cross, nevertheless they presented identical viscous and elastic modulus' values for frequencies till 1 Hz, in comparison to GNEX, EPS-g and EPs-s.

5.2 Film-forming capacity

The polysaccharides synthesized by *Enterobacter* A47 have been also the subject of characterization for renewable materials into bioplastics trends, as they show non-toxic and eco-friendly film forming capacities (Nagarajan et al. 2013).

In order to assess this ability, a preliminary test was performed for all polymers, in which the outcome is shown in Figure 5.3.

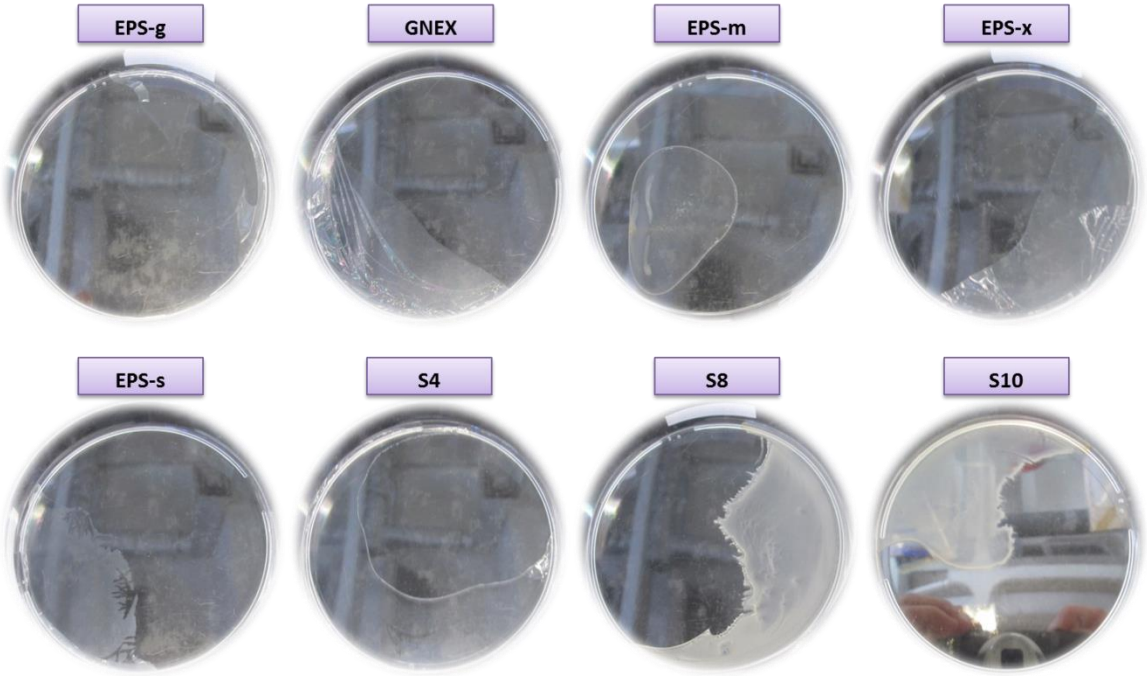

Fig. 5.3 - Preliminary test for film-forming capacity of the different exopolysaccharides produced by *Enterobacter* A47, in petri-dishes, using an aqueous solution of 1% (w/v).

First and foremost, it is possible to verify that most of the polymers were capable of forming films without the addition of any sort of plasticizer. As it can be seen by EPs-g, GNEX, EPS-x, EPS-s, EPS-m and S4 polymers, transparent films with notorious plastic appearance were obtained. S8 and S10 polymers were unable to form films (Figure 5.3)

With these results in perspective, the most promising film-forming EPS were chosen to perform further films characterization, namely, the study of their mechanical properties and water vapour permeability. The selected polymers were: EPs-g, EPs-x and EPs-s (Figure 5.4). For this film-forming characterization, a widely used plasticizer, glycerol, was used in order to overcome the films' brittleness usually associated with polysaccharide-based films, and also to increase their workability and flexibility (Tajik et. al. 2013). The glycerol was added into all EPS

solution in the same concentration (0.3 $g_{glycerol}$ $g_{polymer}^{-1}$) and the water content was measured, to ensure equal conditions.

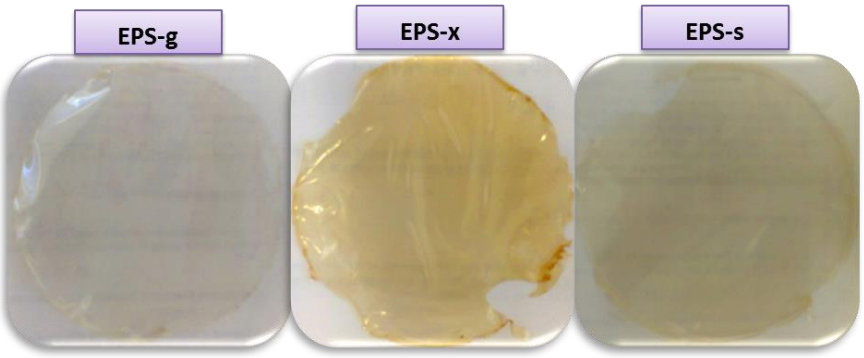

Fig. 5.4 - Films made from specific exopolysaccharides produced by *Enterobacter* A47, which presented film-forming capacity using an aqueous solution of 1.25 % (w/v), with presence of 30% of glycerol 99% as plasticizer.

At first sight both EPs-g and EPs-s exhibited thin, transparent, but somewhat fragile films, while EPS-x presented a colored and more elastic film.

The films were maintained under a controlled relative humidity before performing any others tests. The water content of the conditioned films from, EPs-g, EPs-x and EPs-s was: 18 ± 0.6, 15 ± 3.0 and 20 ± 0.2, respectively, describing an average of 18-20% for all three polymers. In addition to glycerol, a common plasticizer (Tajik et. al. 2013), the adsorbed water also acts like it, conditioning the films properties.

5.2.1 Tensile tests

The mechanical properties of a polymer allow to perceive the resilience of these type of biomaterials and, consequently, establish if they accommodate the desired applications' characteristics. In order to assess these properties, polymers were subjected to tensile stress to evaluate their elastic or plastic behavior. With elastic behavior, a polymer can suffer elongation and return to its original shape and size, upon removal of the tension, while with plastic (or ductile) behavior its deformation is permanent. After continuous stress the ductile material eventually ruptures, step also known as ductile fracture (He et al. 2013).

The selected polymers were then tested through tensile tests, as shown in the next figure:

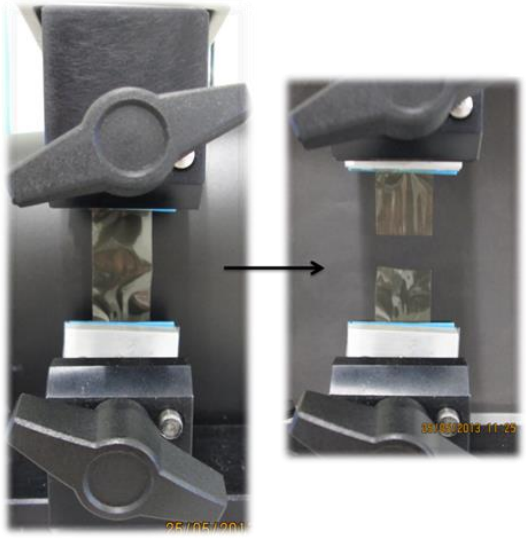

Fig. 5.5 - Assembly of the film in the TA-Xtplus texture analyser at rest and visualization of film's rupture as result of the tensile experiments.

As the films were stretching due to the applied force, their capacity to endure such strength was recorded *online* until they were no longer capable of stretch and break, as shown by Figure 5.5.

It was evidently observed that EPs-s and EPS-g films had similar mechanical properties (Figure 5.6), in relation to the deformation tolerated till break occurred. These two polymer films had identical strain at break, 4 and 5 % (Table 9), respectively, since for both of them an elongation of almost 3-4 mm was only possible with a force of approximately 8-10 N. On the contrary, for EPS-x polymer's break to occur it was necessary much less applied force, around 3 N, and it was stretched till 9 mm, which was more than double comparatively to the other polymers.

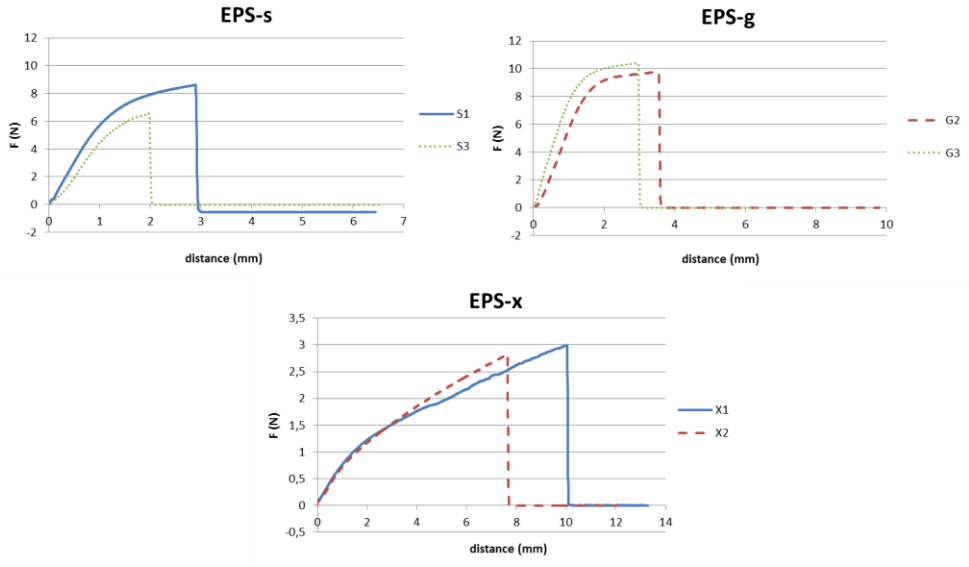

Fig. 5.6 - Representation of the tensile tests, F *vs* distance, of the EPS-s, EPs-g and EPS-x films.

Once EPs-x films required less stress to break (3.84 MPa) and in simultaneous demonstrated a higher strain at break (15%), nearly triple, it illustrated a significant reduction on the mechanical resistance when compared to the other films tested.

These facts were corroborated by the data presented in Table 9, whereas the lower mechanical resistance of EPS-x film is suggested by the lowest Young's modulus value, as opposed to the higher mechanical resistance of the EPS-s and EPs-g films.

Table 9 –Mechanical properties for the three tested biopolymers, EPs-s, EPS-g and EPS-x, and literature data, with 30% (w/w) of glycerol content.

Film	**Thickness** (μm)	**Young's modulus** (MPa)	**Stress at break** (MPa)	**Strain at break** (%)
EPs-s	32.0 ± 5.7	303	11.64	4
EPS-g	40.0 ± 8.5	425	15.54	5
EPS-x	57.0 ± 14.1	15	3.84	15
Soluble soybean polysaccharide (*)	73.0 ± 5.0	40	11.27	30

(*)Tajik et. al. 2013

Also, when compared to the reported data by Tajik et. al., 2013 (Table 9), it was possible to observe that only EPS-x described higher elongation with considerable reduction of the applied force, which represented a significant diminution of the mechanical resistance. Concerning EPS-s and EPS-g films, although they demonstrated higher mechanical resistance than the soluble soybean polysaccharide film, they consequently presented significant reduction in the ability to deform, a characteristic that doesn't meet the required standards. All these results can be probably due to the lesser film's thickness in comparison to the soluble soybean polysaccharide film.

The different characteristics of EPS-x film might be due to its different chemical composition in terms of galactose content that was much lower than the other two polymers. Besides that, EPS-x average molecular weight was lower ($1.7x10^6$) than the others (> $4.0x10^6$) (Table 2).

5.2.2 Water Vapour Permeability

This parameter indicates the easiness how water molecules diffuse through the dense film matrix, which are moving according to a concentration gradient (or the driving force).

The EPS-s, EPS-g and EPS-x films were also subjected to this characterization.

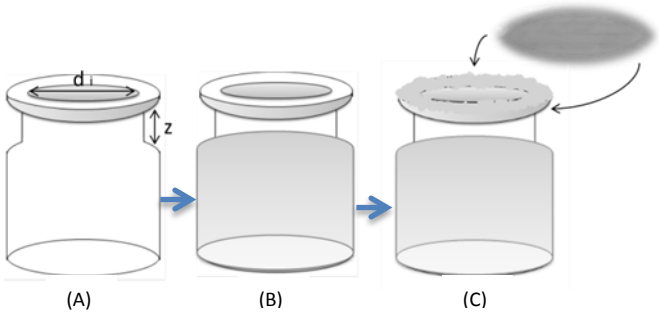

Fig. 5.7 - Schematics of the water vapour permeability's equipment: (A) vial; (B) vial with saturated $Mg(NO_3)_2.6H_2O$ solution and (C) assembly of a EPS film circle in (B), with silicone.

After approximately two days of experiment, it was determined the water vapour permeability, accordingly to equation 7 (Table 10).

Table 10 – Water Vapour Permeability data for the three tested biopolymers, EPs-s, EPS-g and EPS-x, and literature data, incorporated with 30% (w/w) of glycerol content.

Film	**WVP** (mol m^{-1} s^{-1} Pa^{-1}) **average**	**WVP** (mol m^{-1} s^{-1} Pa^{-1}) **standard deviation**
EPs-s	1.75E-11	7,290E-12
EPS-g	1.73E-11	3,994E-12
EPS-x	2.26E-11	5,186E-12
Soluble soybean polysaccharide (*)	1.13E-11	3.889E-12

(*)Tajik et. al. 2013

The water vapour permeability values for EPS-s and EPS-g films, with 1.75E-11 and 1.73E-11 mol m^{-1} s^{-1} Pa^{-1}, respectively, were identical to each other, while EPS-x film presented a higher permeability, of 2.26E-11 mol m^{-1} s^{-1} Pa^{-1}.

In literature, this characteristic has also been found for other polysaccharides. For example, in Tajik et. al. 2013, soluble soybean polysaccharide films (which have a pectin-like structure) presented a water vapour permeability of 1.13E-11 mol m^{-1} s^{-1} Pa^{-1}, with the same glycerol plasticizer concentration (30% (w/w)) and 73 ± 5.0 µm of film thickness. Regarding

EPS films produced by *Enterobacter* A47, the water permeability results do not meet the required parameters into food applications, just because the water vapour permeability in the biofilm should be as low as possible in order to retard the passage of moisture (Bourbon et al. 2011).

To conclude, all three EPS were able to form transparent stand-alone films, whose with mechanical and barrier properties must be enhanced. It is expected that EPS films produced by *Enterobacter* A47 can be tailor-made to meet the specifications of certain food products.

5.3 Emulsifying capacity

The newly demand of emulsifying properties has been increasing over the past years, in several different areas, from personal care products and food processing and even to oil industries, among others (Freitas et al. 2009b).

In response to these needs, microbial polysaccharides have attracted considerable attention over the synthetic products. Aside from their eco-friendly features, such as, biodegradability and low toxicity, microbial polysaccharides also display many attributes required in food applications: higher foaming, specific activity at extreme temperature, pH and salinity, without forgetting their ability to be synthesized from renewable and low cost sources (Banat et al. 2000).

For the purpose of assessing all polymers emulsion formation and stabilizing capacities, tests at different temperatures were performed, over a certain time period. The emulsions were made with 0.5 %(w/v) EPS aqueous solution and sunflower oil in a 2:3 (v/v) ratio and stirred in the vortex for 2 min.

In first place, as seen by Figure 5.8, all polymers were able to form emulsions at room temperature (23 ± 1.0 °C), with an emulsification index of 70% or higher (for the first 24 h), the exception being for EPs-x that wasn't able to form stable emulsions. After this positive preliminary test, additional emulsions tests were executed, at different temperatures, to evaluate their stabilizing capacity over time.

The polymers showed different behavior in terms of their emulsion forming and stabilizing capacity at the different temperatures of the tests. Equally to the emulsions at room temperature, the emulsification index at 24 h for all polymers at 4 and 37 °C were in average above 70%. From that point forward, each polymer emulsion behaved differently, as it will be explained next.

To begin with, it was observed that at room temperature, the emulsions prepared with either EPs-s or S10 polymer solutions showed the lowest decrease in emulsification, 15-20%, for a time period of 21 days. And, with an identical behavior, also GNEX polymer solution's stabilizing capacity presented a reduction of 20% in 17 days. After that, EPS-s and S7 solution polymers presented the highest emulsion stabilizing capacity (above 50%) for another 21-28 days.

At lower temperature, 4 °C, only GNEX and EPS-s polymer solutions possessed a stabilizing capacity above 60% for the first 14 days, while EPS-s polymer solution emulsification remained constant at 60% for another 4 weeks.

With higher temperatures, 37 °C, S10 and GNEX polymer solutions showed the highest stabilizing capacity within 21 days, which was above 60%. On the contrary, for EPs-s and EPs-g, both polymers described an inability to stabilize emulsions at higher temperatures, since it was continuously diminishing in the first 21 days, till eventually breakdown.

Finally, for S8 polymer solutions, they weren't able to stabilize the emulsions at any of the temperatures tested since the emulsification broke down in only 3-7 days, as well as for EPs-x polymer solutions, that in a matter of hours, all three emulsions broke.

This panoply of polymers resulted in a highly variability of results that can be fitted into numerous applications, thus because either demulsification either emulsification are parameters required for different industries. A demulsifier, as the EPS-g polymer, is able to reduce the interfacial shear viscosity of an oil-water interface, and promote the aggregation and coalescence of the water droplets. For this reason, EPS-g polymer, for instance, can compete with commercial polymeric surfactants in oilfileds, and be used for pipelining (Kang et al. 2013).

For the ones whose emulsions were kept stable for time periods longer than a month (4-5 weeks), they can be widely used in products that are expected to have longer lifetime. On the other hand, for those which exhibited less variance after exposure at different temperatures, they can be introduced into products that are expected to maintain their quality during and after transportation and/or storage. For GNEX and S10 polymer solutions, the emulsion stabilizing capacity is still needed to be studied for a longer period in order to conclude if they really promote emulsification above 50% for more than those initial 3 weeks.

To compare the results in Figure 5.8, only S7 and EPS-s polymer solutions were revealed as good emulsifiers (≥50%) at room temperatures and for more than 42 days, while EPS-s polymer solution also provided an emulsification above 50%, at 4 °C, for the same time period.

Relatively to the emulsification index (for 24 h) showed in emulsions at room temperature, only S4, S10 and EPS-g polymers presented an equal value, 80%, to the one showed with rice brain oil, as reported by Freitas et al. 2011b. These results were very promising once the EPS:oil ratio in these experiments, was lower (2:3) than in Freitas et al., 2011b (3:2), meaning that a smaller quantity of EPS solution is capable of efficiently stabilize emulsions. Moreover, EPS-s, S7, GNEX and EPS-m polymers exhibited similar or even higher emulsification indexes (≥90%) in relation to the synthetic surfactant Triton X-100 (90%) and other natural polysaccharides, such as xanthan, alginate and pectin (50-60%) emulsion stabilizing capacities, in water-rice bran oil mixture (Freitas et al. 2011b).

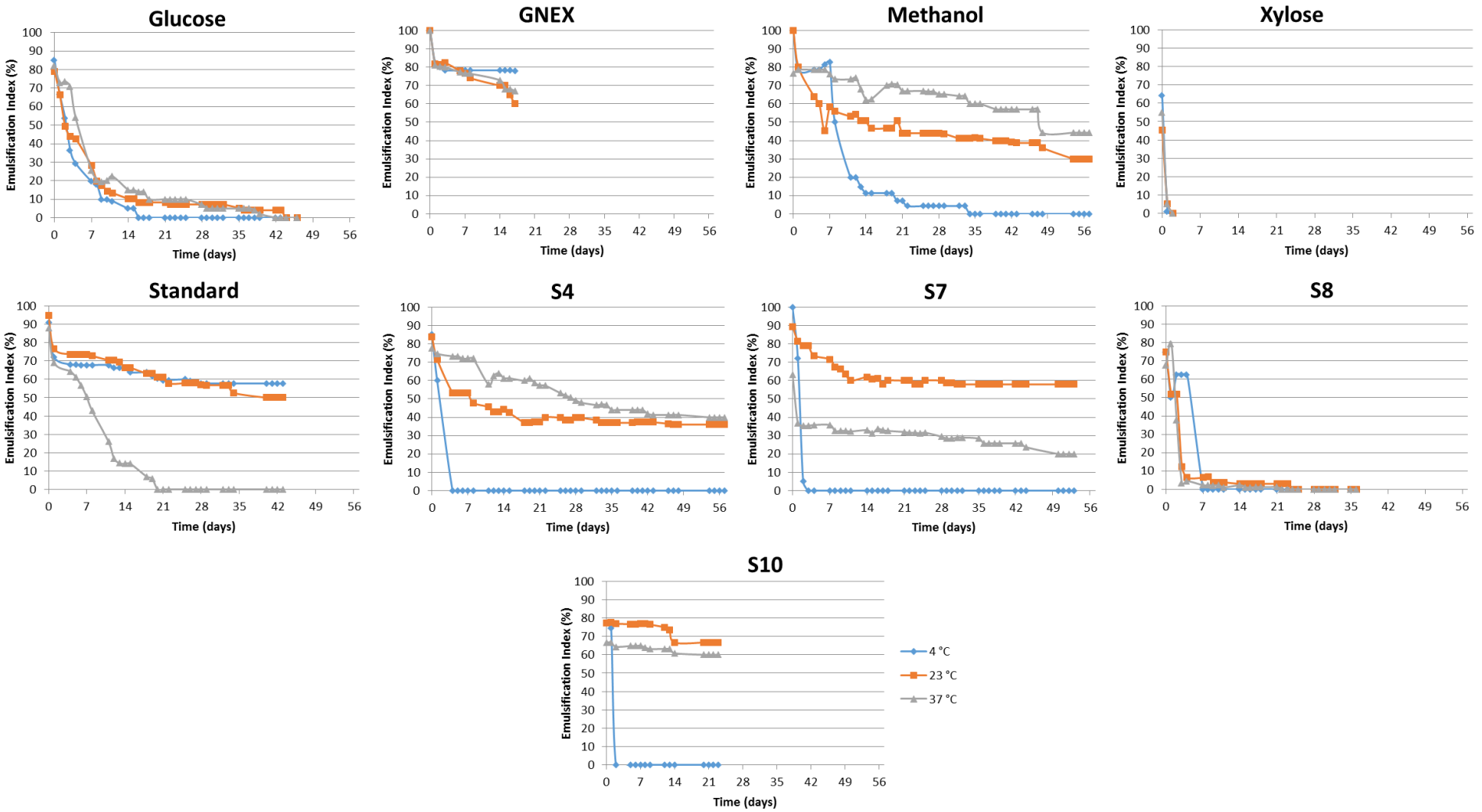

Fig. 5.8 - Representation of the emulsification index at different temperatures: 4, 23 and 37 °C, with 0.5% (w/v), for the different exopolysaccharides produced by *Enterobacter* A47.

Now, considering a time period of 17 days (Figure 5.9), in which all polymers could be equally compared, it was possible to verify that GNEX showed the most promising stable emulsions for all three different temperatures. These highest results are in agreement with (Willumsen et al. 1997), in which is established that a stable emulsion is defined by an emulsification index after 24 h, of 50%.

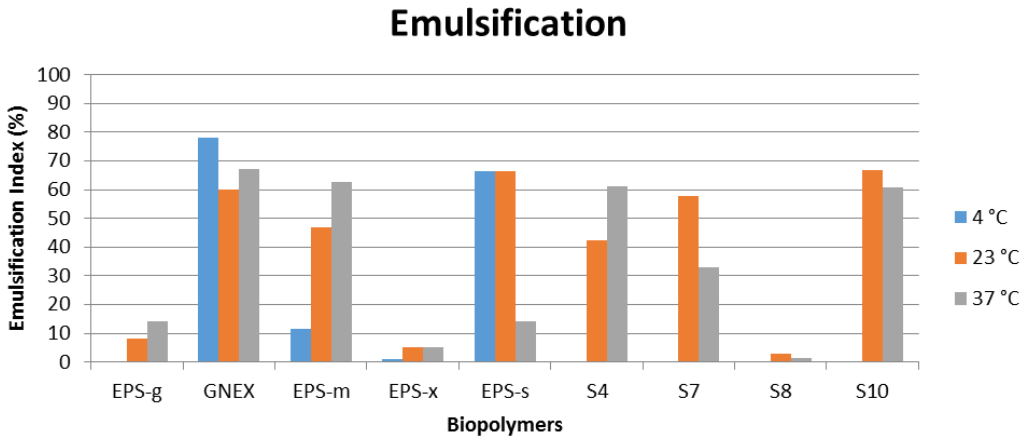

Fig. 5.9 - Emulsification index at different temperatures: 4, 23 and 37 °C within 17 days, of different exopolysaccharides produced by *Enterobacter* A47

Since GNEX fulfills this parameter, it can probably be explained by its lower molecular weight, 7.2×10^5 (Table 2).

Then, EPS-s polymer had the same profile as GNEX but only for emulsions exposed at 4 and 23 °C, possibly due to its higher acetate content. For S10 polymer solution, good emulsions were obtained for 23 and 37 °C, in which its higher rhamnose relative concentration may be the origin of it. For EPS-m and S4 polymer solutions, an only acceptable emulsion were produced at 23 °C, maybe because its glucose content is quite similar to the glucose content in S10, while for S7, a reasonable emulsion at 37 °C was caused by its absence of succinate content, commonly to S10.

As for EPS-g, EPS-x and S8, no emulsions were found at low temperatures, 4 °C, and residual emulsions were produced at 23 and 37 °C.

In spite this last data, it's conclusive that *Enterobacter* A47 is a good emulsifier-producing strain because the majority of its polymers' emulsions have emulsification indexes equal to 50%, 24 h after shaking.

5.3.1 Freezing and Thawing Stability

From the previous analysis on the emulsion stability over time, it was set aside EPS-x and S8 polymers into further assessments because of their absent emulsion stabilizing capacity.

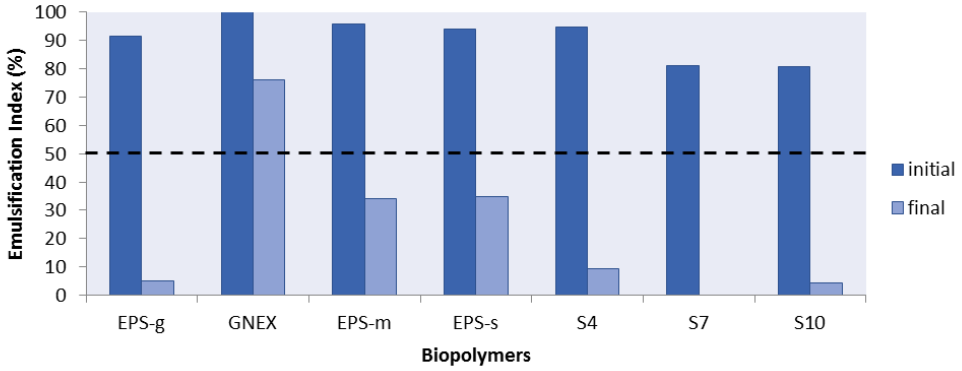

Fig. 5.10 - Emulsification index relative to freezing at -20°C for 24 h (initial) and sequential thawing at room temperature for 24 h (final).

After preliminary tests on emulsions at 4, 23 and 37 °C, it was performed an evaluation on their stability upon a freezing-thawing cycle (Figure 5.10). This test consisted on freezing the prepared emulsions at -20 °C for 24 h, followed by thawing those emulsions at room temperature, 23 °C, for another 24 h. After freezing at -20°C, all polymers described an excellent ability to stabilize highly stable emulsions (≥ 80%), but only GNEX, also presented great emulsion stabilization in sequential thawing at room temperature (24% reduction of emulsification index). From EPS-m and EPS-s polymers it was possible to achieve satisfactory emulsions, with only 30%, while from S4, EPS-g, S10 and S7, those were entirely disrupted.

Taking into consideration the high emulsion stabilizing capacity for all EPS solutions polymers (Figure 5.10) after freezing at -20 °C, their integration into frozen-food applications (e.g. pre-prepared meals, sugar syrups), can be applied. They can act as inhibitors of crystal formation, like xanthan is used to it (Sutherland 1998). In addition to this, GNEX polymer solution showed the highest emulsification upon freezing-thawing cycle, and consequently has the highest potential to be used for example in food applications wherein the products are frozen or stored at room temperature.

5.3.2 Thermal Stability

In addition to exposure at lower temperatures, the emulsion capacity to endure exposure to high temperatures was also measured. The emulsions prepared with the EPS polymers previously selected, were subjected to increasing temperatures, from 40 to 100 °C, as described in Figure 5.11.

In opposition to previous emulsification results, EPS-g turned out to be an excellent emulsifying agent at high temperatures, since its emulsification index was always close to 100%, decreasing slightly to 89 and 70% at 90 and 100 °C, respectively. Hence, they may be considered as thermostable emulsions.

For the emulsions prepared with S4, identical results were achieved with temperatures from 40 to 80, emulsification indexes varying between 100 and 80%, but as the temperature rose to 100 °C, the emulsification index dropped significantly to 35% as result of stability diminution. With S7 polymer, it was noted a good and constant stabilizing capacity at 70% for the increasing temperatures, even for 100 °C in which only EPS-g polymer was able to endure it too.

The emulsions prepared with EPS-s, GNEX, EPS-m and S10 polymers were less thermostable, as they were disrupted for temperatures above 50 °C (Figure 5.11).

As postulated in literature (Sutherland 2001), the polymers properties are influenced by many factors, such as, the sugar and acyl composition, primary, secondary and tertiary polymeric structure, molecular weight, presence of contaminants, among others. With this in perspective, and since all polymers were produced at different cultivations conditions, it could be the origin of such emulsion formation and stabilizing variability (Figures 5.8 – 5.11), in relation to these polymers solutions. For instance, proteins can be responsible for these differences in thermostability. If this contaminant isn't effectively removed during the polymer's purification step, it could act as surfactant, and in the presence of high temperatures (above 37 °C), protein denaturation may occur changing their surfactant ability and changing the stability of the emulsions.

To compare, some other microbial polysaccharides, like gellan and xanthan, have also been recognized as emulsion former and stabilizers agents (Imam et al. 2012). For instance, like xanthan (Sutherland 1998), the EPS polymer produced by *Enterobacter* A47, can be used to foam stabilization in fire-fighting fluids, wherein high temperatures are achieved.

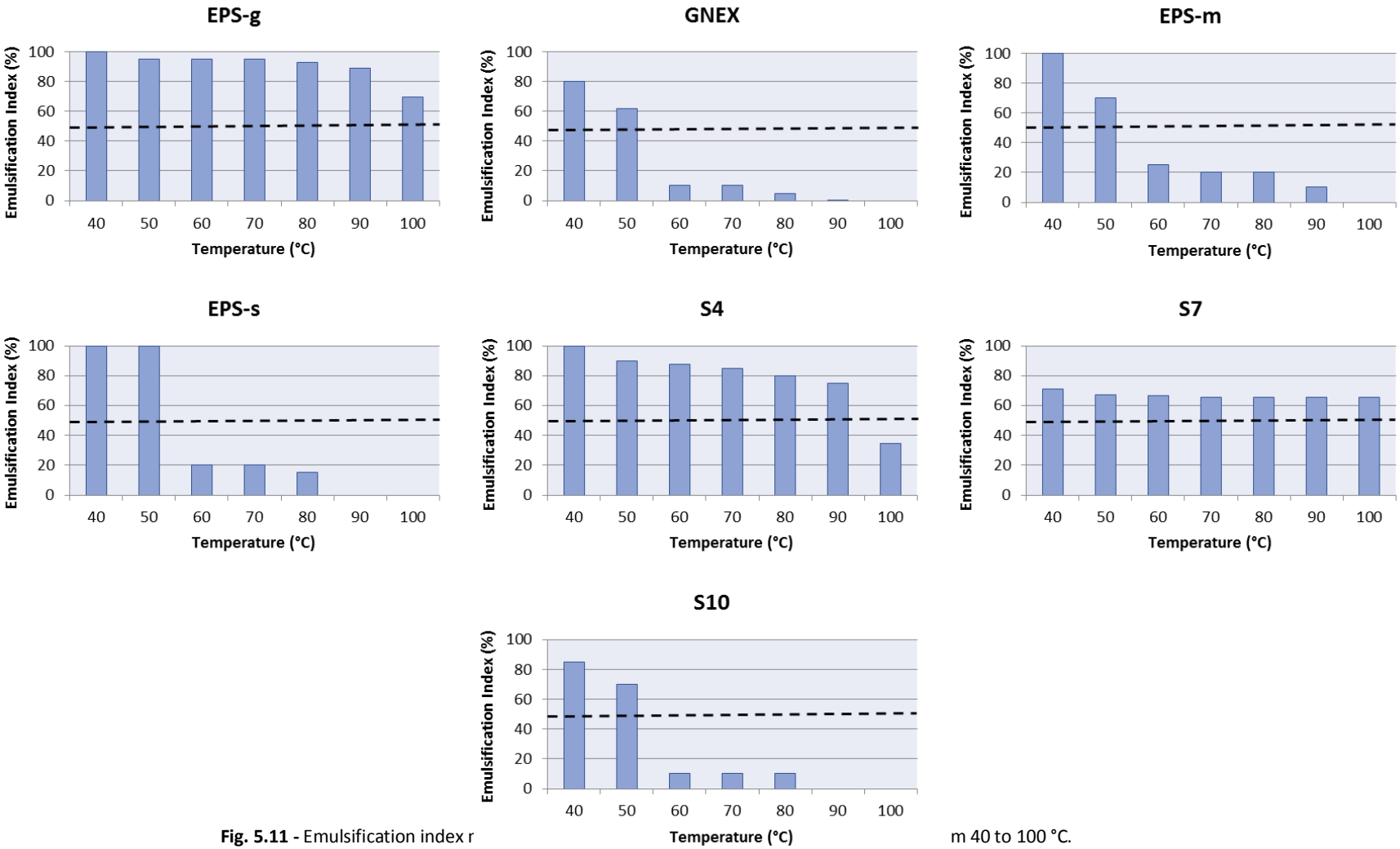

Fig. 5.11 - Emulsification index r m 40 to 100 °C.

5.4 Flocculating capacity

The main objective of this evaluation experiments was to assess the flocculating activity of the *Enterobacter* A47 polysaccharides, at room temperatures and neutral pH.

In order to determine this physical property, an EPS aqueous solution that acts as a flocculating agent, was added to a kaolin clay suspension. This flocculant agent can promote the flocculation activity after mechanical agitation, wherein the dispersed clay platelets spontaneously form flocs and are separated from the liquid phase (Chaiwong et al. 2008).

Two sets of experiments were performed, namely using EPS solutions with different concentration 0.01 and 0.001% (Figure 5.12). At this concentration, EPs-x immediately stands out for its superior flocculating capacity, in which 43% of the particles dispersed into the solution were separated from the liquid phase. In similar way, also S7 and EPS-m polymers were capable of flocculating 39 and 37% of the clay particles, respectively. However, EPS-s had the lowest flocculating capacity (<20%).

As demonstrated in Freitas et al. 2011b, considering the flocculants concentration of 0.01%, the most efficient inorganic flocculating agent, $Al_2(SO_4)_3$ (at 30%), was evidently exceeded by EPS-g < S8 < EPs-m < S7 < EPS-x polymers (in ascending order).

With a 10% lower concentration of EPS (Figure 5.12), almost all biopolymers' flocculating activity were dramatically reduced, especially for S8 and EPS-g polymers whose flocculating activity was extinguished.

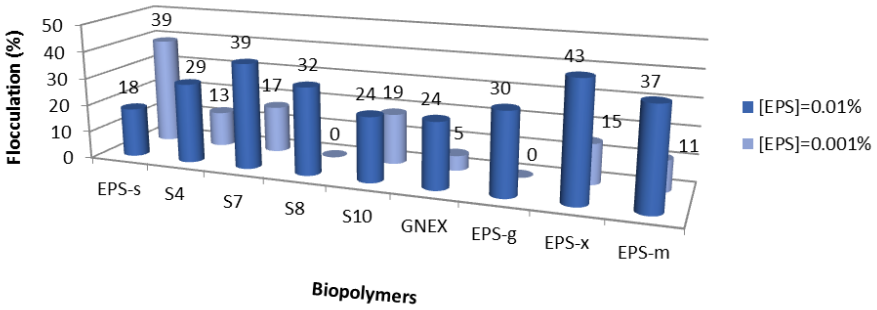

Fig. 5.12 - Flocculation capacity of different exopolysaccharides, produced by *Enterobacter* A47, at two distinct exopolysaccharide concentrations: 0.01 and 0.001%.

For EPS-x, EPS-m and S7 polymers that exhibited the highest flocculating activity (with 0.01%), it was diminished between 26-29%. However, S10 showed a similar flocculating activity for both EPS concentrations. And, for EPS-s, it was doubly enhanced, turning into the most economical promising biopolymer, since with fewer EPS concentration it was able to aggregate the same amount of particles presented in solution.

In conclusion, polysaccharides produced by *Enterobacter* A47 can be broadly used for specific market needs, not only because their flocculating capacity meet the high standards imposed by synthetic polymers, but also because their higher biodegrability, eco-friendly compatibility and low toxicity can compete with the low cost and not shear resistant synthetic polymers.

6 Conclusion and Future Work

Enterobacter A47 was able to grow and synthesize EPS under the different experimental conditions performed, namely, with DO at 10, 30 and 60%, as well as with reduced phosphate concentrations, 5.25 and 3.81 g L^{-1}. However, the culture's ability to synthesize EPS and the polymer's composition were affected by the conditions tested.

The optimal control of DO concentration for FucoPol production was determined to be 10% of air saturation value, due to its superior EPS production (6.11 g L^{-1}) and volumetric productivity (0.080 g L^{-1} h^{-1}), in comparison to the assays wherein DO was controlled at 30 and 60%.

The reduction of phosphate concentration in the cultivation medium to a quarter of its value in the standard cultivation, did not affect the culture's EPS production capacity. In fact, an EPS production of 7.38 g L^{-1} was achieved, a value identical to the standard conditions (7.50 g L^{-1}) previously reported. Moreover, this reduction implied a considerable diminution of the overall FucoPol's cost production, since phosphate sources are expensive.

In future studies, the FucoPol production can be further improved by assessing the influence of other inorganic salts present in the cultivation media, namely, magnesium, calcium, sodium, potassium, among others, as suggested by studies performed with other microorganisms.

The study about the functional properties of the distinct EPS synthesized by *Enterobacter* A47 revealed that their different physical-chemical characteristics have conferred them different interesting properties.

In terms of rheology, all polymers demonstrated shear-thinning behavior, the exception being for S8 and S10 in which a Newtonian plateau was described. It would be interesting to complement this part of the study, with an evaluation of the polymers' apparent viscosity at various temperatures, and also an assessment of the polymers' intrinsic viscosity, the latter being because they all present different molecular weights and possibly different chain rigidities.

Concerning the film-forming capacity, EPS-s, EPS-g and EPS-x films presented high water vapour permeability and, in addition to this, it was clearly observed that EPS-x films demonstrated a superior elasticity. However, it is still necessary to examine the mechanical resistance of these three films with variable glycerol concentrations and without glycerol as plasticizer, in order to correlate its influence on the films behavior. Moreover, all of these film-forming tests are yet to be performed for S4 and GNEX polymers, who also demonstrated film-forming capacity.

Regarding the emulsion formation capacity, all polymers possessed this functional characteristic. Nevertheless, emulsion stabilizing capacity was not present in all of them, more precisely to S8 and EPS-x. Instead, EPS-s polymer solutions showed higher stability (at 4 and 23 °C) within 42 days, while S7 polymer solution stabilizing capacity (at 23°C) was extended for another 14 days. With freezing-thawing cycle evaluation, only GNEX showed excellent emulsion stabilizing capacity, while at increasing temperatures, it was EPS-g who evidently had superior stabilizing capacity. This work could also be optimized by varying the EPS:oil ratio in the mixtures, and assessing the minimal quantity of EPS needed to form and stabilize emulsions.

Additionally, all polymers presented different flocculating capacities for both 0.01 and 0.001% of EPS. The EPS-x polymer showed highest flocculating capacity, 43% (at 0.01%), and EPS-s increased its flocculating capacity up to 38%, with a lower concentration (0.001%). Thus, this capacity should also be studied within different pH ranges and with different cations, for example.

In conclusion, *Enterobacter* A47 is able to produce various polymers with many different functional properties. This versatility is very interesting thus because it is possible to produce tailor-made polymers (to meet specific market demands), and also because it can have a wide range of applications. Those applications can be implemented in food, pharmaceutical, oil and petroleum industries, painting manufacture, and others, as emulsifier, stabilizer, gelling agents, thickening agents, film formers, and even to be incorporated as antimicrobial or antioxidant agents, for example.

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