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Biological conversion of industrial by-products/wastes into value-added bacterial exopolysaccharides

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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To my men, Nuno and Benjamim

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Abstract

This thesis is focused on the production of value-added bacterial exopolysaccharides (EPS) by the bacterium *Enterobacter* A47 (DSM 23139), using industrial by-products/wastes as substrates. *Enterobacter* A47 has demonstrated the ability to synthesize a high molecular weight (M_w) fucose-rich EPS, namely, the heteropolysacharide named FucoPol. This new EPS is composed of fucose, glucose, galactose and glucuronic acid, which present interesting functional properties.

Cheese whey was the first industrial by-product studied. Under standard controlled parameters for FucoPol's production, *Enterobacter* A47 successfully grew using lactose as carbon source and an EPS concentration of 6.40 g L^{-1} was reached within 3.2 days of cultivation, corresponding to a volumetric productivity of 2.00 g L^{-1} d^{-1}. The produced EPS was mainly composed of glucuronic acid and fucose, which confers it a great potential for use in high-value applications, such as cosmetics and pharmaceuticals.

The use of out-of-specification tomato paste as substrate resulted in the highest production (8.77 g L^{-1}) and overall volumetric productivity (2.92 g L^{-1} d^{-1}), which were obtained with continuous substrate feeding at a constant flow rate of 11 g h^{-1}. The polymer produced had the typical FucoPol composition: 30-36 mol% of fucose; 22-29 mol% of galactose; 25-34 mol% of glucose; 9-10 mol% of glucuronic acid and 12-22 wt.% of acyl groups.

The ability of *Enterobacter* A47 to grow and produce EPS using as carbon source a mixture of glucose and xylose (75:25%) was also tested using pure sugars and a brewer's spent grain (BSG) hydrolysate. The use of BSG as substrate resulted in an EPS concentration of 2.30 g L^{-1} with a low volumetric productivity (0.57 g L^{-1} d^{-1}). The produced EPS was mainly composed of glucose. On the other hand, 5.71 g L^{-1} of EPS was achieved after 4 days using commercial glucose/xylose mixture, giving an overall volumetric productivity of 1.43 g L^{-1} d^{-1}. The EPS produced revealed to be similar to FucoPol composition. Although that the ability of *Enterobacter* A47 to use BSG hydrolysate as sole substrate to grow and produce EPS was demonstrated, further studies need to be developed to increase the polymer's productivity.

In order to evaluate the potential of the different EPS produced by *Enterobacter* A47 in this study, their properties in aqueous solutions, emulsion forming and stabilizing capacity, and film-forming capacity were studied. EPS_{CW} presented the lowest apparent viscosity (η_a), but it was able to stabilize emulsions for over 4 weeks with olive oil, cedarwood oil and paraffin oil

and its films had higher elongation capacity. EPS_{TP} showed the lowest intrinsic viscosity ([η]) and angular frequency at dependence of storage (*G*') and loss moduli (*G*'') cross-over, good emulsion capacity and stabilization with peanut oil, almond oil and olive oil, and the produced films are slightly more rigid. In contrast, EPS_{GX} reached the highest [η] and η_a, the emulsions formed were very strong with most of the tested oils at low O/W ratios, although having low stability. Moreover, EPS_{GX} films were slight stiffer.

Keywords: Exopolysaccharides (EPS), *Enterobacter* A47, Industrial by-products/wastes, Fucose, Glucuronic acid, Functional properties.

Resumo

Esta tese é focada na produção de exopolissacáridos (EPS) bacterianos de valor acrescentado pela bactéria *Enterobacter* A47 (DSM 23139), utilizando como substracto subprodutos/resíduos industriais. *Enterobacter* A47 demonstrou a capacidade de sintetizar um EPS rico em fucose de elevado peso molecular (M_w), nomeadamente, o heteropolissacárido denominado FucoPol. Este novo EPS é composto de fucose, glucose, galactose e ácido glucurónico, que apresentam propriedades funcionais interessantes.

O soro de leite foi o primeiro subproduto industrial estudado. Sob os parâmetros controlados típicos da produção de FucoPol, *Enterobacter* A47 cresceu com sucesso utilizando lactose como fonte de carbono e uma concentração de 6,40 g L^{-1} foi alcançada em 3,2 dias de cultivo, correspondendo a uma produtividade volumétrica de 2,00 g L^{-1} d^{-1}. O EPS produzido era composto principalmente de ácido glucurónico e fucose, o que lhe confere um grande potencial para uso em aplicações de elevado valor, como cosméticos e produtos farmacéuticos.

A utilização de pasta de tomate não conforme como substrato resultou numa elevada produção (8,77 g L^{-1}) e productividade volumétrica global (2,92 g L^{-1} d^{-1}), à qual foram obtidas usando alimentação contínua de substrato a um caudal constante de 11 g h^{-1}. O polímero produzido tinha a composição típica do FucoPol: 30-36 mol% de fucose; 22-29 mol% de galactose; 25-34 mol% de glucose; 9-10 mol% de ácido glucurónico e 12-22 wt.% de grupos acilo.

A capacidade da *Enterobacter* A47 para crescer e produzir EPS usando como fonte de carbono uma mistura de glucose e xilose (75: 25%) também foi testada usando açúcares puros e dreche cervejeira hidrolisada. O uso de dreche como substrato resultou numa concentração de 2,30 g L^{-1} com baixa produtividade volumétrica (0,57 g L^{-1} d^{-1}). O EPS produzido era composto principalmente por glucose. Por outro lado, obtiveram-se 5,71 g L^{-1} de EPS após 4 dias usando uma mistura comercial de glucose/xilose, dando uma produtividade volumétrica global de 1,43 g L^{-1} d^{-1}. O EPS produzido revelou ser semelhante à composição do FucoPol. Embora tenha sido demonstrada a capacidade da *Enterobacter* A47 para usar a dreche cervejeira hidrolisada como único substrato para crescer e produzir EPS, é necessário desenvolver mais estudos para aumentar a produtividade em polímero.

Para avaliar o potencial dos diferentes EPS produzidos pela *Enterobacter* A47 neste estudo, as suas propriedades em soluções aquosas, capacidade de formar e estabilizar emulsões e

capacidade de formar filmes, foram estudadas. O EPS_{CW} apresentou a menor viscosidade aparente (η_a), mas conseguiu estabilizar emulsões durante mais de 4 semanas com azeite, óleo de cedro e óleo de parafina e seus filmes apresentaram maior capacidade de alongamento. O EPS_{TP} mostrou a menor viscosidade intrínseca ([η]) e a menor frequência angular de cruzamento do Módulo Elástico (G') com o Módulo Viscoso (G''), boa capacidade da formar emulsões e estabilizá-las com óleo de amendoim, óleo de amêndoa e azeite, e os filmes produzidos são um pouco mais rígidos. Em contraste, o EPS_{GX} atingiu a maior [η] e η_a, as emulsões formadas com baixo rácio O/W foram muito fortes com a maioria dos óleos testados, embora com baixa estabilidade. Além disso, os filmes EPS_{GX} eram ligeiramente mais rígidos.

Palavras-chave: Exopolissacáridos (EPS), *Enterobacter* A47, Subprodutos/resíduos industriais, Fucose, Ácido glucurónico, Propriedades funcionais.

Nomenclature

Abbreviations

ADP	Adenosine diphosphate
BSA	Bovine serum albumin
BSG	Brewer's spent grains
CDM	Cell dry mass
CDW	Cell dry weight
CMC	Carboxymethyl cellulose
DO	Dissolved oxygen level
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulture
EB	Elongation at break (%)
EM	Elastic Modulus (MPa)
EPS	Extracellular polysaccharide(s) or Exopolysaccharide(s)
EPS_{CW}	Exopolysaccharide produced by *Enterobacter* A47 using cheese whey as substrate
EPS-g	Exopolysaccharide produced by *Enterobacter* A47 using commercial glucose as substrate
EPS_{GX}	Exopolysaccharide produced by *Enterobacter* A47 using commercial glucose/xylose mixture as substrate
EPS_{lac}	Exopolysaccharide produced by *Enterobacter* A47 using commercial lactose as substrate
EPS-s	Exopolysaccharide produced by *Enterobacter* A47 using commercial glycerol as substrate

EPS_{TP}	Exopolysaccharide produced by *Enterobacter* A47 using out-of-specification tomato paste as substrate
EPS-x	Exopolysaccharide produced by *Enterobacter* A47 using commercial xylose as substrate
GDP	Guanosine diphosphate
HIV	Human immunodeficiency virus
HMF	Hydroxymethylfurfural
HPLC	High performance liquid chromatography
HPMC	Hydroxypropylmethylcellulose
LAB	Lactic acid bacteria
LB	Luria Bertani
LDPs	Lignin degradation products
MA	Massachusetts
MALS	Multi-angle light scattering
MWCO	Molecular weight cut-off
N	Nitrogen
O/W	Oil/water ratio
OECD	Organization for Economic Co-operation and Development
P	Phosphate
PEG	Polyethylene glycol
PS	Puncture strength at break (kPa)
RI	Refractive Index
SEC	Size exclusion chromatography
sp.	Specie(s)
TDP	Thymidine diphosphate
TFA	Trifluoroacetic acid
TS	Tensile strength at break (MPa)

UDP	Uridine diphosphate
UK	United Kingdom
US	United States
USA	United States of America
UV	Ultraviolet spectroscopy
UV/VIS	Ultraviolet-visible spectroscopy

Variables

Acet	Acetyl content (wt.%)
C	Polymer concentration (g dL^{-1})
CDM_{max}	Maximum CDM concentration (g L^{-1})
CDW_{max}	Maximum CDW concentration (g L^{-1})
dn dc^{-1}	Refractive index increment (mL g^{-1})
E_{24}	Emulsification index after 24 hours (%)
EI	Emulsification index (%)
EPS_{max}	Maximum EPS concentration (g L^{-1})
Fuc	Fucose content (mol%)
G’	Storage modulus (Pa)
G’’	Loss modulus (Pa)
Gal	Galactose content (mol%)
Glc	Glucose content (mol%)
GlcA	Glucuronic acid content (mol%)
he	Height of the emulsion layer (mm)
ht	Overall height of the liquid column (mm)
k_H	Huggins coefficient (dimensionless)
k_K	Kramer coefficient (dimensionless)

Mn	Number average molecular weight
M_w	Average molecular weight
PDI	Polydispersity index (dimensionless)
Pyr	Pyruvyl content (wt.%)
r_P	EPS volumetric productivity (g L^{-1} d^{-1})
Succ	Succinyl content (wt.%)
T	Temperature (°C)
t	Efflux time of the solution
t_0	Efflux time of the solvent
Xyl	Xylose content (mol%)

Greek letters

$\dot{y}$	Shear rate (s^{-1})
[η]	Intrinsic viscosity (dL g^{-1})
η_0	Zero-shear rate viscosity (Pa·s)
η_a	Apparent viscosity (Pa·s)
η_{sp}	Specific viscosity (dimensionless)
η_{rel}	Relative viscosity (dimensionless)
μ_{max}	Maximum specific growth rate (h^{-1})

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CHAPTER 1

Background and Motivation

1.1. Background

Since 1990, the scientific concept of sustainable chemistry seeks to *"improve the efficiency with which natural resources are used to meet human needs for chemical products and services. Sustainable chemistry encompasses the design, manufacture and use of efficient, effective, safe and more environmentally benign chemical products and processes. Sustainable chemistry is also a process that stimulates innovation across all sectors to design and discover new chemicals, production processes, and product stewardship practices that will provide increased performance and increased value while meeting the goals of protecting and enhancing human health and the environment."* (Organization for Economic Co-operation and Development (OECD)).

The development of biopolymers production and applications is a remarkable example of sustainable chemistry. A substantial number of biopolymers (polysaccharides, polyesters, polyamides, etc.) can be obtained from several natural sources, namely, plants, algae, animals and microorganisms. To gradually have more polysaccharides on the market is essential to develop new biodegradable biopolymers with superior material properties suitable for high-value applications.

1.1.1. Polysaccharides

Polysaccharides are high molecular weight carbohydrates (10^4-10^7 Da) occurred in Nature that perform important roles in several biological mechanisms such as adhesion, infection, immune response and signal transduction (Öner, 2013). Those biopolymers, that cover the surface of the most cells, are renewable materials, being generally non-toxic and biodegradable (Freitas et al., 2009).

Polysaccharides are composed of monomers connected through glycosidic linkages, often forming repeating units whose repetition would produce the complete polymer chain. Besides neutral sugars (e.g. galactose, glucose, mannose), acidic sugars (e.g. glucuronic acid, galacturonic acid) or amino-sugars (e.g. N-acetyl-glucosamine, N-acetyl-galactosamine), many of these polymers can also contain non-sugar components, such as organic groups (e.g. acetate, pyruvate, succinyl) or inorganic residues (e.g. sulphate) (Reis et al., 2008; Sutherland, 2001).

Their physicochemical properties allow a wide array of applications, for example, they can be used as thickeners, bioadhesives, stabilizers, prebiotic, and gelling agents in food and cosmetic products and as emulsifier, biosorbent, and bioflocculant in the environmental sector (Freitas et al., 2011 ; Öner, 2013).

1.1.2. Microbial Polysaccharides

Microorganisms synthesize a varied range of polysaccharides that have different cell functions, including:

- Intracellular polysaccharides that provide carbon or energy storage reserves (e.g. glycogen);
- Structural polysaccharides that are components of the cell structures, such as cell wall components (e.g. chitin);
- Extracellular polysaccharides (exopolysaccharides, EPS) that are secreted by the cells and accomplish a diversity of functions including adhesion, cell to-cell interactions, biofilm formation, and cell protection against environmental stresses (Öner, 2013). The EPS can form either a capsule that remains associated with the cell surface (e.g. K30 antigen) or a slime that is loosely bound to the cell surface (e.g. xanthan, gellan) (Rehm, 2010; Kumar and Mody, 2009).

EPS have been obtained from different genera of Archaea, Bacteria, Fungi and Algae, belonging to mesophilic (such as *Bacillus* species (sp.) and *Lactobacillus bulgaricus*), thermophilic (such as *Thermococcus* and *Bacillus thermantarcticus*) and halophilic groups (such as *Haloferax* and *Halobacterium*) (Laplagia and Hartzell, 1997; Nicolaus et al., 1993; Parolis et al., 1996; Rinker and Kelly, 1996; Sutherland, 1982). EPS-producing microorganisms are found in various ecological niches. Habitats having high carbon/nitrogen ratios are known to contain polysaccharides-producing microorganisms, for example, effluents from the sugar, paper or food industries as well as wastewater plants. In general, EPS producing bacteria are found in environments rich in organic compounds (Morin, 1998).

EPS production is influenced by cultivation conditions, such as medium composition (carbon, nitrogen and/or salt concentration), pH and temperature (Kumar et al., 2007; Torres et al., 2012, 2014). EPS biosynthesis is related to the primary carbohydrate metabolism of the producing cells and is expected to take place during active sugar consumption, as it requires large numbers

of activated nucleotide sugars, energy for building the repeating units, for polymerization and transmembrane translocation and is usually a growth-associated product (Figure 1.1) (De Vuyst and Degeest, 1999; Levander et al., 2002). The synthesis of polysaccharides with different composition and structures requires the recruitment of different enzymes and proteins, which is reflected in the varied organizations of the biosynthesis gene clusters. Induction of EPS biosynthesis is often associated with biofilm formation, during which EPS are important matrix components. The direct precursors for EPS biosynthesis, such nucleoside diphosphate sugars (e.g. ADP-glucose), nucleoside diphosphate sugar acids (e.g. GDP-mannuronic acid) and nucleoside diphosphate sugar derivatives (e.g. UDP-N-acetyl glucosamine) are described in Table 1.1 for several commercial bacterial polysaccharides (Rehm, 2010).

Based on their monomeric composition, polysaccharides are commonly classified as homopolysaccharides or heteropolysaccharides. Polysaccharides composed of only one type of monosaccharide repeating unit are classified as homopolysaccharides (pullulan, levan or dextran). Heteropolysaccharides are composed of two or more types of monosaccharides and usually present as multiple copies of oligosaccharides, containing three to eight residues (gellan or xanthan) (Freitas et al., 2011b; Öner, 2013). Table 1.1 also summarizes the chemical characteristics, main properties and applications of major bacterial and fungal commercial polysaccharides.

Several EPS produced by microorganisms are interesting to use in food, pharmaceutical, biomedical, bioremediation, waste water treatment and bioleaching fields due to their wide chemical, structural diversity and their physical, rheological and other unique properties. EPS may function as viscosifying agents, stabilizers, emulsifiers, gelling agents, or water-binding agents in food (Freitas et al., 2011b; Öner, 2013; Rehm, 2010).

Xanthan and gellan, which have widespread legislative approval for food use in the USA and Europe (Garcia-Ochoa et al., 2000), owe their success to their improved physical properties compared to traditional polysaccharides, such as alginate or carrageenan. Xanthan is an excellent thickening or suspending agent with high stability under a range of pH and temperature conditions (Garcia-Ochoa et al., 2000), while gellan is a gelling agent (Bajaj et al., 2007).

Hyaluronic acid, on the other hand, has found medical, pharmaceutical and cosmetic applications due to their similarity to eukaryotic polymers (Sutherland, 2001). Homopolymeric β-D-glucans, such as bacterial cellulose, are immune stimulants or tumour suppressive agents (Chawla et al., 2009).

Furthermore, in healthcare, some EPS act as antimicrobial compounds against pathogenic bacteria and have been used as natural food preservative and prebiotics (Salas-Jara et al., 2016). In biomedical applications, EPS could have antitumor, antiviral and immune stimulant activities (Moscovici, 2015).

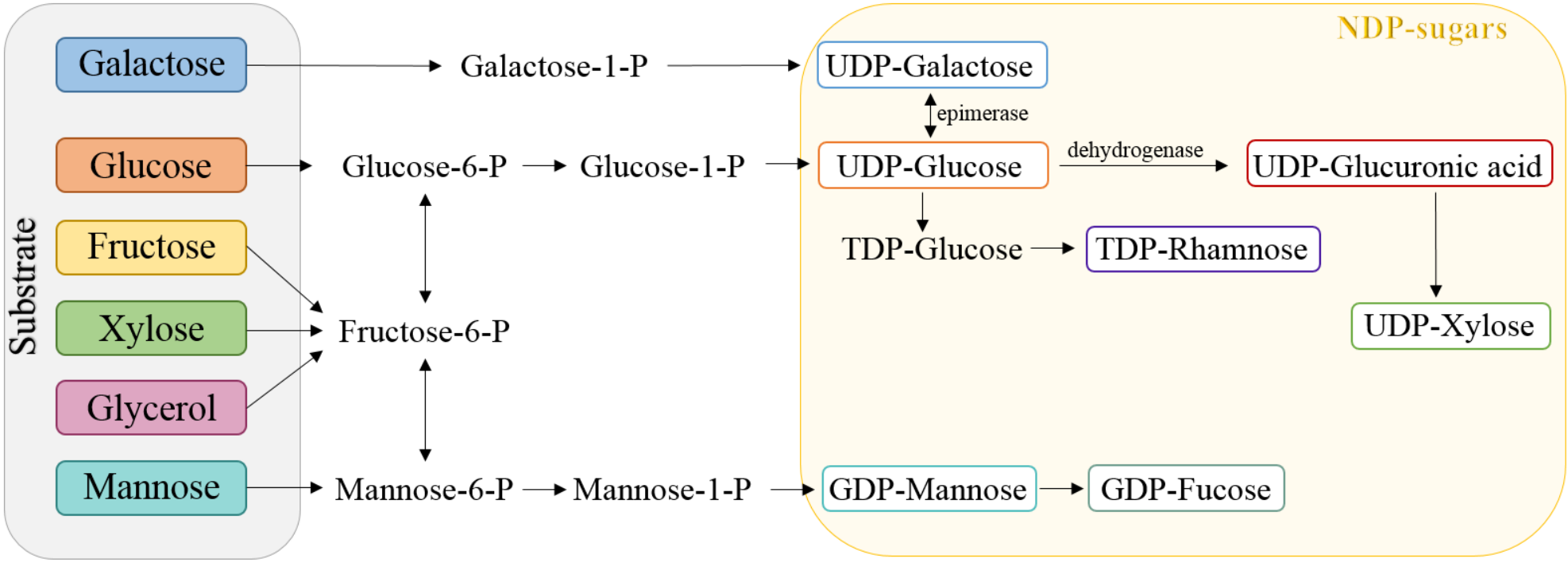

Figure 1.1: Representative scheme of the sugar nucleotide biosynthesis pathways for EPS producing microorganisms (P: phosphate; GDP: guanosine diphosphate; TDP: thymidine diphosphate; UDP: uridine diphosphate (adapted from Freitas et al., 2011b; Kumar et al., 2007; Ankit et al., 2012).

Table 1.1: Commercial microbial EPS: overview of the most relevant physicochemical and functional properties and main areas of application.

Polysaccharide	Producing strain	Composition	Precursors	Main properties	Main applications	References
Xanthan	• *Xanthomonas campestris*	Glucose Mannose Glucuronic acid Acetate Pyruvate	UDP-glucose GDP-mannose UDP-glucuronate	• High viscosity hydrocolloid - Stability over wide temperature, pH and salt concentrations ranges • Emulsifier	• Foods • Petroleum industry • Pharmaceuticals • Cosmetics and personal care products • Agriculture • Drilling muds	Garcia-Ochoa et al., 2000 Sutherland, 2002 Rehm, 2010 Freitas et al., 2011b
Gellan gum	• *Sphingomonas paucimobilis*	Glucose Rhamnose Glucuronic acid Acetate Glycerate	UDP-glucose TDP-rhamnose UDP-glucuronate	• Hydrocolloid - Stability over wide pH range • Gelling capacity - Thermoreversible gels - Thickening agent, gelling agent, stabilizer	• Foods • Pet food • Pharmaceuticals • Plant biotechnology • Research: agar substitute and gel electrophoresis	Sutherland, 2002 Bajaj et al., 2007 Banik et al., 2007 Rehm, 2010 Freitas et al., 2011b
Dextran	• *Leuconostoc mesenteroides* • *Streptococcus* sp. • *Lactobacillus* sp.	Glucose	Saccharose	• Non-ionic • Good stability • Newtonian • fluid behavior	• Foods • Pharmaceutical industry: - Blood volume expander - Chromatographic media	Naessens et al., 2005 Qader et al., 2005 Rehm, 2010 Freitas et al., 2011b
Bacterial Cellulose	• *Acetobacter* sp.	Glucose	UDP-glucose	• High crystallinity • Insolubility • in most solvents • High tensile strength • Moldability	• Foods (indigestible fiber) • Biomedical: - Wound healing - Tissue engineered blood vessels - Audio speaker diaphragms	Sutherland, 2002 Chawla et al., 2009 Rehm, 2010 Freitas et al., 2011b
Hyaluronic acid	• *Streptococcus* sp. • *Pasteurella multocida*	Glucuronic acid Acetylglucosamine	UDP-glucuronate UDP-N-acetyl glucosamine	• Biological activity • Highly hydrophilic • Biocompatible	• Medicine • Solid culture media • Cosmetics	Rinaudo, 2008 Liu et al., 2009 Rehm, 2010 Freitas et al., 2011b

Polysaccharide	Producing strain	Composition	Precursors	Main properties	Main applications	References
Alginate	• *Pseudomonas* • *Azotobacter*	Guluronic acid Mannuronic acid Acetate	GDP–mannuronic acid	• Hydrocolloid • Gelling capacity • Film-forming	• Food hydrocolloid • Biomedical: - Wound management - Controlled drug release - Surgical dressings	Rehm, 2010
Curdlan	• *Alcaligenes faecalis* • *Agrobacetrium* sp.	Glucose	UDP–glucose	• Gelling capacity • Thermoreversible gels • Water insolubility • Edible and non-toxic • Biological, anti-tumor and anti-HIV activity	• Foods • Pharmaceutical industry • Heavy metal removal • Concrete additive	Wu et al., 2012 Donot et al., 2012 Rehm, 2010 Freitas et al., 2011b
Levan	• *Bacillus subtilis* • *Zymomonas mobilis* • *Halomonas* sp. • *Erwinia* sp.	Fructose Sucrose Glucose	Levansucrase	• Low viscosity • High water solubility • Biological activity: • Anti-tumor activity • Anti-inflammatory • Adhesive strength • Film-forming capacity	• Food (prebiotic) • Feed • Medicines • Cosmetics • Industry	Donot et al., 2012 Franken et al., 2013 Freitas et al., 2011b
Pullulan	• *Aureobasidium pullulans*	Maltotriose Sucrose	UDP–glucose isomaltose	• Viscous non-hygroscopic solutions • Adhesiveness • Film formability • Enzymatically mediated degradability • Film-forming agent	• Tumor target drug delivery • Food: - low-viscosity filler in beverages and sauces • Plasma expander • Medical imaging • Tissue engineering and grafting	Prajapati et al., 2013 Donot et al., 2012

An expanding area of biotechnology is the application of EPS producing microorganisms in the remediation of environmental effluents produced by the mining industry (More et al., 2014). In general, biofilm reactors are used to treat hydrocarbons, heavy metals and large volumes of dilute aqueous solutions, such as industrial and municipal waste water (Pal and Paul, 2008; Singh et al., 2006). The potential role of EPS in the removal of heavy metals from the environment is due to their involvement in flocculation and ability to bind metal ions from solutions (Pal and Paul, 2008). Some bacteria, such as the sulphate reducing bacteria, have shown to be highly efficient in the anaerobic degradation of many organic pollutants and in the precipitation of heavy metals from waste water (Singh and Cameotra, 2004). Other bacteria exhibiting biosorption of toxic heavy metals in bioremediation processes include *Enterobacter* and *Pseudomonas* sp. (Pal and Paul, 2008)

The commercial value of a polysaccharide will depend on its composition, on the amount produced and the ease of extraction and processing (Reis et al., 2008). Extracellular products present the considerable advantage of having relatively easy extraction processes, comparing to polysaccharides that are plant or algae cell-wall constituents. The global hydrocolloid market dominated by algal and plant polysaccharides like starch, galactomannans, pectin, carrageenan, and alginate reached 3.9 billion US dollars by 2012 (Öner, 2013). Xanthan gum is the only significant bacterial EPS, which accounted for 6% of the total market value (Freitas et al., 2011b and 2014).

The sustainable and economical production of microbial polysaccharides requires innovative approaches and considerable progress has been made in discovering and developing new microbial extracellular polysaccharides that possess novel industrial significance (Öner, 2013). Furthermore, microbial sources are preferred since they enable fast and high yielding production processes under fully controlled fermentation conditions.

The main factor limiting the commercial production of microbial polysaccharides is the high cost of carbon sources, especially sugars (e.g. glucose, fructose, sucrose), that have a direct impact on production costs (Freitas et al., 2014; Sutherland 2001). From an economical point of view, the demand for cheaper substrates, with comparable incomes, is crucial to reduce production costs, since the substrate represents about 20 - 40% of those costs (Kumar et al., 2007). In order to reduce microbial polysaccharide production costs, it is desirable to use abundant and less expensive carbon sources, such as agro-food and industrial wastes and by-products.

1.1.3. Industrial Wastes/By-products Valorisation

In order to reach high production titers at reasonable costs, fermentation medium should be carefully designed to make the end product compatible with its synthetic petrochemical equivalent. Although, complex fermentation medium applied for growth and biopolymer production are not economically satisfactory due to their high amount of expensive nutrients such as peptone, yeast extract and salts and could represent almost 30% of the total cost of microbial cultivation (Öner, 2013). Until 20 years ago, studies were mostly focused on using defined culture conditions in order to recover pure biopolymers with the lowest variation between batches and free of contaminants that would interfere with their chemical and biological properties (Öner, 2013). Fortunately, the recent studies tend to use cheaper alternatives such olive mill wastewater, syrups, and molasses to maximize the cost effectiveness of the process (Nicolaus et al., 2010; Sutherland, 2007).

In developed countries, the agro-food industry generates large amount of wastes or by-products annually around the world from a variety of sources. Gradually, industrial ecology concepts such as cradle to cradle and circular economy are considered leading principle for sustainable innovation, aiming at "zero waste economy" in which waste or by-product are an excellent source of nutraceuticals, bioactive, inherently functional compounds that are good for human health (Mirabella et al., 2014). Nowadays, the process improvement of using wastes/by-products as substrates for industrial fermentations, such as sugarcane molasses, cheese whey, waste sugar beet pulp and glycerol from the biodiesel industry not just decrease the biopolymer production costs but also add value to that wastes/by-products that previously were incinerated, disposal in landfills or fed to animals being and an environmental concern. In addition, modern sustainable technologies, promote the use of food waste to obtain biopolymers that can be re-used in the same sector of the raw materials (Liguori et al., 2013).

Syrups and molasses have been tested as substrates for fermentative production of commercial polysaccharides such as pullulan (Göksungur et al., 2004; Israilides et al., 1998; Lazaridou et al., 2002; Roukas, 1998), dextran (Vedyashkina et al., 2005), levan (Küçükaşik et al., 2011; Oliveira et al., 2007), and gellan (Banik et al., 2007), mainly due to their high sucrose and other nutrient contents, ready availability and ease of storage (Öner, 2013). In particular, xanthan production is being tested as substrate sugar cane molasses, cheese whey, waste sugar beet pulp and peach pulp and achieved competitive results. Nevertheless, the industrial process is still based on glucose and sucrose due to the higher production yields and product quality they enable (Kalogiannis et al., 2003; Rosalam and England, 2006).

In addition, large amounts of lignocellulosic by-products/wastes are generated by the agro-industry, including: winery wastes (grape bagasse, grape stalks); rice husk and straw; tomato

pomace and nut shells, among others (Duarte et al., 2007). There have been several reports on the use of lignocellulosic materials or their hydrolysates as substrates for the production of different microbial products, including: hydrogen (Kapdan and Kargi, 2006), ethanol (Senthilkumar and Gunasekaran, 2005), lactic acid (Bi et al., 2009; Wang et al., 2010), xylitol (Rivas et al., 2009; Zeid et al., 2008), polyhydroxyalkanoates (Silva et al., 2007) and polysaccharides (Meade et al., 1994 and 1995).

1.1.4. FucoPol Production

Alves et al. (2010) reported for the first time the production of a new fucose-rich EPS patented by the Portuguese company 73100, Lda. (WO 2011/073874 A2) produced by *Enterobacter* A47, a short rod, gram-negative motile bacterium with a Patent deposit at Deutsche Sammlung von Mikroorganismen und Zellkulture (DSMZ) with accession number 23139 (Reis et al., 2011). *Enterobacter* A47 has demonstrated the ability to synthesize a novel exopolysaccharide, named FucoPol, when grown on glycerol by-product from the biodiesel industry (Alves et al., 2010). This new EPS is a fucose-containing high molecular weight heteropolysaccharide, characterized by interesting rheological, flocculating and emulsifying properties, which make it a good alternative to other polysaccharides in several applications (Alves et al., 2010; Cruz et al., 2011; Freitas et al., 2011a; Torres et al., 2011). On the other hand, the presence of fucose renders this biopolymer an enormous potential for use in pharmaceuticals (e.g. anti-cancer or anti-inflammatory agent, wound healing) and cosmetics (e.g. skin moisturizing and anti-agent) (Freitas et al., 2011b). FucoPol may also be used as a source of fuco-oligosaccharides and fucose monomers, presenting themselves added value applications (Hidari et al., 2008; Péterszegi et al., 2003; Vanhooren and Vandamme, 1999).

Previously, reported by Alves et al. (2010) and Freitas et al. (2011) a typical FucoPol production by *Enterobacter* A47, using glycerol by-product as carbon source, occur at 30 °C, pH 6.8 and C:N ratio of 14:1 (w/w). During the first 24 h, *Enterobacter* A47 grow exponentially and initiate the EPS production. Then, by imposing a nitrogen limiting condition (<0.1 g NH_4^+ L^{-1}) the feeding solution begin to enter into the bioreactor at a constant flow rate of 2.5 mL h^{-1} L^{-1}. Moreover, dissolved oxygen concentration is controlled at 10% by the automatic variation of the stirring rate between 300 – 800 rpm and it's when the FucoPol production reaches their maximum. Using the same industrial by-product, Torres et al. (2012) reported that the FucoPol production was maximized at 30 °C and pH 7.0. Later, Torres et al. (2014) reported that EPS synthesis by

Enterobacter A47 is influenced by both the initial glycerol and nitrogen concentrations and by the nutrients' feeding rate during the fed-batch phase.

Furthermore, *Enterobacter* A47 demonstrated the ability to grow and synthesize FucoPol using different sugars, including glucose and xylose, which are components of several agro-food industry wastes/by-products. In view of this, *Enterobacter* A47 has great potential to be used for the biotechnological valorisation of such feedstocks (Freitas et al., 2014).

1.2. Motivation and Thesis Outline

1.2.1. Motivation

Regarding the fact that renewable resources, like agro-food industrial wastes/by-products, may be a potential alternative substrate sources for biopolymers production, and that the bacterium *Enterobacter* A47 demonstrate the ability to use different sugars as carbon source, in this Ph.D. thesis, the development of production of EPS using agro-food industry by-products as substrate was studied.

The following main objectives were envisaged:

1- Screening of agro-food industrial wastes/by-products as substrates for *Enterobacter* A47 cultivation. Based on the literature and on the industrial partners, study and characterize different renewable materials;

2- Perform bioreactor cultivations to optimize the stoichiometric and kinetic parameters, such as cell growth rates, EPS productivities and yields of polymer on substrate;

3- Evaluate the impact of the different substrates tested upon polymer physical-chemical characteristics by the analysis of the chemical composition, molecular weight and intrinsic viscosity. Also, assess some functional properties, which will determine the final polymer applications, including the rheological properties, emulsion forming and stabilizing ability and film-forming capacity.

1.2.2. Thesis Outline

This thesis is divided into six chapters, describing the work performed during this Ph.D. project. The methodology used in each individual chapter is detailed in the context of the respective subject and, when applicable, is related to that used in previous chapters. Chapters 2, 3 and 4 are dedicated to EPS production using different agro-food industry by-products and process optimization. Chapter 5 describe the physicochemical properties of the EPS produced by *Enterobacter* A47 using the different wastes/by-products.

The work performed during this Ph.D. resulted in 2 scientific papers, which have been published in international scientific publications, while two more manuscripts are being prepared.

- Chapter 1 describes the background (state of the art) and motivation of this Ph.D. thesis.
- Chapter 2 presents the production of EPS by *Enterobacter* A47 using as carbon source commercial lactose and cheese whey. The chemical composition and M_w of the produced EPS (EPS_{CW}) are also described.
- Chapter 3 describes the production of EPS using out-of-specification tomato paste and optimization of the feeding strategy. The chemical composition and M_w of the produced EPS (EPS_{TP}) are presented.
- Chapter 4 reports the optimization of the brewer spent grains (BSG) hydrolysis for the bioreactor cultivation of *Enterobacter* A47 and EPS production. Also, cultivation with a mixture of glucose and xylose commercial sugars are described to mimic the use of hydrolysate from lignocellulosic materials and infer the ability of *Enterobacter* A47 to growth and produce EPS using this carbon source. The chemical composition and M_w of the produced EPS (EPS_{GX}) are also described.
- Chapter 5 describes the properties in aqueous solutions, ability to emulsify and stabilize mixtures of water and hydrophobic compounds and film-forming capacity of the EPS produced by *Enterobacter* A47 using cheese whey (EPS_{CW}) (Chapter 2), out-of-specification tomato paste (EPS_{TP}) (Chapter 3) and a glucose/xylose mixture (EPS_{GX}) (Chapter 4).
- Chapter 6 presents the final remarks and main conclusions of this thesis, along with some suggestions for future research are also proposed.

The scientific work developed in this Ph.D. project is described in Chapters 2 to 5. These chapters are written in the format of scientific papers, Chapters 2 and 3 being already published, while Chapter 4 and 5 are the basis of two manuscripts to be published. The methodology used in each individual chapter is detailed in the context of the respective subject and, when appropriate, is related to that used in previous chapters.

CHAPTER 2

Extracellular Polysaccharides Production: Cheese whey

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

Antunes, S., Freitas, F., Alves, V.D., Grandfils, C., Reis, M.A.M., 2015. Conversion of cheese whey into a fucose- and glucuronic acid-rich extracellular polysaccharide by *Enterobacter* A47. Journal of Biotechnology. 210, 1-7.

2.1. Summary

Cheese whey was used as the sole substrate for the production of EPS by *Enterobacter* A47. An EPS concentration of 6.40 g L^{-1} was reached within 3.2 days of cultivation, corresponding to a volumetric productivity of 2.00 g L^{-1} d^{-1}. The produced EPS was mainly composed of glucuronic acid (29 mol%) and fucose (29 mol%), with lower contents of glucose and galactose (21 mol% each) and a total acyl groups content of 32 wt.% . The polymer had an M_w of 1.8×10^6 Da, with a polydispersity index (PDI) of 1.2. This novel glucuronic acid-rich polymer with high content of glucuronic acid and fucose, two bioactive sugar monomers, confers it a great potential for use in high-value applications, such as cosmetics and pharmaceuticals.

2.2. Introduction

Cheese whey is a lactose-rich by-product generated in large amounts by the dairy industry, representing about 85-95% of the milk's volume. Worldwide, whey production averages around 1.15×10^8 to 1.40×10^8 tons per year, with an estimated annual increase of 1-2 % (Koller et al., 2012). This by-product consists mainly of lactose (70-80%), soluble proteins (8-14%), minerals (12-15%), lactic acid (0.8-12%) and fats (1-7%), possessing a high oxygen demand that needs to be treated before discharged into the environment (Koller et al., 2012; Prazeres et al., 2012; Siso, 1996). To overcome this, over the years, several approaches have been proposed aiming to treat or valorise cheese whey. Some of whey products utilization, mostly as food supplements, e.g. sweets, nutraceuticals, additives for processed food and baby food, are limited due to human lactose intolerance (Koller et al., 2012). Additional alternatives for cheese whey's valorisation include its use as a biotechnological resource for the generation of added-value products, such as bioethanol (Ozmihci and Kargi, 2007), biogas (Davila-Vazquez et al., 2009), organic acids (Roukas and Kotzekidou, 1998), electricity (Kassongo and Togo, 2010), proteins (Morr and Ha, 1993), polyhydroxyalkanoates (Koller et al., 2012; Pais et al., 2014) and microbial polysaccharides (Fialho et al., 1999; Khanafari and Sepahei, 2007; Savvides et al., 2012).

Bacterial EPS are carbohydrate polymers secreted by the cells that either remain attached to the cell envelope (capsular polysaccharides) or form a slime that is loosely bound to the cell surface (Freitas et al., 2011b; Rehm, 2010). Depending on their subunit composition, structure and molecular mass, EPS can have commercially relevant material properties that are attractive

for applications ranging from pharmaceuticals and cosmetics to industrial uses (Prazeres et al., 2012).

Most wild-type EPS producing bacteria are unable to efficiently using lactose as a carbon source because of their low β-galactosidase activity (Siso, 1996). To overcome this issue, it is often necessary to convert the disaccharide lactose into the constituent monosaccharides, glucose and galactose, either chemically or enzymatically, which increases the processes costs. An alternative is the use of genetically modified or adapted bacteria able to utilize lactose as carbon source (Audic et al., 2003). The first hypothesis increases the costs of the process since one additional step is required, while the second has implications on the microorganism genetic stability and transfer of recombinant genes with resistance to other bacteria. Thus, it is of interest to find microorganisms that can directly use lactose and convert it into the bioproduct (Davison, 2005).

Enterobacter A47 (DSM 23139) is an EPS-producer that synthesizes high molecular weight heteropolysaccharides composed of fucose, glucose, galactose and glucuronic acid, which present interesting functional properties (Cruz et al., 2011; Dhadge et al., 2014; Ferreira et al., 2014; Freitas et al., 2011a, 2014). *Enterobacter* A47 has demonstrated to be highly versatile due to its ability to use a wide range of substrates, including glycerol, glucose and xylose (Alves et al., 2010; Freitas et al., 2014), but lactose was not previously tested. Hence, in this work, the ability of *Enterobacter* A47 to use lactose and cheese whey as sole carbon sources for EPS production was assessed in fed-batch bioreactor cultivations. The produced polysaccharides were analysed in terms of sugar and acyl groups composition and M_w.

2.3. Materials and Methods

2.3.1. Exopolysaccharide production

Microorganism and media

The bacterium *Enterobacter* A47 (DSM 23139) was cryopreserved at -80 °C, in 20% (v/v) glycerol. Reactivation from the stocks culture was performed in Luria Bertani (LB) medium (bacto-tryptone, 10 g L^{-1}; yeast extract, 5 g L^{-1}; sodium chloride, 10 g L^{-1}; pH 6.8) that was also the medium used for inocula preparation.

In the bioreactor assays, *Enterobacter* A47 was grown on a slightly modified Medium E* (pH 7.0), with the following composition (per liter): $(NH_4)_2HPO_4$, 3.3 g; K_2HPO_4, 5.8 g; KH_2PO_4, 3.7g; 10 mL of a 100 mM $MgSO_4$ solution and 1 mL of a micronutrient solution (Freitas et al., 2014). The micronutrients solution had the following composition (per liter of 1N HCl): $FeSO_4 \cdot 7H_2O$, 2.78 g; $MnCl_2 \cdot 4H_2O$, 1.98 g; $CoSO_4 \cdot 7H_2O$, 2.81 g; $CaCl_2 \cdot 2H_2O$, 1.67 g; $CuCl_2 \cdot 2H_2O$, 0.17 g; $ZnSO_4 \cdot 7H_2O$, 0.29 g). Medium E* was supplemented with lactose (Scharlau) or cheese whey (supplied by Lactogal Produtos Alimentares S.A, Portugal) to give a lactose concentration of approximately 70 g L^{-1}. The cheese whey had a lactose content of 78.40 wt.%, as well as 13.62 wt.% protein, 1.21 wt.% fat and 0.89 wt.% lactic acid. Concentrated lactose and cheese whey aqueous solutions (125 g L^{-1}) were prepared and autoclaved separately (121 ºC, 2 atm, for 30 min). The autoclaved cheese whey mixture was further centrifuged, under aseptic conditions, at 10,375 × *g* during 15 min, to separate undissolved constituents and precipitated proteins. The resulting lactose-rich supernatant was used to supplement the cultivation medium.

Bioreactor cultivation

The experiments were performed in 5 L bioreactors (BioStat B, Sartorius, Germany) with initial working volumes of 2.5 L. Inocula for the assays were prepared by inoculating 20 mL of LB medium grown cells into 200 mL fresh LB medium and incubation in an orbital shaker for 65 h (at 30 °C, and 200 rpm). The culture obtained was centrifuged (4,053 × *g*, for 5 min) and the cells were resuspended in 50 mL phosphate buffer (K_2HPO_4, 5.8 g L^{-1}; KH_2PO_4, 3.7g L^{-1}; pH 7.0) for inoculation in the bioreactor.

All assays took 4 days, under controlled temperature and pH conditions of 30 ± 0.2 ºC and 7.0 ± 0.02, respectively. The aeration rate (0.125 vvm - volume of air per volume of reactor per minute) was kept constant throughout all cultivation runs. The dissolved oxygen level (DO) was controlled below 10% by the automatic variation of the stirrer speed (300-800 rpm). In the fed-batch phase, the bioreactor was fed with Medium E* supplemented with either lactose or cheese whey (lactose concentration of ~120 g L^{-1}) at a constant lactose feeding rate of 5 g L^{-1} h^{-1}. Samples (25 mL) were periodically withdrawn from the bioreactor for quantification of biomass, lactose, ammonium and EPS concentration, as well as measurement of the broth's viscosity.

Analytical techniques

The viscosity of culture broth samples was measured immediately after collection from the bioreactor, using a digital viscometer (Brookfield Engineering Laboratories Inc., Stoughton, MA, USA), for shear rates between 0.28 and 55.8 s^{-1}.

Culture broth samples were centrifuged at 13,000 × *g*, for 15 min, for cell separation. Viscous samples (>100 cPs) were diluted (1:2, v/v) with deionized water for viscosity reduction. The cell-free supernatant was used for the determination of nutrients concentration, and for EPS quantification. The cell pellet was used for the gravimetric determination of the cell dry weight (CDW), after washing twice with deionised water (resuspension in water, centrifugation at 13,000 × *g*, for 15 min, and, finally, resuspension in deionised water) and drying at 100 ºC until constant weight.

Sugars (lactose, glucose and galactose) and organic acids (citric and lactic acids) in the cell-free supernatant samples were determined by high performance liquid chromatography (HPLC), with a MetaCarb 87H column (Varian) coupled to a refractive index detector. The analysis was performed at 50 ºC, with sulphuric acid (0.01 N) as eluent, at a flow rate of 0.6 mL min^{-1}. Lactose (Scharlau), glucose (Sigma), galactose (Alfa Aesar), citric acid (Panreac) and lactic acid (Sigma) were used as standards at concentrations between 0.062 and 1.0 g L^{-1}. Ammonium concentration was determined using a potentiometric sensor (Thermo Electron Corporation, Orion 9512). Ammonium chloride (Sigma) was used as standard at concentrations between 0.006 and 1.8 g L^{-1}.

The EPS was extracted from the cultivation broth by the procedure described by Freitas et al. (2014), with minor modifications. Briefly, the cell-free supernatant was subjected to thermal treatment (70 °C, during 30 min) to denature remaining cheese whey and/or bacterial proteins. The precipitated proteins were removed by centrifugation (13,000 × *g*, for 15 min) and the treated supernatant was dialyzed with a 10,000 molecular weight cut-off (MWCO) membrane (Snake-Skin™ Pleated Dialysis Tubing, Thermo Scientific), against deionized water, over 48 h at 4 °C, and, finally, freeze dried. A cheese whey solution (125 g L^{-1}) was subjected to the same procedure to quantify the content in high molecular weight compounds present in the cheese whey powder.

All analyses were performed in duplicate.

Calculations

The overall volumetric EPS productivity (r_P, g L^{-1} d^{-1}) was determined as follows:

$$r_P = \frac{\Delta EPS}{\Delta t} \qquad (1)$$

where ΔEPS is the concentration of EPS produced (g L^{-1}) within the time period Δ*t* (d).

2.3.2. Polymer characterization

Chemical composition

For the EPS compositional analysis, polymer samples (~5 mg) dissolved in deionized water (5 mL) were hydrolyzed with trifluoroacetic acid (TFA) (0.1 mL TFA 99%) at 120 °C, for 2 h. The hydrolysate was used for the identification and quantification of the constituent monosaccharides by HPLC, using a CarboPac PA10 column (Dionex), equipped with an amperometric detector (Dionex), as described by Freitas et al. (2014). The acid hydrolysates were also used for the identification and quantification of acyl groups by HPLC, with an Aminex HPX-87H column (BioRad), coupled to an ultraviolet (UV), as described by Freitas et al. (2014).

Molecular weight

Number and weight average molecular weights (Mn and Mw, respectively), as well as the polydispersity index (PDI=Mw/Mn), were obtained by size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS). The latter combines a HPLC pump (Hewlett Packard quaternary 1050), an autoinjector (Hitachi-Merck, Lachrom L7200, model 1405 – 040), and a set of two analytical SEC linear columns (PL Aquagel-OH mixed 8 µm, 300 x 7.5 mm) protected by a guard column (Polymer Laboratory; 50 x 7.5 mm). UV (Beckman UV model 266 fixed at 254 nm) signals were recorded together with MALS and refractive index (RI) signals (the same MALS and RI detector as described above were used) in order to follow the purity and molecular mass distribution of the polysaccharide.

The EPS produced from lactose by *Enterobacter* A47 and the polysaccharide fraction recovered from the cheese whey powder were dissolved first in Tris–HCl 0.1 M; NaCl (0.2 M), pH 8.1 buffer, at a concentration of 0.2 g d L^{-1}. These solutions were warmed for 1 h at 80 °C in a water bath under lateral agitation. Dissolution of the polymer was continued for 35 h under rolling agitation at room temperature. A different dissolution protocol was used for the EPS produced from cheese whey because the Tris–HCl buffer solution was not suitable for its solubilisation. Hence, this EPS was dissolved overnight at room temperature under magnetic stirring (300 rpm) in NaCl (0.1 M). NaN_3 (10 ppm) was added to prevent microbial contamination. After dissolution, the pH was adjusted to 8.0.

EPS solutions were filtered with 0.45 µm polysulfone membranes (Whatman; Puradisc 25AS) before their injection on the SEC-MALS system. Tris-HCl buffer solution was used as eluent with a flow rate of 0.7 mL min^{-1} at room temperature. The analysis was performed by

injection of 100 µL of EPS solution (0.2 g d L^{-1}). Each analysis was conducted in duplicate. Signals from MALS (Wyatt Technology Corporation Dawn Model mounted with an uniphase argon laser (488 nm; 10 mW; K5 cell Flow cell) and RI signals (Optilab DSP, Wyatt Technology Corporation 488.0 nm, K5 cell at 30 °C) were recorded in parallel and treated with Astra (V 4.73.04) in order to follow the purity and molecular mass distribution of the polysaccharide. A dn dc^{-1} of 0.190 mL g^{-1} was adopted to calculate the Mw of the EPS. Quality control of the MALS installation was verified with polyethylene glycol (PEG) standards and bovine serum albumin (BSA).

2.4. Results and discussion

2.4.1. Cultivation of *Enterobacter* A47 on lactose as the sole substrate

In order to determine the ability of *Enterobacter* A47 to use lactose as sole carbon source for cell growth and EPS synthesis, without the potential interference of the cheese whey constituents, fed-batch bioreactor cultivations were conducted using lactose as model substrate (Figure 2.1).

Although the culture was able to use lactose for cell growth, the maximum specific growth rate (0.06 h^{-1}) was considerably lower than that obtained in previous studies with glycerol (0.26-0.32 h^{-1}), glucose (0.35 h^{-1}) or xylose (0.25 h^{-1}), under similar cultivation conditions (Table 2.1). A CDW of 4.56 g L^{-1} was attained at the end of the batch phase (2.3 days), when cell growth was limited by ammonium exhaustion and the fed-batch phase was initiated (Figure 2.1).

Concomitant with cell growth, 25 g L^{-1} of lactose were consumed out of the initial 74 g L^{-1}. No EPS production was detected during the batch phase. During the fed-batch phase, lactose concentration was kept above 40 g L^{-1} by supplying the bioreactor with a lactose containing medium. These conditions of lactose availability are required for lac operon's positive regulation. In gram-negative bacteria, lactose is converted to allolactose, acting as an inducer of lac operon's transcription into β-galactosidase (Juers et al., 2012).

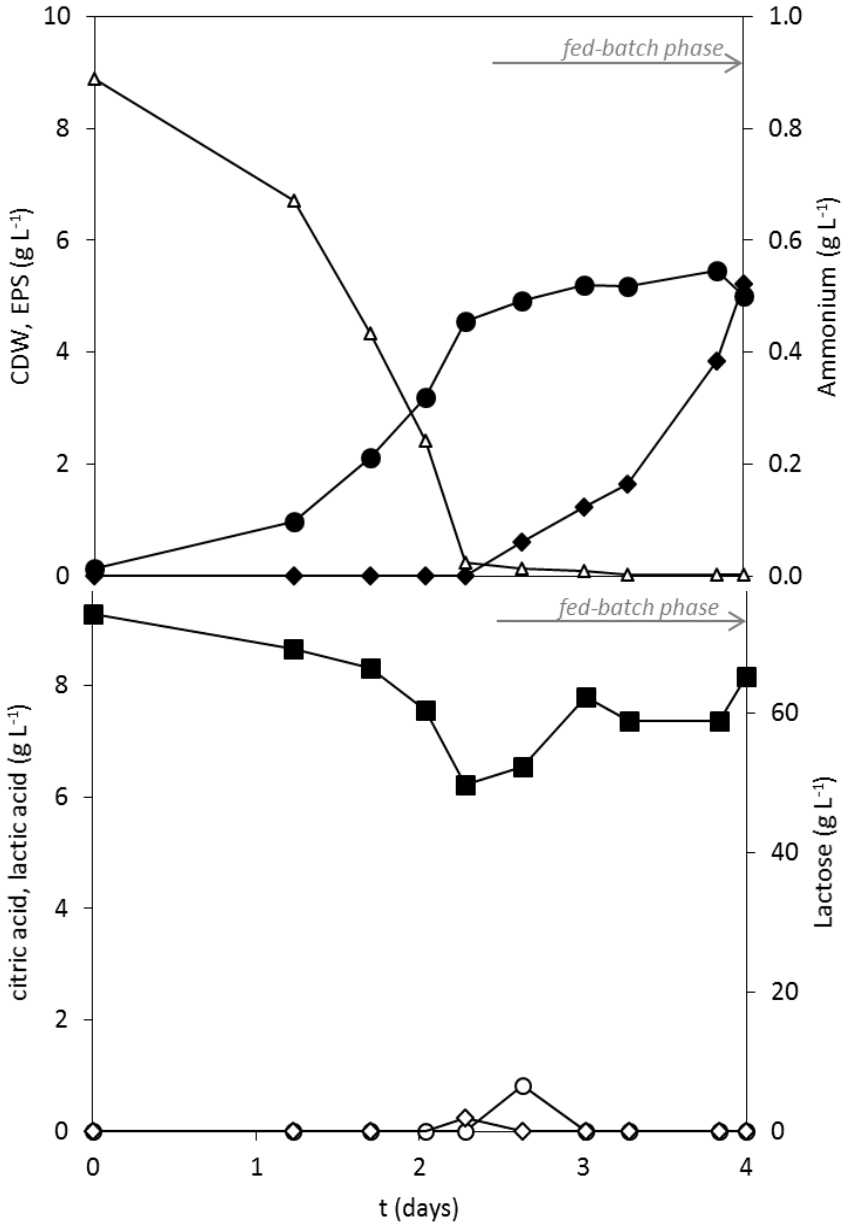

Figure 2.1: Cultivation profile of *Enterobacter* A47 using lactose as carbon source (●) CDW; (◆) EPS concentration; (△) ammonium concentration; (■) lactose concentration; (○) citric acid concentration; (◇) lactic acid concentration).

In the fed-batch phase, lactose was mostly used for EPS synthesis (Figure 2.1), since cell growth was limited by the low ammonium concentration available (< 0.1 g L^{-1}). At the end of the 4.0 days cultivation run, 5.22 g L^{-1} EPS were produced, which corresponds to an overall volumetric productivity of 1.31 g L^{-1} d^{-1} (Table 2.1). These values are similar to the ones obtained for *Enterobacter* A47 cultivation on xylose as the sole carbon source (5.39 g L^{-1} and 1.39 g L^{-1} d^{-1}, respectively) (Freitas et al., 2014), but considerably lower than those obtained from glycerol (7.50-7.97 g L^{-1} and 2.52 g L^{-1} d^{-1}, respectively) (Alves et al., 2010; Torres et al., 2011) or glucose (13.4 g L^{-1} and 3.78 g L^{-1} d^{-1}, respectively) (Freitas et al., 2014) (Table 2.1). EPS production by *Enterobacter* A47 from lactose was also lower than the values reported for xanthan and gellan gum produced by adapted wild-type strains using lactose as sole carbon source (12.5 g L^{-1} and 10.2 g L^{-1}, respectively) (Table 2.1).

Table 2.1: Kinetic parameters obtained in the bioreactor cultivation of *Enterobacter* A47 using different substrates and productivity of commercial bacterial polysaccharides tested with lactose or cheese whey as substrate.

Bacterium	Polysaccharide	Substrate	μ_{max} (h^{-1})	CDW_{max} ($g\ L^{-1}$)	EPS_{max} ($g\ L^{-1}$)	r_P ($g\ L^{-1}\ d^{-1}$)	References
Enterobacter A47	EPS_{lac}	Lactose	0.06	5.33	5.22	1.31	This study
	EPS_{cw}	Cheese whey	0.15	8.60	6.40	2.00	This study
	FucoPol	Glycerol	0.26-0.32	5.80-6.75	7.50-7.97	2.52	Alves et al., 2010; Torres et al., 2011
	EPS-g	Glucose	0.35	8.14	13.40	3.78	Freitas et al., 2014
	EPS-x	Xylose	0.25	3.92	5.39	1.39	Freitas et al., 2014
Xanthomonas campestris	Xanthan	Lactose	n.a.	n.a.	12.5	3.00	Savvides et al., 2012
		Cheese whey	n.a.	n.a.	8.7	2.09	Savvides et al., 2012
Sphingomonas paucimobilis	Gellan gum	Lactose	n.a.	n.a.	10.2	2.45	Fialho et al., 1999
		Cheese whey	n.a.	n.a.	7.9	1.90	Fialho et al., 1999
Streptococcus thermophiles YIT 2084	Hyaluronic acid	Lactose	0.68	n.a.	1.2	1.11	Izawa et al., 2011
Azotobacter chroococcum	Bacterial alginate	Cheese whey	n.a.	n.a.	1.0	0.17	Khanafari and Sepahei, 2007

n.a. – data not available

2.4.2. Cultivation of *Enterobacter* A47 on cheese whey as the sole substrate

Considering that *Enterobacter* A47 was able to grow on lactose as sole carbon source and synthesize EPS, the use of cheese whey as substrate was then tested under similar cultivation conditions (Figure 2.2).

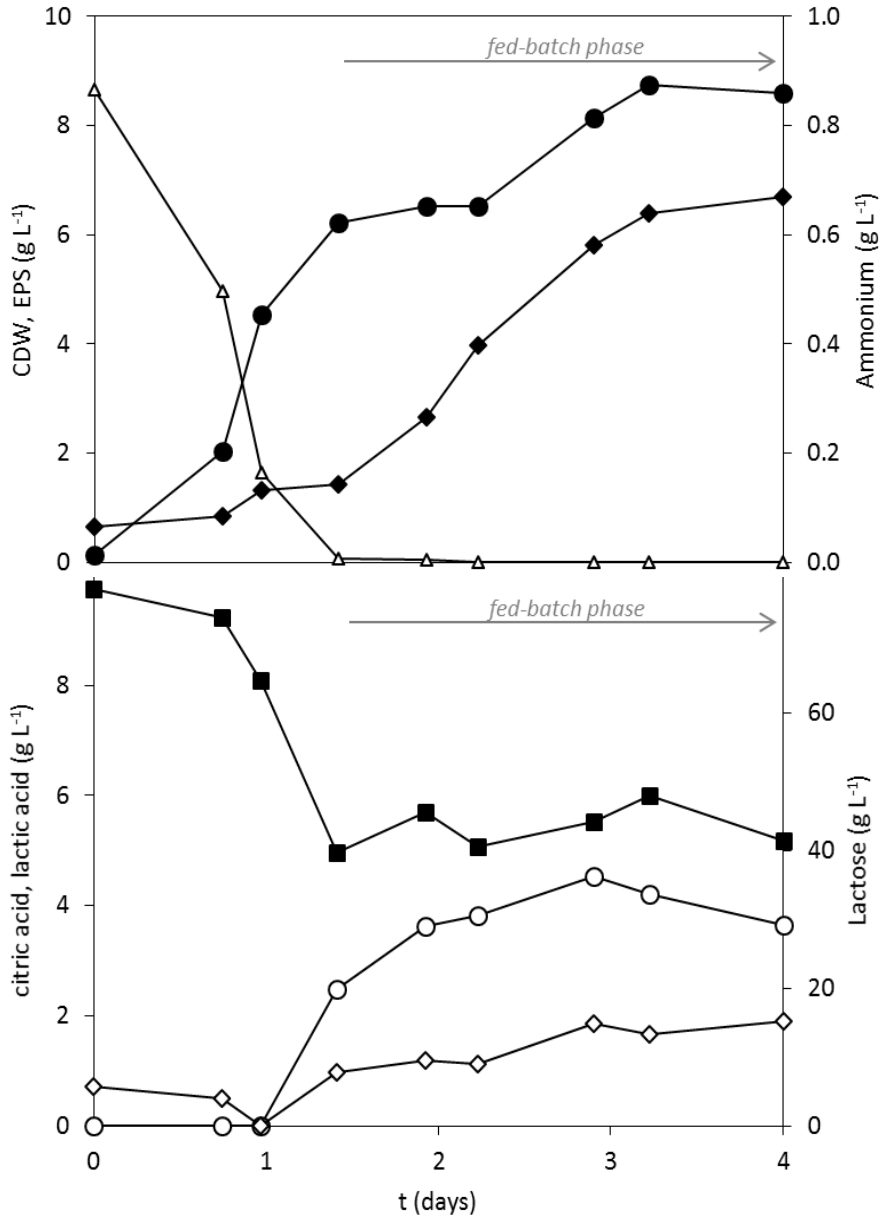

Figure 2.2: Cultivation profile of *Enterobacter* A47 using cheese whey as carbon source ((●) CDW; (◆) EPS concentration; (△) ammonium concentration; (■) lactose concentration; (○) citric acid concentration; (◇) lactic acid concentration).

The specific cell growth rate was considerably higher (0.15 h^{-1}) than with lactose (0.06 h^{-1}) (Table 2.1). This result may be related to the cultivation medium provided by cheese whey, which, in addition to lactose, contained remaining proteins, vitamins and mineral salts that may have stimulated cell growth. During the batch phase (1.4 days), the culture attained a CDW of 6.22 g L^{-1}, but still continued to grow, reaching a maximum CDW of 8.74 g L^{-1} at 3.2 days of cultivation. Since the ammonium concentration supplied during the fed-batch phase was not sufficient to support cell growth (< 0.1 g L^{-1}), the observed cell growth can be assigned to the consumption of organic nitrogen compounds present in the cheese whey feeding solution.

A low EPS concentration was detected at the beginning of the run (0.66 g L^{-1}), but its concentration remained practically unchanged until the fed-batch phase was initiated, increasing considerably thereafter (Figure 2.2). The initial polymer content in this assay was mainly due to a polysaccharide originally present in the cheese whey, which represented 0.8 wt.% of the cheese whey powder. This EPS was probably produced by lactic acid bacteria (LAB) present in fermented dairy products, such as cheese (Harutoshi, 2013).

An EPS concentration of 6.40 g L^{-1} was obtained at 3.2 days, corresponding to a volumetric productivity of 2.00 g L^{-1} d^{-1}, which is lower than the values obtained for EPS production by *Enterobacter* A47 using glycerol or glucose as substrates (Table 2.1). Nevertheless, the obtained EPS concentration was similar to that reported for xanthan and gellan gum using cheese whey as substrate (8.70 and 7.90 g L^{-1}, respectively) and considerably higher than the values reported for hyaluronic acid (1.20 g L^{-1}) and bacterial alginate (1.00 L^{-1}) using the same substrate (Table 2.1). In those studies, both xanthan and gellan gum were recovered from the broth by solvent precipitation, without purification step (Fialho et al., 1999; Savvides et al., 2012), thus resulting in higher yields of less pure products. In this work, simple dialysis was successful to produce pure EPS from *Enterobacter* A47.

Significant amounts of citric and lactic acids were detected (4.53 g L^{-1} and 1.90 g L^{-1}, respectively) in the culture broth during cultivation of *Enterobacter* A47 on cheese whey (Figure 2.2). The production of these organic acids was not observed in previous studies using other carbon sources, namely, glycerol, glucose or xylose (Freitas et al., 2014; Torres et al., 2011). In the lactose fed experiment (Figure 2.1), citric and lactic acids were detected during the fed-batch phase, but their concentrations were very low (<1.0 g L^{-1}). The higher organic acids synthesis may be related to the presence of proteins, vitamins and mineral salts in the cheese whey medium. Such components, besides stimulating cell growth, may also have induced the metabolic pathways leading to the synthesis of citric and lactic acids. In the cheese whey assay, a fraction of the available carbon was used by the culture for the synthesis of organic acids through metabolic pathways different from those leading to polysaccharide synthesis. Therefore, EPS production by

Enterobacter A47 from cheese whey might be optimized by minimizing the synthesis of lactic and citric acids, which might increase polymer's productivity. Strategies to reduce the production of organic acids by *Enterobacter* A47 grown on cheese whey may include, for example, the proton suicide method (Pais et al., 2014) for selection of mutants with diminished or depleted organic acids production.

2.4.3. Exopolysaccharide composition and average molecular weight

The EPS synthesized by *Enterobacter* A47 using commercial lactose, EPS_{lac}, was composed of fucose (25 mol%), galactose (22 mol%), glucose (24 mol%) and glucuronic acid (29 mol%). A similar composition was found for the EPS synthesized from cheese whey, EPS_{CW}: fucose (29 mol%), galactose (21 mol%), glucose (21 mol%) and glucuronic acid (29 mol%) (Table 2.2). Although the same sugar monomers were previously reported as residues of the EPS synthesized by *Enterobacter* A47 from other carbon sources, their relative proportion was not the same (Table 2.2). The main difference was the considerably higher glucuronic acid content (29 mol%), that was two to three times higher than the EPS produced from glycerol (9-10 mol%), glucose (16 mol%) or xylose (17 mol%). Conversely, both EPS_{lac} and EPS_{CW} had lower contents in fucose, glucose and galactose compared to EPS produced from glycerol (Table 2.2).

The presence of fucose and glucuronic acid as main components of EPS_{lac} and EPS_{CW} is worth to mention due to their uncommon presence and bioactive properties. In particular antitumor, anti-inflammatory and immune-enhancing activities have been reported for fucose-containing polysaccharides, suggesting their valorisation for the development of new pharmaceuticals (Hidari et al., 2008; Péterszegi et al., 2003). On the other hand, glucuronic acid has been reported as a detoxifying agent in liver, but also for the treatment of osteoarthritis (Yavari et al., 2011). Glucuronic acid-based polysaccharides, such as hyaluronic acid and heparin, have proven biomedical applications in surgery, regenerative medicine, tissue engineering and as active ingredients in anti-thrombotic and anti-arthritic drugs (Cimini et al., 2012).

Table 2.2: Sugar (Fuc: fucose; Gal: galactose; Glc: glucose; GlcA: glucuronic acid) and acyl groups (Pyr: pyruvyl; Succ: succinyl; Acet: acetyl) composition, average molecular weight (Mw) and polydispersity index (PDI) of the extracellular polysaccharides produced by *Enterobacter* A47 from different substrates.

Substrate	Sugar composition (mol%)				Acyl groups composition (wt.%)			M_w (Da) ($\times 10^6$)	PDI	References
	Fuc	Gal	Glc	GlcA	Pyr	Succ	Acet			
Lactose	25	22	24	29	22	10	4	4.7	1.7	This study
Cheese whey	29	21	21	29	24	1	7	1.8	1.2	This study
Glycerol	32-36	25-26	28-34	9-10	13-14	3	3-5	5.7	1.3	Alves et al., 2010; Torres et al., 2012
Glucose	29	29	26	16	15	2	5	4.2	1.4	Freitas et al., 2014
Xylose	38	18	27	17	15	2	0	1.7	1.4	Freitas et al., 2014

Non-carbohydrate components, namely, the organic acyl groups pyruvyl, succinyl and acetyl, were also detected as constituents of both EPS_{lac} and EPS_{CW}, accounting for 32 – 34 wt.% of the polymers' dry weight (Table 2.2). The main acyl substituent present in both EPS_{lac} and EPS_{CW} was pyruvate, whose content was considerably higher (22 and 24 wt.%, respectively) than previously reported for the polymers obtained from glycerol, glucose or xylose (13-15 wt.%). EPS_{lac} had a high succinate content of 10 wt.%, while EPS_{CW} had only trace amounts of that acyl group (1 wt.%).

EPS_{lac} and EPS_{CW} were both high molecular weight biopolymers (4.7×10^6 Da and 1.8×10^6 Da, respectively) with relatively low polydispersity indexes (1.7 and 1.2, respectively) (Table 2.2). These Mw values are within the ranges reported for other EPS synthesized by *Enterobacter* A47 using different substrates ($1.7\text{-}5.7\times10^6$ Da) and also for EPS synthesized by other bacteria, such as xanthan ($2.0\text{-}50\times10^6$ Da), alginate ($0.3\text{-}1.3\times10^6$ Da), GalactoPol ($1.0\text{-}5.0\times10^6$ Da) and hyaluronic acid (2.0×10^6 Da) (Freitas et al., 2011b).

To confirm if any high molecular weight compounds present in the cheese whey were carried over to EPS_{CW}, cheese whey powder was analysed for its content in high molecular weight compounds by subjecting a cheese whey solution to dialysis with a 10,000 MWCO. A polysaccharide fraction, accounting for 0.8 wt.% of the cheese whey powder, was recovered. This polysaccharide's molecular weight (4.6×10^4 Da) was significantly lower than that of EPS_{CW} (1.8×10^6 Da). It was mainly composed of rhamnose (43 mol%), galactose (35 mol%) and glucose (18 mol%), with a minor fucose content (3.6 mol%). The polysaccharide recovered from the cheese whey powder also had a low acyl groups content, 9.23 wt.%, including pyruvyl, acetyl and succinyl. Heteropolysaccharides composed of glucose, galactose and rhamnose have been reported to be synthesized by lactic acid bacteria used in the dairy industry, namely, *Streptococcus thermophilus* and *Lactobacillus* sp., with productions that vary widely, between 0.05 and 2.8 g L^{-1} (Harutoshi, 2013).

The rhamnose-rich polysaccharide recovered from the cheese whey powder had a composition and a Mw distinct of those of the EPS recovered from the culture broth at the end of the cultivation of *Enterobacter* A47 in cheese whey. Hence, we may conclude that the polysaccharide EPS_{CW} was indeed a product synthesized by *Enterobacter* A47 and that no rhamnose-rich polysaccharide from the cheese whey powder were present in the final product.

2.5. Conclusions

Cheese whey, an inexpensive and abundant dairy by-product, was converted into a novel fucose- and glucuronic acid-rich polysaccharide by the bacterium *Enterobacter* A47. One interesting feature of this bioprocess is the use of a culture that is able to directly use lactose, without any pretreatment, as the substrate for the synthesis of a novel value-added polysaccharide with valuable properties. The combination of the two bioactive sugar monomers, fucose and glucuronic acid, in EPS_{cw}, confers it a huge potential for application in different areas, including cosmetics, pharmaceuticals and food products.

CHAPTER 3

Extracellular Polysaccharides Production: Tomato paste

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

Antunes, S., Freitas, F., Sevrin, C., Grandfils, C., Reis, M.A.M., 2017. Production of FucoPol by *Enterobacter* A47 using waste tomato paste by-product as sole carbon source. Bioresource Technology. 227, 66–73.

3.1. Summary

Out-of-specification tomato paste, a by-product from the tomato processing industry, was used as the sole substrate for cultivation of the bacterium *Enterobacter* A47 and production of FucoPol, a value-added fucose-rich extracellular polysaccharide. Among the different tested fed-batch strategies, pH-stat, DO-stat and continuous substrate feeding, the highest production (8.77 g L^{-1}) and overall volumetric productivity (2.92 g L^{-1} d^{-1}) were obtained with continuous substrate feeding at a constant flow rate of 11 g h^{-1}. The polymer produced had the typical FucoPol composition (37 mol% fucose, 27 mol% galactose, 23 mol% glucose and 12 mol% glucuronic acid, with an acyl groups content of 13 wt.%). The average molecular weight was 4.4×10^6 Da and the PDI was 1.2. This study demonstrated that out-of-specification tomato paste is a suitable low-cost substrate for the production of FucoPol, thus providing a route for the valorisation of this by-product into a high-value microbial product.

3.2. Introduction

Enterobacter A47 (DSM 23139) is a gram-negative bacterium that synthesizes FucoPol, a fucose-rich EPS (Alves et al., 2010; Freitas et al., 2014; Reis et al., 2011; Torres et al., 2011, 2012, 2014). FucoPol is a high molecular weight biopolymer composed of fucose, glucose, galactose and glucuronic acid, and acyl groups substituents (Freitas et al., 2014; Torres et al., 2012), which presents interesting functional properties. FucoPol forms viscous aqueous solutions with shear thinning behaviour (Cruz et al., 2011; Torres et al., 2015), it has film-forming capacity (Ferreira et al., 2014, 2016; Freitas et al., 2014), flocculating activity, and emulsion forming and stabilizing capacity (Freitas et al., 2011a, 2014). Furthermore, the presence of fucose, a rare sugar reported to confer biological activity to fucose-containing polymers (Péterszegi et al., 2003; Roca et al., 2015; Wijesinghe and Jeon, 2012), renders FucoPol increased value.

The bacterium *Enterobacter* A47 demonstrated the ability to use a wide range of substrates, including glycerol (Alves et al., 2010; Reis et al., 2011; Torres et al., 2011, 2012, 2014), glucose, xylose (Freitas et al., 2014) and lactose (Chapter 2; Antunes et al., 2015), as sole carbon sources. This substrate multiplicity makes the bioprocess more robust and versatile, but the composition of the polymer synthesized is influenced by the type of carbon source used. In fact, the use of xylose as carbon source, for example, results in increased fucose content in the polysaccharide (Freitas et al., 2014), while lactose induces the synthesis of a biopolymer enriched in glucuronic

acid (Chapter 2; Antunes et al., 2015). This interesting flexibility allows to tune the bioprocess towards the production of tailor-made biopolymers with potentially distinct valuable properties. In this perspective, it is relevant to search for alternative feedstocks, especially wastes and by-products generated by agro-industrial activities, which can not only lower the production costs but also result in biopolymers with novel valuable properties. Within this context, the suitability of glycerol by-product from the biodiesel industry (Alves et al., 2010; Reis et al., 2011; Torres et al., 2011) and cheese whey from the dairy industry (Chapter 2; Antunes et al., 2015) as substrates for the cultivation of *Enterobacter* A47 has already been demonstrated. Similarly, other sugar-rich materials, like fruit and vegetable wastes and by-products, may also be valorised into valuable polysaccharides with this bioprocess.

Tomato is the second largest produced vegetable in the world (faostat.fao.org). Tomato processing generates a large amount of wastes and by-products (e.g., discarded tomatoes, pulp, seeds and peels) that require adequate treatment/disposal procedures (Grassino et al., 2016; Sánchez et al., 2002). Currently, tomato processing wastes represent 10–40% of total processed tomatoes, being mainly used as animal feed or as soil fertilizers (Strati and Oreopoulou, 2011). Some alternative environmentally friendly valorisation strategies have been proposed for tomato processing wastes, including the recovery of different value-added products, such as lycopene and β-carotene (Choudhari and Ananthanarayan, 2007; Nobre et al., 2009; Strati and Oreopoulou, 2011), sterols, tocopherols, terpenes and polyphenols (Kalogeropoulos et al., 2012), hydrolytic enzymes (Umsza-Guez et al., 2011), polysaccharides (Grassino et al., 2016; Romano et al., 2004; Tommonaro et al., 2008), oligosaccharides (Suzuki et al., 2002) and seed oil (Botinestean et al., 2015). Other strategies include the production of fuel-like material (Toscano et al., 2015), biomethane (Calabrò et al., 2015), energy (Bacenetti et al., 2015) and for heat recovery (Amón et al., 2015).

A huge amount of tomato is processed into tomato paste, an ingredient of many products, such as ketchup, soups and sauces (Sánchez et al., 2002). Tomato paste is obtained by tomato pulp concentration, after removal of skins and seeds (Grassino et al., 2016; Sánchez et al., 2002). In 2013, 1.54 Mt of tomato paste were produced worldwide (faostat.fao.org). Part of this product resulted in out-of-specification tomato paste, which is tomato paste whose test results that fall outside the specifications or acceptance criteria established for commercialization. This tomato processing by-product is mainly incinerated or disposed in landfills, with associated costs for the manufacturer and environmental impact due to the generation of liquid and gaseous emissions (Calabrò et al., 2015). Since out-of-specification tomato paste is a sugar-rich complex mixture (Calabrò et al., 2015), it has potential to be used as a biotechnological resource for the generation

of value-added microbial products, such as biopolymers. However, this strategy has received little attention.

In this work, out-of-specification tomato paste was tested for the first time as substrate for cultivation of the bacterium *Enterobacter* A47 to assess its suitability for the production of value-added extracellular polysaccharides. Fed-batch bioreactor experiments were performed under different cultivation modes to evaluate the best strategy to reach high substrate conversion into polysaccharide and high volumetric productivities. The polymer synthesized was recovered from the cultivation broth and characterized in terms of composition and molecular mass distribution.

3.3. Materials and Methods

3.3.1. Characterization of tomato paste

Out-of-specification tomato paste was supplied by HIT Group (www.hit-tomato.com), a tomato processing industry. The physical-chemical characterization of tomato paste included the determination of its pH, viscosity, content in solids, moisture, and concentration of phosphate, ammonium, simple sugars and high molecular weight soluble compounds.

The viscosity was measured using a rotational viscometer (Fungilab, S.A., Spain), for shear rates between 0.005 and 1.667 s^{-1}, at room temperature (20 °C). The moisture content of the tomato paste was determined by subjecting a sample (50 mg) to a temperature of 100 ºC, until constant weight was attained. The dried sample was further subjected to pyrolysis at a temperature of 550 ºC, for 24 hours, to determine its content in inorganic salts.

A modified Lowry method (Lowry et al., 1951) was used to determine the total protein content of tomato paste, as described by Freitas et al. (2009). Briefly, 5.5 mL of a tomato paste aqueous suspension (250 g L^{-1}) was mixed with 1 mL NaOH 20% (w/v) and heated at 100 °C for 5 minutes. After cooling on ice, 170 µL of copper sulfate pentahydratate 25% (w/v) were added. The samples were centrifuged (3500×*g*, for 5 min) and the optical density of the supernatant was measured at 560 nm. Albumin solutions (0.05 - 3.0 g L^{-1}) were used as standards.

Ammonium and phosphate concentrations were determined by colorimetry, as implemented in a flow segmented analyser (Skalar 5100, Skalar Analytical, The Netherlands). Ammonium chloride and sodium phosphate were used as standards at concentrations between of 5 and 20 mg L^{-1}. Glucose and fructose concentrations were determined by HPLC, with a MetaCarb 87H

column (Varian) coupled to a RI detector. The analysis was performed at 30 °C, with sulphuric acid (0.01 N) as eluent, at a flow rate of 0.5 mL min^{-1}. Glucose and fructose were used as standards at concentrations between 0.062 and 2.0 g L^{-1}.

For quantification of tomato paste's content in high molecular weight compounds, a suspension (82 g L^{-1}) was centrifuged (10 000×*g*, for 15 min), for removal of suspended solids, and the clarified supernatant was subjected to thermal treatment (70 °C, for 1 h) to denature proteins that were removed by centrifugation (10 000×*g*, for 15 min). The treated supernatant was then submitted to a diafiltration process, using a crossflow membrane cassette (Hydrosart® Ultrafiltration Cassettes, Sartorius Stedim Biotech GmbH, Germany) with a 100 kDa cut-off and a surface area of 0.6 m², operated at transmembrane pressure of 0.4 bar. The membrane module was switched to an ultrafiltration mode to concentrate the treated supernatant (5:1). The solution was freeze dried and the obtained polymer was stored at room temperature.

3.3.2. Exopolysaccharide production

Microorganism and media

Enterobacter A47 (DSM 23139) was used in all experiments. The culture was reactivated from a cryopreserved stock by inoculation in LB medium agar plates (bacto-tryptone, 10 g L^{-1}; yeast extract, 5 g L^{-1}; sodium chloride, 10 g L^{-1}; agar, 20 g L^{-1} ; pH 6.8). Liquid LB medium (without agar) was used for inocula preparation.

In the bioreactor assays, *Enterobacter* A47 was grown on a mineral medium with the following composition (per liter): $(NH_4)_2HPO_4$, 3.3 g; 10 mL of a 100 mM $MgSO_4$ solution and 1 mL of a micronutrient solution. The micronutrients solution had the following composition (per liter of 1 N HCl): $FeSO_4 \cdot 7H_2O$, 2.78 g; $MnCl_2 \cdot 4H_2O$, 1.98 g; $CoSO_4 \cdot 7H_2O$, 2.81 g; $CaCl_2 \cdot 2H_2O$, 1.67 g; $CuCl_2 \cdot 2H_2O$, 0.17 g; $ZnSO_4 \cdot 7H_2O$, 0.29 g). The mineral medium (1 L) was supplemented with approximately 82 g of tomato paste.

Bioreactor cultivation

Inocula for the assays were prepared by inoculating a single colony, grown on LB agar plates at 30 ºC, into 20 mL LB medium and incubation in an orbital shaker, at 30 ºC and 200 rpm, for 16 hours. The LB grown cells (20 mL) were transferred into 200 mL fresh LB medium and

further incubated for 8 h, under the same conditions. The broth was centrifuged (4 053×*g*, for 5 min) and the cells were resuspended in 50 mL deionized water and used as inoculum for the bioreactor experiments.

The experiments were performed in a 5 L bioreactor (BioStat B, Sartorius, Germany) with an initial working volume of 1.5 L. All assays took around 3 days, under controlled temperature and pH conditions of 30 ± 0.2 ºC and 7.0 ± 0.05, respectively. An air flow rate of 0.4 SLPM (standard liters per minute) was kept constant throughout all cultivation runs. The dissolved oxygen level (DO) was controlled at 10% by the automatic variation of the stirrer speed (300-800 rpm).

After an initial 9 h batch phase, the bioreactor was fed with substrate, a suspension prepared by diluting approximately 250 g of tomato paste in 0.75 L of deionized water. Three feeding strategies were tested, namely, pH-stat mode, DO-stat mode and continuous feeding. The pH-stat mode (Experiment A) was implemented by taking advantage of the low pH (4.0) value of tomato paste, that was used to automatically control the pH during the cultivation (i.e. tomato paste was fed to the culture when the pH rose above 7.05). In the DO-stat mode (Experiment B), the substrate feeding flow rate was controlled as a function of DO level (under a constant stirring of 680 rpm), i.e., when the DO level rose above 10% of the air saturation, tomato paste suspension was automatically fed to the bioreactor until the DO dropped to the set point. For the continuous feeding experiments, three different constant substrate feeding rates were tested, namely, 4, 6 and 11 g h^{-1} (Experiments C, D and E, respectively).

Samples (25 mL) were periodically withdrawn from the bioreactor for biomass, polysaccharide and nutrients quantification.

Analytical techniques

Cell growth was monitored by measuring the optical density at 450 nm (OD450). The cell dry mass (CDM) was estimated considering that one unit of OD450 is equivalent to a CDM of 0.26 g L^{-1}. This ratio was experimentally determined by measuring the OD450 of cell suspensions containing different CDM values. To obtain such suspensions, an *Enterobacter* A47 LB grown culture was centrifuged and the cell pellet was suspended in Medium E* supplemented with tomato paste at the same concentration used in the bioreactor experiments. This strategy was adopted because the insoluble materials content in tomato paste made it impossible to gravimetrically determine the CDM.

The cell-free supernatant samples obtained by centrifugation of the cultivation broth (13 000×*g*, for 15 min) were used for the quantification of sugars (glucose and fructose), ammonium and phosphate, as described above section.

All analyses were performed in duplicate.

Extraction and purification of extracellular polysaccharides

The culture broth was diluted with deionized water for viscosity reduction and centrifuged at 10 000×*g*, for 15 min, for cell separation. The cell-free supernatant thus obtained was subjected to thermal treatment (70 °C, for 1 h) to denature proteins that were removed by centrifugation (10 000×*g*, for 15 min). The treated supernatant was submitted to a diafiltration process, as described above for quantification of high molecular weight compounds in tomato paste. The solutions thus obtained were further purified by dialysis with 1 000 000 MWCO membranes (Spectra/Por® Float-A-Lyzer® G2, Spectrum Laboratories, Inc.) against deionized water, at room temperature. Finally, the solutions were freeze dried and the polymers were stored at room temperature.

3.3.2. Polymer characterization

Chemical composition

For the compositional analysis, polymer samples (~5 mg) dissolved in deionized water (5 mL) were hydrolysed with trifluoroacetic acid (TFA) (0.1 mL TFA 99%) at 120 °C, for 2 h. The hydrolysate was used for the identification and quantification of the constituent monosaccharides by HPLC, using a CarboPac PA10 column (Thermo Dionex), equipped with pulsed amperometric detector (Dionex ICS3000, ThermoFisher Scientific Inc.), the analysis was performed at 30 °C, at an eluent flow rate of 1.0 mL min^{-1}, with the following eluent gradient: 0-20 min, sodium hydroxide 18 mM; 20-40 min, sodium hydroxide (50 mM) and sodium acetate (170 mM). Glucuronic acid, galacturonic acid, glucose, galactose, rhamnose, xylose, arabinose and fucose at concentrations between 5 and 100 ppm were used as standards. The hydrolysates were also used for quantification of the organic acyl groups by HPLC, with a Biorad Aminex 87H column, using sulphuric acid (H_2SO_4 0.01 N) as eluent, at 30 °C with a flow rate of 0.6 mL min^{-1}. The ultraviolet-visible (UV/VIS) detection was performed at 210 nm. Acetic, succinic and pyruvic acids at concentrations between 25 and 500 ppm were used as standards.

Molecular mass distribution

Number and weight average molecular weights (Mn and Mw, respectively), as well as the polydispersity index (PDI=Mw/Mn), were obtained by size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS). The latter combines a HPLC pump (Hewlett Packard quaternary 1050), an autoinjector (Hitachi-Merck, Lachrom L7200, model 1405 – 040), and a set of two analytical SEC linear columns (PL Aquagel-OH mixed 8 µm, 300 × 7.5 mm) protected by a guard column (Polymer Laboratory; 50 × 7.5 mm). UV (Beckman UV model 266 fixed at 254 nm) signals were recorded together with MALS and RI signals (the same MALS and RI detector as described above were used) in order to follow the purity and molecular mass distribution of the polysaccharide.

The polysaccharide samples were dissolved in Tris–HCl 0.1 M; NaCl (0.2 M), pH 8.1, buffer, at a concentration of 0.2 g dL^{-1}. The solutions were warmed for 1 h at 80 °C in a water bath under lateral agitation. Dissolution of the polymers was continued for 35 h under rolling agitation at room temperature. The solutions were filtered with 0.45 µm polysulfone membranes (Whatman; Puradisc 25AS) before their injection on the SEC-MALLS system. Tris-HCl buffer solution was used as eluent with a flow rate of 1.0 mL min^{-1} at 20 °C. The analysis was performed by injection of 100 µL of EPS solution (0.2 g dL^{-1}). Each analysis was conducted in duplicate. Signals from MALLS (Wyatt Technology Corporation Dawn Model mounted with an uniphase argon laser (488 nm; 10 mW; K5 cell Flow cell) and refractive index (RI) signals (Optilab DSP, Wyatt Technology Corporation 488.0 nm, K5 cell at 30 °C) were recorded in parallel and treated with Astra (V 4.73.04) in order to follow the purity and molecular mass distribution of the polysaccharide. A dn dc^{-1} of 0.190 mL g^{-1} was adopted to calculate the Mw of the polymers. Quality control of the MALS installation was verified with PEG standards, egg albumin and BSA.

3.4. Results and discussion

3.4.1. Characterization of tomato paste

The out-of-specification tomato paste used in this study was a viscous suspension (384 Pa s, measured at 20 °C, for a shear rate of 0.005 s^{-1}). It had a non-Newtonian and shear-thinning fluid behavior, with the apparent viscosity (η_a, Pa·s) decreasing as the shear rate was increased. This behavior was probably due to its high content in total solids (27.2 wt.%) (Abu-Jdayil et al., 2004; Bayod et al., 2008; Sánchez et al., 2003). The suspension had a pH value of 4.0, which is within the typical pH range (3.8 – 4.3) reported for tomato paste (Sobowale et al., 2012), important for preventing the growth of pathogenic microorganisms in the product. The tomato paste had a moisture of 72.8 wt.%, an inorganic salts content of 3.5 wt.% and a total protein content of 4.3 wt.%.

A high molecular weight fraction, with an average molecular weight of 1.38×10^5 Da and a PDI of 3.07, was recovered from tomato paste by diafiltration and accounted for 1.0 wt.% of its mass. This fraction was mainly composed of galacturonic acid (46 mol%), galactose (20 mol%), glucose (13 mol%) and arabinose (11 mol%), with minor rhamnose and xylose contents (6 and 4 mol%, respectively). This galacturonic acid-rich fraction was probably a mixture of polysaccharides, including pectins that are high molecular weight polysaccharides (2×10^5 Da) composed mainly of galacturonic acid (up to 78 wt.%) and smaller amounts of galactose, glucose, arabinose, rhamnose and/or xylose (Chou and Kokini, 1987; Makarova et al., 2015; Müller-Maatsch et al., 2016).

Considering the composition of out-of-specification tomato paste, it may be an interesting source for *Enterobacter* A47 cultivation mainly because of its high content in simple sugars, namely, glucose (5.9 wt.%) and fructose (6.9 wt.%). As reported by Freitas et al. (2014), *Enterobacter* A47 reaches high exopolysaccharide production using glucose as sole carbon source, but fructose was not previously tested. Additionally, the content of ammonium (0.5 wt.%) and phosphate (0.4 wt.%) detected in out-of-specification tomato paste rendered it also an interesting source of nitrogen and phosphorus.

3.4.2. Cultivation of *Enterobacter* A47 using tomato paste as sole substrate

Fed-Batch Cultivation under pH-stat Mode

Taking advantage of the low pH value (4.0) of out-of-specification tomato paste, the pH-stat mode was evaluated for cultivation of *Enterobacter* A47 and production of extracellular polysaccharides in Experiment A (Figure 3.1). During cultivation of *Enterobacter* A47, a pH raise is usually indicative of substrate exhaustion or limitation. In the pH-stat mode, the substrate was automatically fed to the culture as a function of the pH value that was measured online. In this cultivation strategy, when the pH value rose above the set point (7.00 ± 0.05), the substrate was automatically fed to the bioreactor until the pH reached the set point.

During the batch phase, the culture grew at a maximum specific growth rate of 0.29 h^{-1} (Table 3.1). This value is within the range of those obtained in previous studies (0.25-0.36 h^{-1}) for cultivation on different substrates, including glycerol, glucose and xylose (Freitas et al., 2014; Torres et al., 2011, 2012, 2014), which demonstrates that tomato paste is a suitable substrate for *Enterobacter* A47 cell growth.

A CDM of 7.06 g L^{-1} was attained at the end of the batch phase (Figure 3.1a). Although most of the available ammonium was consumed within the first 9 h of cultivation, remaining in the broth at a limiting concentration (below 0.1 g L^{-1}) during the fed-batch phase, the culture continued to grow (using the ammonium content in feed), reaching a maximum CDM of 10.22 g L^{-1} at 69 h (Figure 3.1a; Table 3.1). This value is higher than those obtained during cultivation with defined carbon sources, such as glycerol, glucose, xylose or lactose (Chapter 2; Antunes et al., 2015; Freitas et al., 2014; Torres et al., 2011, 2012, 2014), and is probably related to the richer cultivation medium provided by tomato paste, which, in addition to glucose and fructose, contained ammonium. Moreover, tomato paste is also rich in proteins (4.3 wt.%) that may have served as additional nitrogen sources for cell growth. Tomato paste also provided an adequate supply of phosphate for cell growth. From the initial 0.80 g L^{-1} present in the cultivation medium, 0.30 g L^{-1} were consumed during the batch phase (Figure 3.1b). A phosphate concentration of 0.25 g L^{-1} was maintained during most of the fed-batch phase, showing that practically all the phosphate provided by tomato paste feeding was consumed by the culture. There was an overall phosphate consumption of 0.62 g L^{-1}.

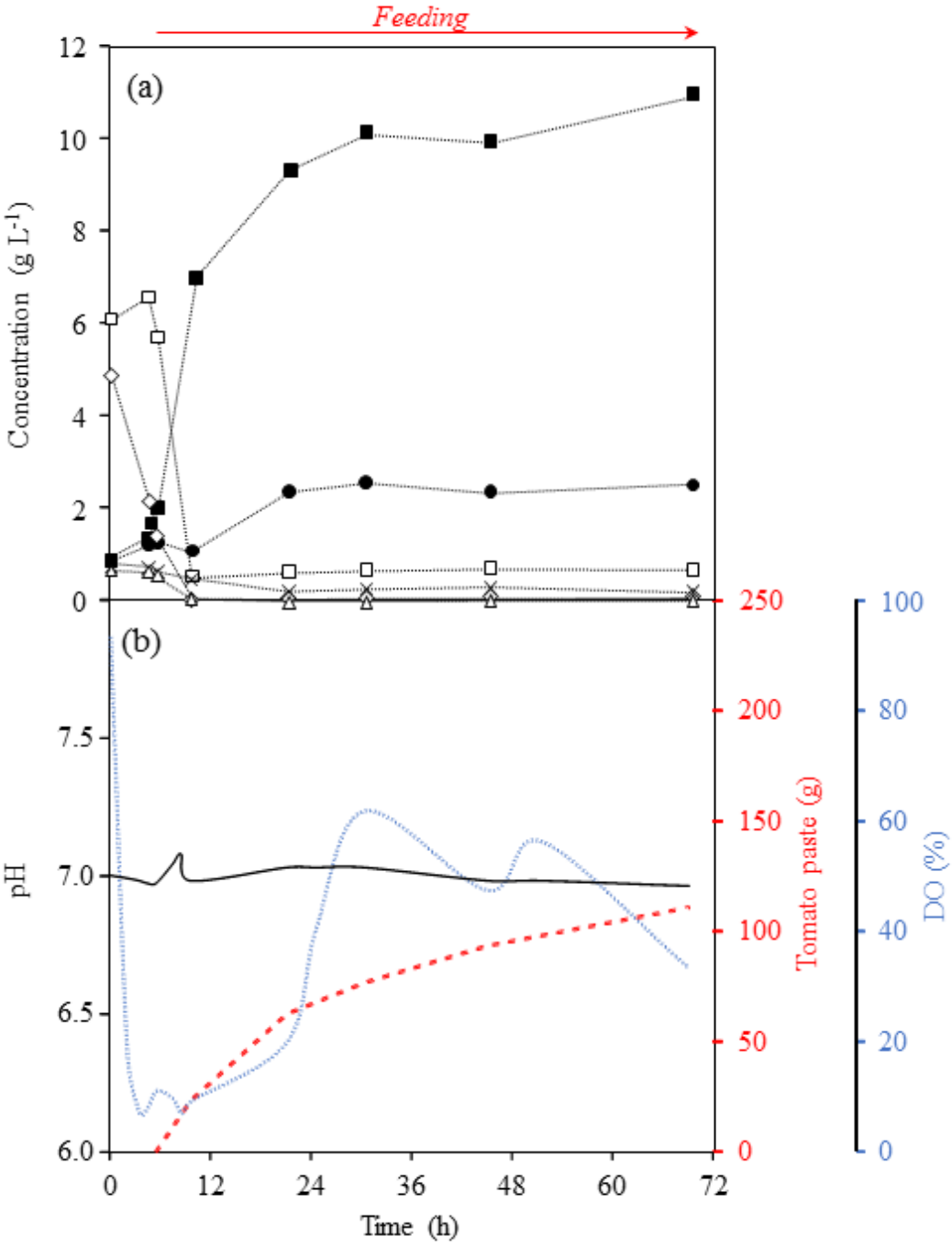

Figure 3.1: Cultivation profile obtained in Experiment A for the cultivation of *Enterobacter* A47 under a pH-stat mode, using tomato paste as sole substrate: (a) glucose (◇), fructose (□), ammonium (△), phosphate (×), CDM (■) and polymer (●); (b) pH (———), DO (·····) and tomato paste feed (– – – –).

Table 3.1: Kinetic and stoichiometric parameters obtained in the bioreactor cultivation of *Enterobacter* A47 using different substrates and cultivation modes (μ_{max}: maximum specific growth rate; CDM: cell dry mass, estimated considering that one unit of OD450 is equivalent to 0.26 g L^{-1} CDM).

Substrate	Cultivation mode	μ_{max} (h^{-1})	CDM_{max} (g L^{-1})	EPS_{max} (g L^{-1})	Productivity (g L^{-1} d^{-1})	References
Glycerol	Continuous feeding	0.27 – 0.36	5.80 – 7.68	7.23 – 7.97	2.04 – 3.72	Freitas et al., 2014; Torres et al., 2011, 2012, 2014
Glucose	DO-stat	0.35	8.14	13.40	3.78	Freitas et al., 2014
Xylose	DO-stat	0.25	3.92	5.39	1.39	Freitas et al., 2014
Lactose	Continuous feeding	0.06	5.33	5.22	1.31	Chapter 2; Antunes et al., 2015
Cheese whey	Continuous feeding	0.15	8.60	6.40	2.00	Chapter 2; Antunes et al., 2015
Tomato paste	pH-stat (Experiment A)	0.29	10.22	1.65	0.57	This study
	DO-stat (Experiment B)	0.27	9.81	3.43	1.16	This study
	Continuous feeding					
	4 g h^{-1} (Experiment C)	0.27	10.14	3.99	1.34	This study
	6 g h^{-1} (Experiment D)	0.32	10.74	4.54	1.56	This study
	11 g h^{-1} (Experiment E)	0.33	13.58	8.77	2.92	This study

As shown in Figure 3.1a, the two simple sugars detected in tomato paste, glucose and fructose, were efficiently used by the culture. However, glucose consumption was initiated right after inoculation, while fructose was only utilized when glucose concentration in the broth was below 2 g L^{-1}. All the available glucose (4.85 g L^{-1}) and most of the fructose (5.57 g L^{-1}) were consumed during the batch phase. At around 8 h of cultivation, there was a raise of the pH value, which was indicative of substrate limitation and triggered substrate feeding under the pH-stat mode. During the fed-batch phase, glucose and fructose concentration in the broth remained practically unchanged (0.08-0.13 and 0.58-0.74 g L^{-1}, respectively) (Figure 3.1a), which shows that the rate of feeding of both hexoses was the same as their rate of consumption. For the first hours of the fed-batch phase, the substrate feeding rate was high (~6 g h^{-1}), but it declined significantly thereafter (Figure 3.1b). In fact, between 21 and 70 h of cultivation, a substrate feeding rate of only ~1 g h^{-1} was provided by the pH-stat mode.

A high molecular weight fraction (0.82 ± 0.10 g L^{-1}) was detected in the cultivation medium at the beginning of the run, which probably corresponded to the presence of the galacturonic acid-rich polysaccharide fraction that accounted for 1 wt.% of tomato paste mass, as described above. During the batch phase, the content in high molecular weight compounds in the cultivation broth remained practically unchanged (Figure 3.1a), indicating that no significant bacterial exopolysaccharide synthesis occurred during that period. Afterwards, during the fed-batch phase, increased production was detected, reaching a final concentration of 1.65 g L^{-1} (Figure 3.1a; Table 3.1). The overall volumetric productivity of exopolysaccharides was 0.57 g L^{-1} d^{-1}, which is lower than that obtained in previous studies with glycerol (2.04 – 3.72 g L^{-1} d^{-1}) (Freitas et al., 2014; Torres et al., 2011, 2012), glucose (3.78 g L^{-1} d^{-1}) (Freitas et al., 2014), xylose (1.39 g L^{-1} d^{-1}) (Freitas et al., 2014), lactose (1.31 g L^{-1} d^{-1}) (Chapter 2; Antunes et al., 2015) or cheese whey (2.00 g L^{-1} d^{-1}) (Chapter 2; Antunes et al., 2015) (Table 3.1).

The presence of ammonium and proteins in tomato paste has probably served as additional nitrogen sources for the culture, thus favouring cell growth over extracellular polysaccharide synthesis. On the other hand, there may have been a limitation of available carbon source, which might have restricted polysaccharide synthesis. Apparently, the amount of substrate required to control the pH at the intended set point during the fed-batch phase was not enough to guaranty an adequate supply of carbon source. This is also supported by the fact that the DO increased at around 21 h and remained above 40% until the end of the experiment (Figure 3.1b), which could be related to the limitation of available substrate in the cultivation medium.

The purified extracellular polysaccharide recovered from the broth at the end of Experiment A had an average molecular weight of 3.6×10^6 Da and was composed of fucose (20 mol%), galactose (45 mol%), glucose (24 mol%) and glucuronic acid (11 mol%) (Table 3.2).

Table 3.2: Sugar composition and acyl groups content of the extracellular polysaccharides produced by *Enterobacter* A47 from various substrates under different cultivation modes (Fuc: fucose; Gal: galactose; Glc: glucose; GlcA: glucuronic acid).

Substrate	Cultivation mode	Sugar composition (mol%)				Acyl groups content (wt.%)	References
		Fuc	Gal	Glc	GlcA		
Glycerol	Continuous feeding	30-36	22-29	25-34	9-10	12-22	Freitas et al., 2011a, 2014; Torres et al., 2011, 2012
Glucose	DO-stat	29	29	26	16	22	Freitas et al., 2014
Xylose	DO-stat	38	18	27	17	17	Freitas et al., 2014
Lactose	Continuous feeding	25	22	24	29	36	Chapter 2; Antunes et al., 2015
Cheese whey	Continuous feeding	29	21	21	29	32	Chapter 2; Antunes et al., 2015
Tomato paste	pH-stat (Experiment A)	20	45	24	11	4	This study
	DO-stat (Experiment B)	28	35	25	12	6	This study
	Continuous feeding						
	4 g h^{-1} (Experiment C)	27	31	32	10	5	This study
	6 g h^{-1} (Experiment D)	33	27	29	11	10	This study
	11 g h^{-1} (Experiment E)	37	27	23	12	13	This study

This sugar monomer profile is rather different from that obtained in previous studies, namely, the polymer had a lower fucose content, while the galactose content was considerably higher. Moreover, the polymer produced in Experiment A had an acyl groups content (4 wt.%) much lower than the exopolysaccharides produced from glycerol, glucose or xylose (12-22 wt.%) as carbon sources (Alves et al., 2010; Torres et al., 2011; Freitas et al., 2014).

These results show that the cultivation conditions used in Experiment A not only affected the polymer's composition, resulting in a polysaccharide significantly different from the typical FucoPol, but also resulted in poor exopolysaccharide synthesis. Therefore, the pH-stat mode was not an adequate strategy to feed tomato paste to the culture.

Fed-Batch Cultivation under DO-stat Mode

In Experiment B (Figure 3.2), the bioreactor was operated under a DO-stat mode during the fed-batch phase. In this cultivation strategy, the feeding flow rate was controlled as a function of the DO concentration, a parameter that was measured online and whose raise is indicative of substrate limitation.

Similarly, to the batch phase of Experiment A (pH-stat mode), in Experiment B, the culture first consumed glucose until a concentration below ~2 g L^{-1} had been reached (Figure 3.2a). Only then (~6 h), fructose started to be consumed. During the batch phase, all the available glucose (8.37 g L^{-1}) and 5.89 g L^{-1} of fructose were consumed. The culture grew at a maximum specific growth rate of 0.27 h^{-1} (Table 3.1) and a CDM of 5.03 g L^{-1} was obtained at around 9 h of cultivation (Figure 3.2a). Starting at 9 h of cultivation, whenever the DO level rose above 10%, the substrate was automatically fed to the bioreactor until the DO reached the set point again (Figure 3.2b). This strategy allowed to control the DO concentration and, simultaneously, supply fresh substrate to the culture during the fed-batch phase. A total of 241 g of tomato paste were fed, supplying the culture with 11.43 g L^{-1} of glucose and 13.49 g L^{-1} of fructose that were consumed for cell growth and exopolysaccharide synthesis. Similarly to Experiment A, the culture continued to grow during the fed-batch phase, despite the limiting ammonium concentration supplied to the culture, achieving a maximum CDM of 9.81 g L^{-1} at the end of the run (Figure 3.2a; Table 3.1). Exopolysaccharide synthesis, on the other hand, was significantly improved, compared to Experiment A. A final exopolysaccharide concentration of 3.43 g L^{-1} was attained (Figure 3.2a), corresponding to an overall volumetric productivity of 1.16 g L^{-1} d^{-1} (Table 3.1). This higher polymer production was due to the higher substrate availability provided by the DO-stat mode strategy (241 g of tomato paste, compared to the 110 g provided with the pH-stat mode strategy).

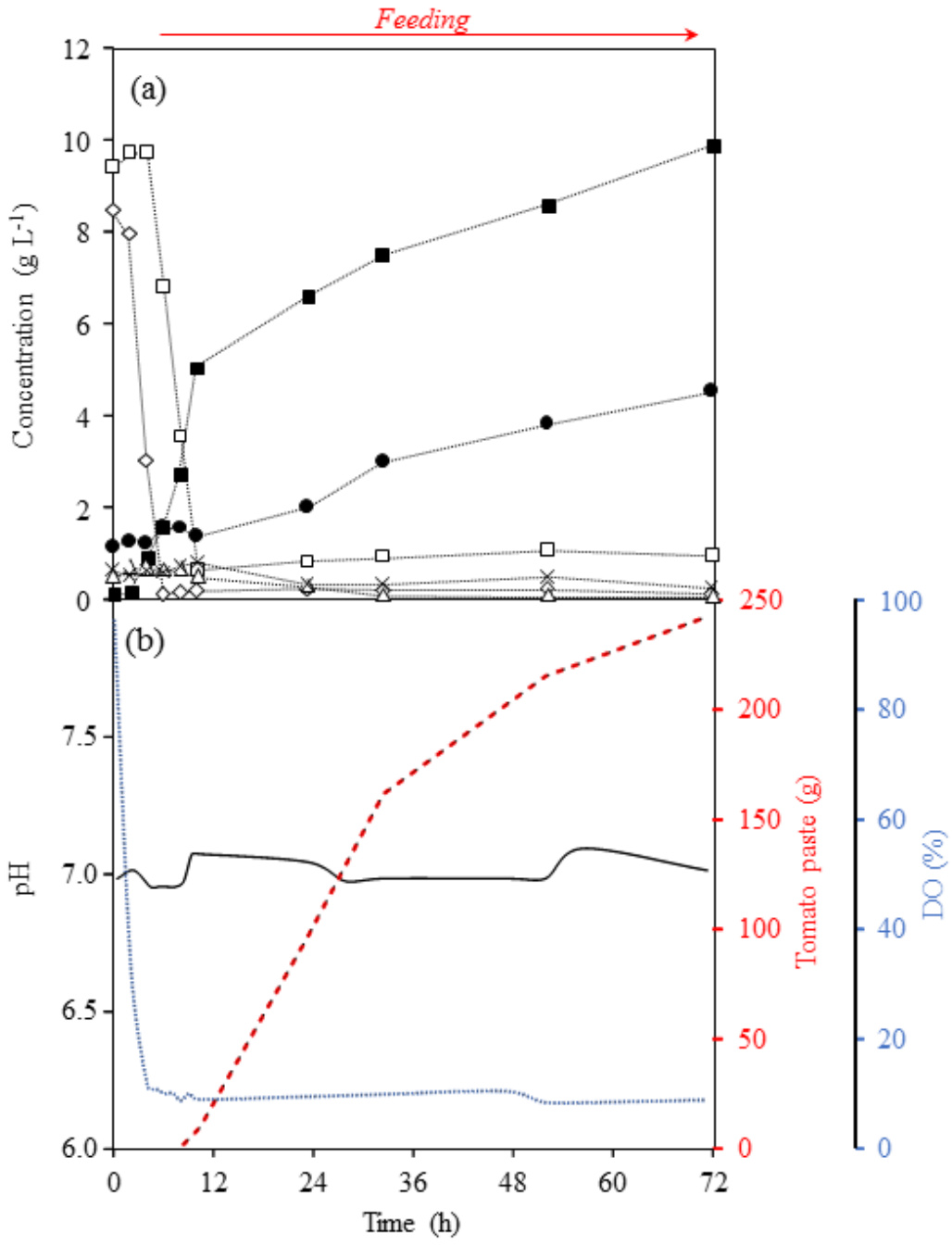

Figure 3.2: Cultivation profile obtained in Experiment B for the cultivation of *Enterobacter* A47 under a DO-stat mode, using tomato paste as sole substrate: (a) glucose (◇), fructose (□), ammonium **(△)**, phosphate (×), CDM **(■)** and polymer **(●)**; (b) pH (———), DO (·····) and tomato paste feed (- - - -).

This feeding strategy resulted in a high substrate feeding rate (~7 g h^{-1}) until around 30 h of cultivation (Figure 3.2b). Afterwards, it gradually decreased to below 3 g h^{-1}.

The purified extracellular polysaccharide recovered from the broth at the end of Experiment B had an average molecular weight of 3.2×10^6 Da and was composed of fucose (28 mol%), galactose (35 mol%), glucose (25 mol%) and glucuronic acid (12 mol%), and had an acyl groups content of 6 wt.% (Table 3.2). This polymer was still enriched in galactose and had a low acyl groups content, similarly to the exopolysaccharide synthesized in Experiment A, but its fucose content was closer to this monomer's content in FucoPol (29-38 mol%) produced from different carbon sources (Table 3.2).

Although the DO-stat mode strategy improved the overall exopolysaccharide production and productivity, compared to the pH-stat mode, both stoichiometric parameters were still lower than those obtained in previous studies with other substrates (Table 3.1). The tested cultivation strategies, pH-stat and DO-stat modes, did not result in high exopolysaccharide production. Moreover, the polymers synthesized had a sugar monomer composition different from that of the typical FucoPol obtained in previous studies.

Fed-Batch Cultivation under continuous substrate feeding

The poor polymer production observed in Experiments A and B might have been related to the limited substrate availability provided by the pH-stat and DO-stat fed-batch cultivation strategies. Therefore, in an attempt to assure a more adequate supply of substrate during the fed-batch phase, a continuous feeding strategy was tested in the following experiments. This cultivation strategy had already been successfully implemented to feed glycerol (Alves et al., 2010; Torres et al., 2011, 2012, 2014) and lactose or cheese whey (Chapter 2; Antunes et al., 2015) in previous studies.

The continuous feeding strategy was evaluated in three experiments (Figure 3.3), with different feed flow rates during the fed-batch phase. In Experiment B, an overall of 241 g of tomato paste entered the bioreactor throughout the 62 h of the fed-batch phase, corresponding to an average flow rate of approximately 4 g h^{-1}. However, the feed flow rate was decreased after around 30 h of cultivation (Figure 3.2b) and this reduction might have impaired polymer synthesis. Hence, in Experiment C, a similar feed flow rate of approximately 4 g h^{-1} was implemented starting at around 9 h of cultivation (Figure 3.3a) and was kept throughout the cultivation run to guaranty a constant rate of supply of substrate. Two higher feed flow rates, 6 g h^{-1} (Experiment D) and 11 g h^{-1} (Experiment E), were also tested to evaluate if a higher substrate availability

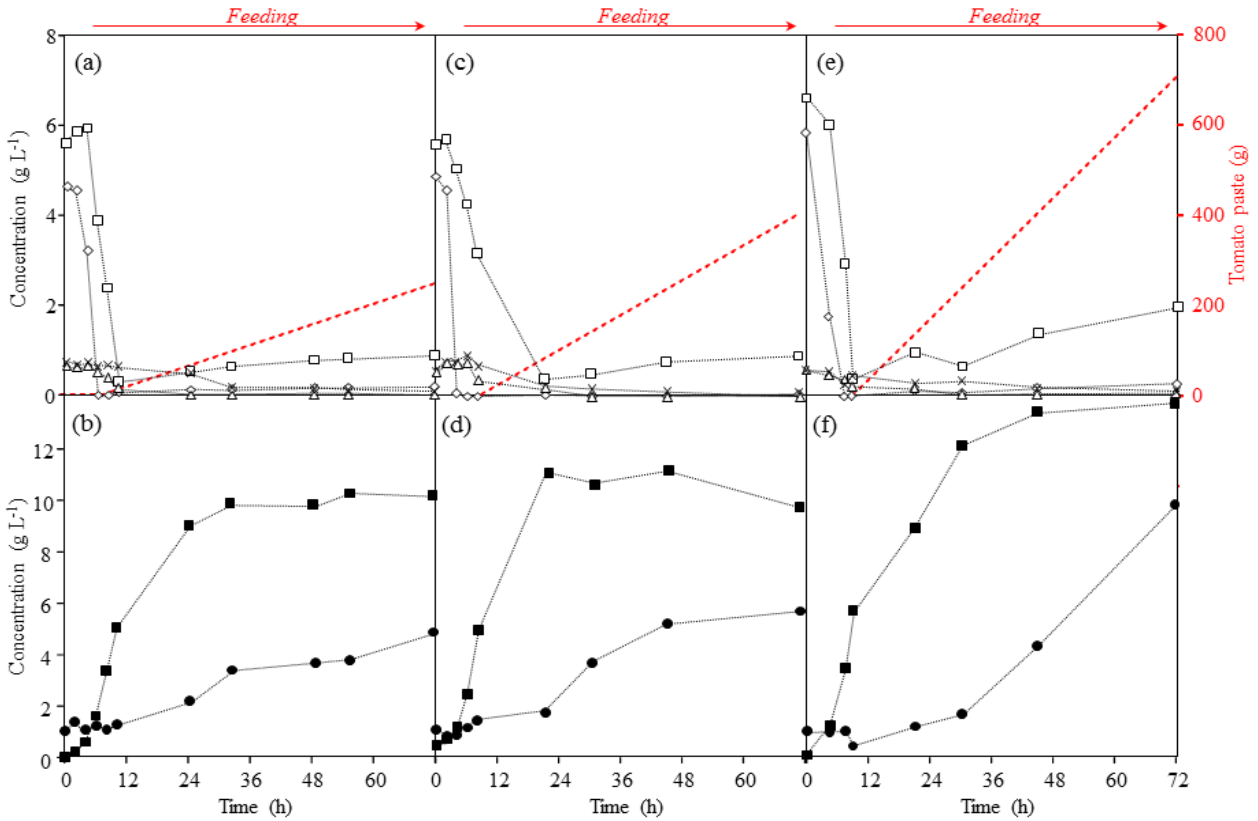

Figure 3.3: Cultivation profiles obtained in Experiment C (a and b), Experiment D (c and d) and Experiment E (e and f) for the cultivation of *Enterobacter* A47 under continuous substrate feeding mode, using tomato paste as sole substrate (◇, glucose; □, fructose; - - - -, tomato paste feed; ■, CDM; ●, polymer; △, ammonium; ×, phosphate concentration).

would improve exopolysaccharide production or, by the contrary, might be inhibitory to the culture.

The feed flow rate tested in Experiment C resulted in an exopolysaccharide production of 3.99 g L^{-1} (Figure 3.3b) and an overall volumetric productivity of 1.34 g L^{-1} d^{-1} (Table 3.1). These values are similar to the ones obtained in Experiment B, wherein the overall amount of substrate fed to the culture during the fed-batch phase was approximately the same. Glucose and fructose were detected in the broth at low concentrations (<0.20 g L^{-1} and <1.0 g L^{-1}, respectively) (Figure 3.3a) during the fed-batch phase, suggesting that exopolysaccharide synthesis might have also been impaired by carbon source limitation.

The exopolysaccharide produced in Experiment C was identical to that obtained in Experiment B, in terms of average molecular weight (4.1×10^6 Da), sugar monomer composition (27 mol% fucose, 31 mol% galactose, 32 mol% glucose and 12 mol% glucuronic acid) and acyl groups content (5 wt.%) (Table 3.2).

Increasing the substrate flow rate and, consequently, the nutrients' availability, in Experiments D and E seemed to stimulate *Enterobacter* A47 cell growth, as seen by the higher maximum CDM obtained (10.74 and 13.58 g L^{-1}, respectively) (Table 3.1; Figure 3.3d and f). Interestingly, the higher substrate availability also resulted in improved exopolysaccharide production, especially in Experiment E, in which a polymer production of 8.77 g L^{-1} (Figure 3.3f) and a volumetric productivity of 2.92 g L^{-1} d^{-1} were achieved (Table 3.1). These values are within the ranges reported for cultivation of *Enterobacter* A47 on glycerol, xylose, lactose or cheese whey (Table 3.1). This reactor operation mode was successful to produce exopolysaccharide from tomato paste. The overall phosphate consumption increased from 0.81 g L^{-1} in Experiment C, to 0.98 and 1.15 g L^{-1} for Experiments D and E, respectively, which might be related to the improved exopolysaccharide synthesis. In fact, the synthesis of polysaccharides in bacteria requires the biosynthesis of activated precursors, nucleoside diphosphate sugars (NDP-sugars), which are derived from phosphorylated sugars (Freitas et al., 2011b). Hence, the increasing phosphate consumption observed in Experiments C, D and E suggests the higher requirement of this nutrient for the biosynthesis of the exopolysaccharides' building blocks.

The higher substrate availability tested in Experiments D and E also resulted in the synthesis of polymers with higher fucose content (33 and 37 mol%, respectively) and lower galactose content (27 mol%), which is in accordance with the typical FucoPol composition (Freitas et al., 2014; Torres at al., 2011, 2012, 2014). Moreover, the exopolysaccharides also had higher acyl groups contents (10 and 13 wt.%) that is also closer to the typical FucoPol content (12 – 22 wt.%) (Table 3.2). The polymer produced in Experiment E had an average molecular weight of 4.4×10^6 Da and a PDI of 1.2.

3.5. Conclusions

Out-of-specification tomato paste was used for the first time as the sole substrate for the production of a microbial polymer. Tomato paste proved to be an adequate source of nutrients, including sugars, ammonium and phosphate, for *Enterobacter* A47 growth and exopolysaccharide synthesis. The best bioprocess performance, in terms of polymer production and volumetric productivity, was higher for the continuous mode operation, which guarantied non-limiting availability of carbon and nutrients. Polymer composition and molecular mass distribution was similar to those produced from other feedstocks.

CHAPTER 4

Extracellular Polysaccharides Production:

Brewer’s spent grain

4.1. Summary

The ability of *Enterobacter* A47 growth and produce extracellular polysaccharides (EPS) using as carbon source a mixture of glucose and xylose (75:25%) was tested using pure sugars and a brewer's spent grains (BSG) hydrolysate. Using as substrate the hydrolysate of a beer manufacturing by-product, BSG, within 4 days the culture reached an EPS concentration of 2.30 g L^{-1}, corresponding to a volumetric productivity of 0.57 g L^{-1} d^{-1}. The produced EPS was mainly composed of glucose (53 mol%), with lower contents of galactose, xylose and fucose (20 mol% ,17 mol% and 10 mol%, respectively), and a very lower acyl groups content (0.5 wt.%). On the other hand, using commercial glucose/xylose mixture, 5.71 g L^{-1} of EPS was achieved after 4 days, giving an overall volumetric productivity of 1.43 g L^{-1} d^{-1}. Evaluating the sugar monomer content, the EPS produced revealed to be similar to FucoPol (36 mol% fucose, 24mol% glucose, 22 mol% galactose and 17 mol% glucuronic acid, with an acyl groups content of 19 wt.%) with an average molecular weight of 3.6×10^6 Da and a PDI of 1.1.

This study demonstrated that the use of glucose/xylose mixture as carbon source is sustainable to produce FucoPol by *Enterobacter* A47. Moreover, although was proved that *Enterobacter* A47 could use BSG hydrolysate as sole substrate to growth and produce EPS, further studies need to be developed to increase the EPS productivity, thus valuing this by-product into microbial value added product.

4.2. Introduction

During the manufacture of the third-most consumed beverage in the world, beer, several residues and by-products are generated, being the most common ones, spent grains, spent hops and surplus yeast (Aliyu and Bala, 2011). Brewer's spent grains (BSG), the residual malted barley grains resulting from wort preparation, correspond to around 85% of the total by-products, which accounts for 30 to 60% of the biochemical oxygen demand (BOD) and suspended solids generated by a typical brewery (Mussatto and Roberto, 2005; Aliyu and Bala, 2011). BSG is rich in cellulose and non-cellulosic polysaccharides, lignin, proteins and lipids and is mainly used as animal feed (Mussatto and Roberto, 2005). Other proposed applications are human nutrition, energy and charcoal production, as a brick component, paper manufacture, adsorbent and biotechnological processes (Mussatto et al., 2006). BSG has been used as substrate for cultivation of

microorganisms such as mushrooms (Wang et al., 2001), production of enzymes such as α-amylase (Francis et al., 2002, 2003) and monosaccharide source to produce ethanol and xylitol, for example (Carvalheiro et al., 2004).

The main processes used for the selective fractionation of hemicelluloses from biomass include the use of acids, water (liquid or steam), organic solvents and alkaline agents (Gírio et al., 2010). Acid catalysed processes can be divided in two general approaches, based on concentrated-acid/low temperature and dilute-acid/high temperature hydrolysis (Gírio et al., 2010). The concentrated-acid hydrolysis has the advantage to allow operating at low/medium temperatures (below 121 °C) leading to a reduction of the operational costs and also extracts some glucose from cellulosic material (Gírio et al., 2010). On the other hand, concentrated-acid hydrolysis has high acid consumption and more costs associated with equipment corrosion (Gírio et al., 2010). The most efficient process to selectively release hemicellulose sugars (xylose and arabinose) is diluted acid hydrolysis but need higher temperatures (above 121 °C) (Mussatto and Roberto, 2005). To prevent the problem of simultaneously hydrolyse the produced monosaccharides into potent inhibitors of cell growth such as furfural, hydroxymethylfurfural (HMF), acetic acid and lignin degradation products (LDPs), it is important to conduct the hydrolysis under adequate reaction conditions. Therefore, it is necessary to establish the best hydrolysis conditions for the lignocellulosic material in order to produce a liquor with a higher amount of fermentable sugars using less acid (Mussatto and Roberto, 2005). The levels of the degradation compounds generated can also be low, under controlled conditions (Gírio et al., 2010).

In previous works, the EPS-producer *Enterobacter* A47 (DSM 23139), demonstrated the ability to consume a wide range of substrates, including glycerol (Alves et al., 2010; Reis et al., 2011; Torres et al., 2011, 2012, 2014), glucose, xylose (Freitas et al., 2014) and lactose (Chapter 2; Antunes et al., 2015) as sole carbon sources. Several by-products were also used as feedstock, namely glycerol generated by the biodiesel industry (Alves et al., 2010; Reis et al., 2011; Torres et al., 2011, 2012, 2014), cheese whey (Chapter 2; Antunes et al., 2015) and out-of-specification tomato paste (Chapter 3; Antunes et al., 2017). The capacity of *Enterobacter* A47 to use a large range of substrates makes the bioprocess more robust and versatile, but the composition of the polymer synthesized could be different according to the type of carbon source used. For example, a polysaccharide with higher fucose content was produced using xylose as carbon source (Freitas et al., 2014) and a biopolymer enriched in glucuronic acid was synthesize using lactose as carbon source (Chapter 2; Antunes et al., 2015). On the other hand, the polysaccharide produced by *Enterobacter* A47 consuming tomato paste as substrate presents similar chemical composition to FucoPol (Chapter 3; Antunes et al., 2017).

From this point of view, the search for alternative feedstocks is very relevant, especially abundant wastes and by-products generated by agro-industrial activities, which can not only lower the production costs but also result in biopolymers with novel valuable properties. Within this context, the potential application of BSG as feedstock for *Enterobacter* A47 growth and EPS production in fed-batch bioreactor cultivations was studied. For that purpose, BSG hydrolysate was obtained using a mild acidic hydrolysis in two steps to infer the best acid concentration to reach high sugar monomers concentration. The produced polysaccharides were analysed in terms of sugar and acyl groups composition.

4.3. Materials and Methods

4.3.1. BSG characterization

From crude BSG, supplied by Unicer Bebidas, S.A. (Matosinhos, Portugal), was determined the moisture by drying a sample (50 mg) at 105 °C,

Before the hydrolisation step, BSG was dried in a 70 °C oven until reaching a moisture content less than 10% (*w/w*). The feedstock material was ground in a hammermill (particle size <1 mm^2) and stored in sealed bags at -20 °C. The dried BSG powder was characterized in terms of particle-size distribution by submitting a milled sample (100 g) through a nest of five different sized sieves (1.0 – 0.125 mm). Also were determined the proteins by Kjeldahl technique, lipids using Soxhlet extraction with hexane and inorganic salts content by incineration at 550 °C.

4.3.2. BSG Hydrolysis

The dry and milled BSG was mixed with sulfuric acid solution at a liquid-to-solid ratio of 8 (*w/w*) in 500 mL Schott® bottles and hydrolysed in an autoclave (121 °C, 20 min). Four different sulphuric acid (Sigma) concentrations (*v/v*) were tested (3, 5, 7 and 9%). After the reaction time and cool down the solution, the liquid phase was filtered with a paper filter (pore size >11 µm). The remaining solid phase was hydrolysed again at the same condition subjected before. After the second hydrolysis, the liquid phase was also filtered and put together with the first liquid phase. BSG hydrolysate was adjusted to pH 7.0 by adding $Ca(OH)_2$ and the precipitate formed was removed by centrifugation (8 000 × *g*, for 10 min). Finally, the BSG hydrolysate was lyophilized.

The BSG hydrolysate sugar content was determined by HPLC, with a Biorad 87H column coupled to a refractive index detector. The analysis was performed at 30 °C, with sulphuric acid (0.01 N) as eluent, at a flow rate of 0.5 mL min^{-1}. Glucose (Sigma) and xylose (Sigma) were used as standards at concentrations between 0.062 and 1.0 g L^{-1}. Total nitrogen concentration was determined using commercial photochemical Hach-Lange test kits (Hach Lange DR 2800, UK). The protein content was determined using a modified Lowry method described at section 3.3.1. - Chapter 3. The method to extract and quantify the BSG hydrolysate's high molecular weight compounds are described at section 3.3.1. – Chapter 3.

4.3.3. Exopolysaccharide production

Microorganism and media

Enterobacter A47 (DSM 23139) reactivation preparation were performed as described in section 2.3.1. - Chapter 2. Also, the bioreactor assays preparation is described in section 2.3.1. - Chapter 2 with some modifications. For the glucose/xylose mixture run, Medium E* (1 L) was supplemented with approximately 40 g of those sugars in a ratio of 75:25 (w/w), while for the BSG assay the ammonium phosphate was withdrawn from Medium E* and supplemented with 40 g of dried BSG hydrolysate.

Bioreactor cultivation

Inocula for the assays were prepared as described in section 3.3.2. - Chapter 3.

The experiments were performed in 2 L bioreactors (BioStat B, Sartorius, Germany) with initial working volume of 1.5 L. The assays took around 4 days, under controlled temperature and pH conditions of 30 ± 0.2 ºC and 7.0 ± 0.02, respectively. An air flow rate of 0.4 SLPM (standard litters per minute) was kept constant throughout the cultivation runs. The DO was controlled at 10% by the automatic variation of the stirrer speed (300-800 rpm).

During the fed-batch phase, the bioreactor was fed with Medium E* supplemented with glucose and xylose to give an overall sugar concentration of 200 g L^{-1} (75:25 w/w) or with BSG hydrolysate (400 g L^{-1}), at constant feeding rates of 8 mL h^{-1} or 4 mL h^{-1}, respectively. Samples (25 mL) were periodically withdrawn from the bioreactor for biomass, polysaccharide and nutrients quantification.

Analytical techniques

The cell growth and CDM were monitored and determined, respectively, as described in section 3.3.2 - Chapter 3.

The cell-free supernatant samples obtained by centrifugation of the cultivation broth (13 000 × *g*, for 15 min) were used for the quantification of sugars (glucose, xylose and arabinose), as described above. Ammonium concentration were determined by colorimetry, as described in section 3.3.1 – Chapter 3.

All analyses were performed in duplicate.

Extraction and purification of EPS

The culture broth from glucose/xylose mixture (75:25 (*w/w*) ratio) bioreactor was extracted and purified as described in section 3.3.2. - Chapter 3 for quantification of high molecular weight compounds. To extract and purify the EPS from BSG bioreactor was applied the procedure described in section 2.3.1. - Chapter 2. Finally, the solutions were freeze dried and the polymers were stored at room temperature.

4.3.4. Polymer characterization

For the compositional analysis, polymer samples were hydrolysed as described in section 3.3.2. - Chapter 3. The identification and quantification of the constituent monosaccharides were also described on the same section in Chapter 3.

Sample preparation and measurement of molecular mass distribution from the EPS purified from glucose/xylose mixture assay (EPS_{GX}) is described in section 3.3.2. – Chapter 3. Intrinsic viscosity, apparent viscosity and viscoelastic properties were determined as described in section 2.3.2. - Chapter 2 but maintaining the native pH of EPS_{GX} solutions.

4.4. Results and Discussion

4.4.1. BSG characterization

BSG composition differs considerably from lot to lot, as it is a by-product resulting from a mixture of several raw materials that can be processed in the brewery under variable conditions. The crude BSG batch used had a moisture of 72.1% (*w/w*) and after drying it had 10% (*w/w*). It was composed of 58.1% (w/w) carbohydrates, 26.9% (*w/w*) proteins, 11.5% (*w/w*) lipids and 3.5% (*w/w*) ash. The crude BSG used in this study strongly agrees with other composition data present in the literature, only with slightly higher ash and fat contents (Table 4.1).

Table 4.1: Chemical composition of the BSG used in this study and comparison with literature data. DW: Dry weight.

Components	**This study**	**Kotlar et al., 2011**	**Pires et al., 2012**	**Mussatto et al., 2006**
Moisture (%, w/w)	72.1	77.4 – 79.7	n.a.	n.a.
Ash (%, w/w) DW	3.5	2.1 – 2.6	2.2	2.4
Protein (%, w/w) DW	26.9	30.0 – 34.6	39.1	24.0
Lipids (%, w/w) DW	11.5	5.7 – 6.7	10.5	10.6
Carbohydrates (%, w/w) DW	58.1	56.3 – 61.6	61.8	59.1

n.a. – data not available

The hydrolysis step could be improved by a previous drying and milling step of BSG, forming small particles that would increase the surface area to volume ratio (Figure 4.1). After the grinding process, the BSG particle-size distribution was mostly constituted of 0.710 to 0.125 mm particles (85% (*w/w*)) (Figure 4.2).

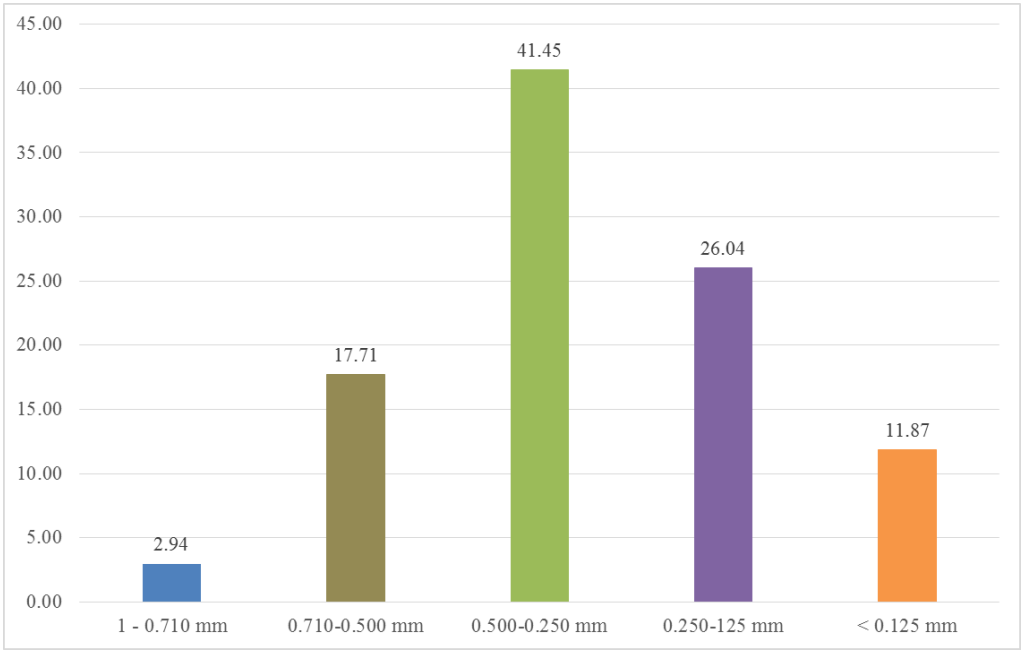

Figure 4.1: Dried BSG before (A) and after milling (B).

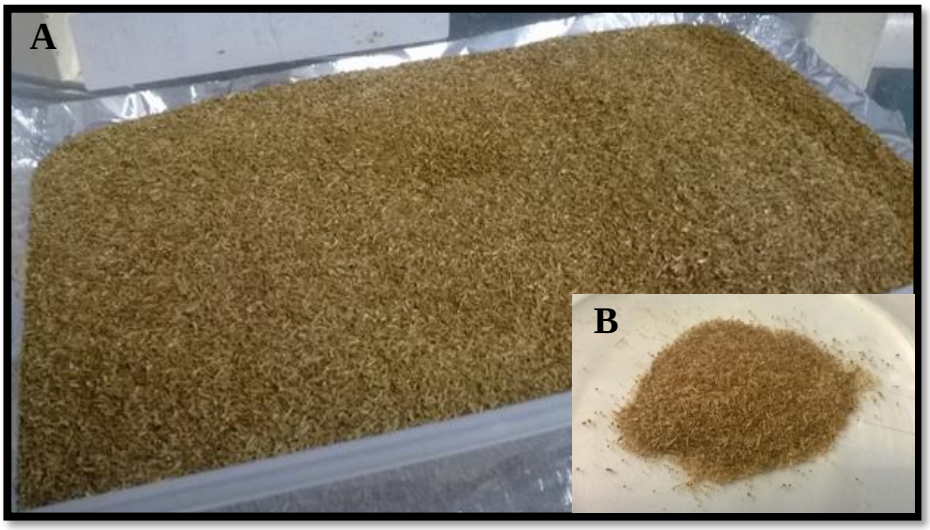

Figure 4.2: Dried BSG particle-size distribution

4.4.2. BSG hydrolysis

The hydrolysis conditions used for this study were based on Carvalheiro et al. (2004), where a dilute-acid hydrolysis of BSG was optimised to obtain a pentose's rich media. In this study, the main difference was that a second hydrolysis was performed to infer if more sugars

could be hydrolysed from the same BSG fraction, improving the final hydrolysis yield (Table 4.2).

Table 4.2: Monosaccharide content and overall hydrolysis yield of BSG acid hydrolysis.

[H_2SO_4] (% *w/w*)	**1st hydrolysis [Sugars] (g L^{-1})**	**2nd hydrolysis [Sugars] (g L^{-1})**	**Combined hydrolysates (g L^{-1})**	**Yield (g $_{sugars}$/g $_{BSG\ hydrolysed}$)**
3	42.05	26.53	58.07	0.55
5	38.75	24.97	54.03	0.51
7	39.71	24.59	54.37	0.51
9	40.21	21.76	51.92	0.50

Table 4.2 demonstrates that the best conditions for hydrolysis were obtained with using 3% (*w/w*) of sulphuric acid. This hydrolysis condition achieved a combined hydrolysate concentration of 58.07 g L^{-1} and a yield of 0.55 g $_{sugars}$/g $_{BSG\ hydrolysed}$ (Table 4.2). On the other hand, the hydrolysis using 9% (*w/w*) of sulphuric acid, thought achieving also high sugar content in the first hydrolysis step (40.21 g L^{-1}), in the second step reached a lower sugar content (21.76 g L^{-1}) causing the lowest combined hydrolysates concentration and yield (51.92 g L^{-1} and 0.50 g $_{sugars}$/g $_{BSG\ hydrolysed}$, respectively) (Table 4.2). This could be related with the fact that with higher acid concentration cellulose could also be hydrolysed but then more quantity of sugar monomers could be converted into furfural or HMF, reducing the sugar amount extracted in the second hydrolysis. Carvalheiro et al. (2004) reported that the optimum conditions to obtain a pentose-rich hydrolysate with dilute-acid hydrolysis of BSG was at temperature of 130 °C during 15 min, obtaining 43.5 g L^{-1} of glucose, xylose and arabinose for just one hydrolysate step. In this study, a concentration of 42.05 g L^{-1} was achieved at a lower temperature, although a higher reaction time was used (121 °C, 20 min). The use of lower acid concentration and temperature of hydrolysis result in less process and operational costs due to lower quantity of acid for the hydrolysis, lower amount of $Ca(OH)_2$ required to neutralize the pH and also less corrosion impact on the equipment.

For the bioreactors experiments, the combined hydrolysates using 3% (*w/w*) of H_2SO_4 was chosen to use as substrate for *Enterobacter* A47 cultivation. This hydrolysate was composed of 41.18 g L^{-1} glucose, 15.66 g L^{-1} xylose and a minor content of arabinose (1.2 g L^{-1}). Besides the simple sugars, the combined hydrolysate also contained a high content of proteins (21.59 g L^{-1}), 0.75 g L^{-1} of ammonium and a total nitrogen concentration of 3.26 g L^{-1}. In addition, a high molecular weight fraction (1.3 g L^{-1}) was extracted from the BSG hydrolysate that was mainly

composed of undergraded hemicellulose and cellulose present in BSG (Mussatto et al., 2006). This polysaccharide was mainly composed of glucose (51 mol%), rhamnose (18 mol%), galactose (16 mol%) and xylose (15 mol%), and an acyl groups content of 0.9 wt.%. It represented 2.25 wt.% of the hydrolysed BSG powder.

4.4.3. Fed-batch cultivation of *Enterobacter* A47

Using commercial glucose/xylose mixture as substrate

Freitas et al. (2014) demonstrated the capacity of *Enterobacter* A47 to use glucose and xylose for the production of EPS (Table 4.3). Using glucose as sole carbon source the culture had the highest EPS productivity (3.78 g L^{-1} d^{-1}) and the polymer had the typical Fucopol composition, namely a fucose content of 29 mol%. On the other hand, the cultivation using xylose achieved a lower EPS productivity (1.39 g L^{-1} d^{-1}), but the polymer richer in fucose (38 mol%). To evaluate the EPS production capacity using mixtures of glucose and xylose, the two sugars were tested at an hexoses:pentoses ratio of approximately 75:25% (*w/w*) as substrate for the cultivation of *Enterobacter* A47 (Figure 4.3).

The cultivation started with a batch phase where the carbon source was used mainly for cell growth (Figure 4.3). *Enterobacter* A47 grew at a specific cell growth rate of 0.39 h^{-1} (Table 4.3) which is higher than those obtained in previous studies for cultivations on different substrates, such as glycerol (0.27 - 0.36 h^{-1}), glucose (0.35 h^{-1}), xylose (0.25 h^{-1}) and lactose (0.06 h^{-1}) (Table 4.3). This result could be explained by the lower glucose/ammonium ratio at batch phase (57 g g^{-1}) comparing with previous cultivation run with glucose as sole carbon source (61 g g^{-1}), meaning that there was more ammonium available for the same amount of glucose, thus increasing the cell growth rate from 0.35 to 0.39 h^{-1}. At the end of batch phase (12 h), just before the ammonium exhaustion, the CDM achieved was 4.42 g L^{-1}, which is very similar to the CDM achieved after 12 h of cultivation by Freitas et al. (2014), using glucose as sole carbon source (Figure 4.3, Table 4.3).

As shown in Figure 4.3, glucose and xylose were efficiently used by the culture. During the batch phase, despite glucose and xylose consumption was initiated right after inoculation, *Enterobacter* A47 consumed almost all the available glucose (31.8 g L^{-1}), but only 3.5 g L^{-1} of xylose out of the 9.3 g L^{-1}available. The sugar consumption profile during the batch phase is in agreement with previous work using the same carbon sources separately (Freitas et al., 2014),

Table 4.3: Kinetic and stoichiometric parameters obtained in the bioreactor cultivation of *Enterobacter* A47 using different substrates and cultivation modes (μ_{max}: maximum specific growth rate; CDM: cell dry mass; BSG: brewer's spent grains.

Substrate	Cultivation mode	μ_{max} (h^{-1})	CDM ($g\ L^{-1}$)	EPS ($g\ L^{-1}$)	Productivity ($g\ L^{-1}\ d^{-1}$)	References
Glycerol	Continuous feeding	0.27 – 0.36	5.80 – 7.68	7.23 – 7.97	2.04 – 3.72	Freitas et al., 2014; Torres et al., 2011, 2012, 2014
Glucose	DO-stat	0.35	8.14	13.40	3.78	Freitas et al., 2014
Xylose	DO-stat	0.25	3.92	5.39	1.39	Freitas et al., 2014
Lactose	Continuous feeding	0.06	5.33	5.22	1.31	Chapter 2; Antunes et al., 2015
Cheese whey	Continuous feeding	0.15	8.60	6.40	2.00	Chapter 2; Antunes et al., 2015
Tomato paste	Continuous feeding	0.27 – 0.33	10.14 – 13.58*	3.99 – 8.77	1.34 – 2.92	Chapter 3; Antunes et al., 2017
Glucose/Xylose mixture	Continuous feeding	0.39	5.91*	5.71	1.43	This study
BSG	Continuous feeding	0.43	17.99*	2.30	0.57	This study

*CDM estimated considering that one unit of OD450 is equivalent to 0.26 $g\ L^{-1}$ CDM).

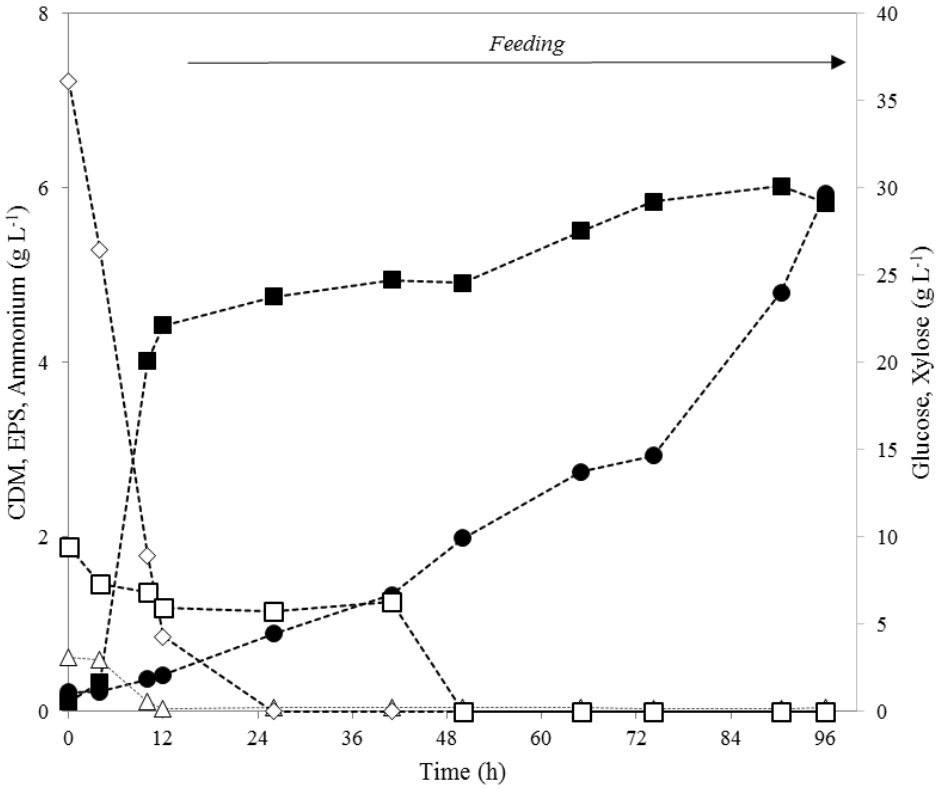

Figure 4.3: Cultivation profile obtained for the bacterium *Enterobacter* A47, using glucose/xylose mixture as carbon source: glucose (◇), xylose (□), ammonium **(△)**, CDM **(■)** and EPS **(●)**.

where glucose was totally used within 24 h and only part of the available xylose (3.48 g L^{-1}) was consumed within the same period of time.

After the fed-batch phase started, although glucose was continuous entering the bioreactor, all concentration continued to decrease, meaning the culture used all the glucose from feed solution, being limited from 24 hours until the end of the run. On the other hand, xylose consumption rate was lower remaining a xylose concentration at 6 g L^{-1} until 50 h of cultivation run and from that point the sugars from the feed were consumed at the same rate that feed was entering the bioreactor (Figure 4.3). Moreover, after 50 h of *Enterobacter* A47 cultivation is notice a slightly increase of CDM concentration that might be explained by evaporation of culture media during the cultivation run.

Before the fed-batch phase was initiated, a small amount of EPS was detected (0.4 g L^{-1}), which indicates that its synthesis is partially growth associated (Figure 4.3). A final EPS production of 5.71 g L^{-1} was obtained at the end of the 96 h cultivation, corresponding to an overall volumetric productivity of 1.43 g L^{-1} d^{-1} (Figure 4.3 and Table 4.3). Comparing with previous work (Freitas et al., 2014), these values are lower than the assay using only glucose as carbon source with DO-stat fed-batch mode (final EPS production of 13.40 g L^{-1} and volumetric productivity of 3.78 g L^{-1} d^{-1}), and slightly higher than the run using xylose as carbon source (final EPS production of 5.39 g L^{-1} and volumetric productivity of 1.39 g L^{-1} d^{-1}). For *Enterobacter* A47 cultivation runs using different carbon sources, the values of EPS concentration and productivity obtained are similar to the ones using lactose (5.22 g L^{-1} and 1.31 g L^{-1} d^{-1}, respectively) (Chapter 2; Antunes et al., 2015), but considerably lower than those obtained from glycerol cultivation runs (7.50-7.97 g L^{-1} and 2.52 g L^{-1} d^{-1}, respectively) (Freitas et al., 2014; Torres et al., 2011, 2012 and 2014).

Although the culture was continuously being fed, during the fed-batch phase the carbon source was depleted in this study after 50 h of culture (Figure 4.3). In opposition, Freitas et al. (2014) reported that under a DO-stat strategy, the carbon source never reached limiting values, which favoured the EPS production achieving the highest productivity value for EPS production by *Enterobacter* A47. In conclusion, the chosen flow rate was probably not sufficient to guaranty the culture's needs to maintain high EPS productivity (Table 4.3) (Freitas et al., 2014).

The EPS recovered from the culture broth at the end of the run (EPS_{GX}) had an average molecular weight of 3.6×10^6 Da and a PDI of 1.1. Comparing with the EPS synthesized with only glucose (4.2×10^6 Da, PDI of 1.4) or xylose (1.7×10^6 Da, PDI of 1.4) as substrate, EPS_{GX} molecular weight is in the range of the Mw from EPS produced using glucose, which might be expected given that glucose was the major sugar in this cultivation run. In addition, EPS_{GX} M_w had the same order of magnitude reported for other substrates, such as the EPS produced from tomato paste (3.2 - 4.4×10^6 Da), lactose, cheese whey and glycerol (1.8 - 5.7×10^6 Da, please see Table 2.2 from section 2.4.3. – Chapter 2).

The polymer was composed of fucose (36 mol%), galactose (22 mol%), glucose (24 mol%) and glucuronic acid (17 mol%) (Table 4.4). This sugar monomer profile is in accordance with the typical FucoPol composition produced using different carbon sources, namely, fucose (27-38 mol%), galactose (18-35 mol%), glucose (23-34 mol%) and glucuronic acid (9-17 mol%) (Table 4.4). Moreover, the acyl groups content (19 wt.%) is similar to that of the FucoPol synthesized using glucose (22 wt.%) or xylose (17 wt.%) as carbon source (Freitas et al., 2014) (Table 4.4). Thus, these results show that the use of the glucose/xylose mixture as substrates for the cultivation of *Enterobacter* A47, had no significant effect on the polymer's sugar and acyl composition.

Table 4.4: Sugar composition and acyl groups content of the extracellular polysaccharides produced by *Enterobacter* A47 from various substrates under different cultivation modes (Fuc: fucose; Gal: galactose; Glc: glucose; GlcA: glucuronic acid; Xyl: xylose).

Substrate	Cultivation mode	Sugar composition (mol%)					Acyl groups content (wt.%)	References
		Fuc	Gal	Glc	GlcA	Xyl		
Glycerol	Continuous feeding	30-36	22-29	25-34	9-10	-	12-22	Freitas et al., 2011a, 2014; Torres et al., 2011, 2012
Glucose	DO-stat	29	29	26	16	-	22	Freitas et al., 2014
Xylose	DO-stat	38	18	27	17	-	17	Freitas et al., 2014
Lactose	Continuous feeding	25	22	24	29	-	36	Chapter 2; Antunes et al., 2015
Cheese whey (EPS_{CW})	Continuous feeding	29	21	21	29	-	32	Chapter 2; Antunes et al., 2015
Tomato paste (EPS_{TP})	Continuous feeding	27 - 37	27 - 31	23 - 32	10 - 12	-	5 - 13	Chapter 3; Antunes et al., 2017
Glucose/Xylose mixture (EPS_{GX})	Continuous feeding	36	22	24	17	-	19	This study
Brewer's spent grains	Continuous feeding	10	20	53	-	17	0.5	This study

Using BSG hydrolysate as substrate

After the results achieved using glucose/xylose (75:25 (*w/w*)) as carbon source have confirmed that the mixture is suitable for bacterial growth and allows to achieve good EPS production by *Enterobacter* A47, with no significant impact on the polymers' composition and M_w, BSG hydrolysate was tested as substrate under similar cultivation conditions (Figure 4.4). BSG hydrolysate had a similar glucose:xylose ratio, although the initial sugar concentration in the bioreactor was lower, 5.66 g L^{-1} and 2.25 g L^{-1} for glucose and xylose, respectively.

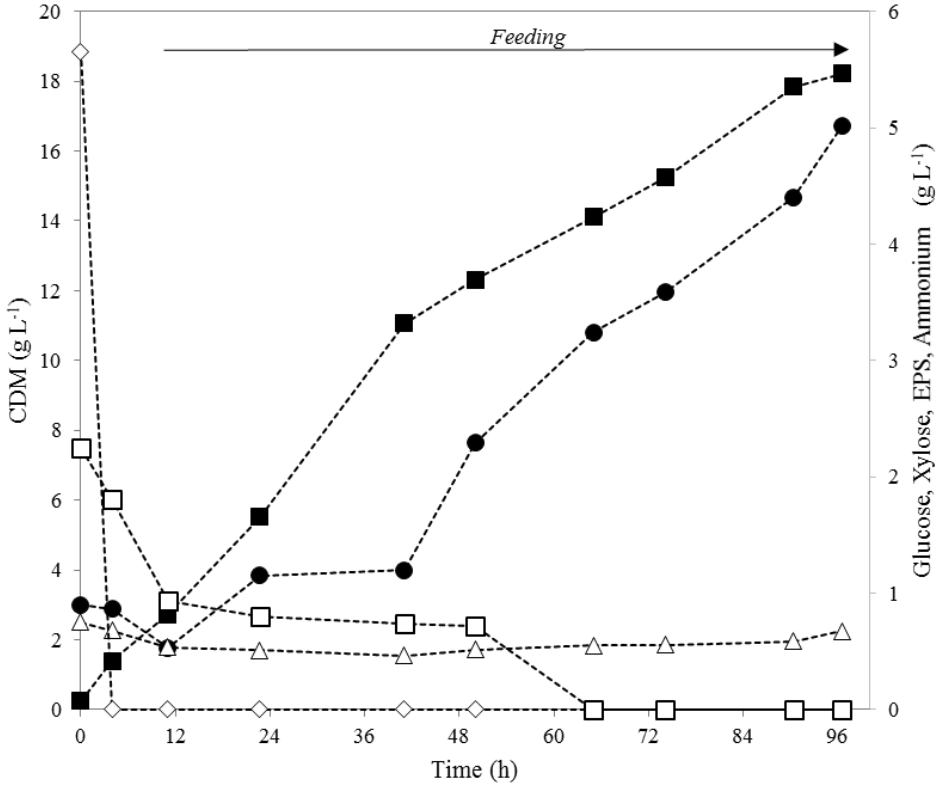

Figure 4.4: Cultivation profile obtained for the bacterium *Enterobacter* A47, using brewer's spent grain hydrolysate as carbon source: glucose (◇), xylose (□), ammonium **(△)**, CDM **(■)** and EPS **(●)**.

The specific cell growth rate obtained with the BSG hydrolysate was higher (0.43 h^{-1}) than with the glucose/xylose mixture (0.39 h^{-1}), attaining also a lower CDM at the end of the batch phase (2.73 g L^{-1}), most likely due to less amount of available sugar monomers from BSG hydrolysate. During the fed-batch phase the culture continued to grow, achieving a maximum CDM of 17.99 g L^{-1} at the end of the run (Figure 4.4, Table 4.3). Since the ammonium concentration supplied during the fed-batch phase was not sufficient to support cell growth (< 0.1 g L^{-1}), the observed CDM is probably related to the cultivation medium provided by BSG hydrolysate,

which, in addition to glucose and xylose, had a high total nitrogen concentration (232.44 mM) and proteins (21.59 g L^{-1}) that could stimulate cell growth (Mussatto et al., 2006).

During the batch phase, all the available glucose (5.66 g L^{-1}) and 1.31 g L^{-1} of xylose were consumed (Figure 4.4). A total of 368 g of BSG hydrolysate solution (400 g L^{-1}) were fed with a total of 14.2 g of glucose and 5.6 g of xylose that were totally consumed, since no sugar was detected during the fed-batch phase. Up to around 40 h of cultivation, no significant EPS synthesis was observed since its content in the broth remained 0.9 – 1.2 g L^{-1} Figure 4.3). When the culture decreases the cell growth rate (40 h), EPS production was initiated, reaching a final concentration of 5.01 g L^{-1}, corresponding to an overall production of 2.30 g L^{-1} of EPS within the 4 days of the run. An overall volumetric productivity of 0.57 g L^{-1} d^{-1} was achieved (Figure 4.3, Table 4.3). The values obtained for EPS production are the lowest values obtained by *Enterobacter* A47 cultivations (Table 4.3). The higher ammonium concentration available during all the assay could explain the reduced EPS production, since the best cultivation strategy to produce EPS requires carbon availability during the fed-batch phase, concomitantly with nitrogen limitation (Freitas et al., 2011b, 2014; Torres et al., 2014). Thus, the results demonstrate that this substrate offered the opposite, which maximised cell growth in detriment of EPS synthesis. Also, the presence of cell growth inhibitors compounds, such as furfural and/or HMF, may have caused a lower EPS production (Gírio et al., 2010; Mussatto and Roberto, 2005).

The polysaccharide fraction recovered from the cell-free supernatant at the end of cultivation run was composed mainly of glucose (53 mol%), galactose (20 mol%), xylose (17 mol%) and fucose (10 mol%), and an acyl groups content of 0.5 wt.% (Table 4.4). This polymer's chemical composition with very low fucose content, no glucuronic acid and presence of xylose monomer also suggests that the recovered EPS was mixed with partially hydrolysed BSG polysaccharides (> 10 kDa) (Mussatto et al., 2006).

Therefore, EPS production by *Enterobacter* A47 from BSG hydrolysate should be optimized by minimizing the nitrogen source and possible cell growth inhibitors compounds, maximizing the sugar monomers hydrolysis. Strategies to produce higher sugar yields and lower decomposition products from BSG may include, for example, an enzymatic hydrolysis after a more diluted acidic prehydrolysis (Hernández et al., 2013).

4.5. Conclusions

Enterobacter A47 demonstrated the capacity to use as substrate a mixture of glucose/xylose, either commercial pure sugars or BSG hydrolysate. The EPS produced using commercial sugars as carbon source has high similarity with FucoPol in terms of chemical composition and molecular mass distribution. The preliminary results of the cultivation of *Enterobacter* A47 using for the first time the hydrolysate from an abundant lignocellulosic by-product, BSG, as substrate, indicate that the culture has the ability to growth and produce EPS, but further studies needs to be developed to improve the EPS productivity.

CHAPTER 5

Extracellular Polysaccharides Functional Properties

Some of the results presented on this chapter were published in a peer reviewed paper.

Antunes, S., Freitas, F., Alves, V.D., Grandfils, C., Reis, M.A.M., 2015. Conversion of cheese whey into a fucose- and glucuronic acid-rich extracellular polysaccharide by *Enterobacter* A47. Journal of Biotechnology. 210, 1-7.

5.1. Summary

The extracellular polysaccharides produced by *Enterobacter* A47 using cheese whey (EPS_{CW}) (Chapter 2), out-of-specification tomato paste (EPS_{TP}) (Chapter 3) and a glucose/xylose mixture (EPS_{GX}) (Chapter 4) were studied to infer their properties in aqueous solutions, their emulsion forming and stabilizing capacity, and their film-forming capacity and film mechanical properties. EPS_{CW} presents the lowest apparent viscosity (0.1 Pa·s) for the same concentration in aqueous solution (1 wt%) and shear rate, stabilize emulsions for more than 4 weeks with olive oil (E_{24} = 70 ± 2%), cedarwood oil (E_{24} = 69 ± 0%) and paraffin oil (E_{24} = 72 ± 1%), and the films formed have higher elongation capacity. EPS_{TP} shows the lowest intrinsic viscosity (4.7 dL g^{-1}) and angular frequency at G' and G'' cross-over (0.18 Hz), good emulsion capacity and stabilization with peanut oil (E_{24} = 74 ± 2%), almond oil (E_{24} = 68 ± 0%) and olive oil (E_{24} = 70 ± 3%) and the produced films are slightly more rigid. In contrast, EPS_{GX} reached the highest intrinsic viscosity (14.7 dL g^{-1}) and apparent viscosity (15 Pa·s), the emulsions formed are very strong with most of the tested oils at low O/W ratios (E_{24} > 90%), but low stability (< 3 weeks) and the films are slight stiffer. Thus, results indicate that the EPS synthesized by *Enterobacter* A47 using the different substrates can be a promising alternative to many synthetic polymers, as well as other natural polysaccharides that are used as thickening, emulsion forming and stabilizing agents and could form films. Potential applications for these EPS are: in the food, pharmaceutical, cosmetic, textile, paper and petroleum industries, since they are biodegradable, harmless to human and environment and produced from renewable resources.

5.2 Introduction

In the domain of water soluble polymers, microbial polysaccharides are particularly important due to their role as thickening, gelling, emulsifying, hydrating and suspending agents (Rinaudo, 2008). Polysaccharides area characterized by a structural diversity that leads to a vast variety of physical chemical properties (Kumar et al., 2007, Freitas et al., 2011b). To predict potential polysaccharides' industrial applications, it is very important to understand their properties in aqueous solution. Therefore, viscometry studies are necessary to obtain information about the molecular characteristics, such as intrinsic viscosity and viscoelastic properties.

The intrinsic viscosity is a direct measure of the solute's contribution to the solution's viscosity for a given solvent. Therefore, it depends on the polymer's molecular mass, chain rigidity and type of solvent, being indicative of the hydrodynamic volume of individual polymer molecules (Freitas et al., 2011a). Consequently, the value of the intrinsic viscosity is indicative of the polysaccharide's conformation and has typical values ranging from 1 dL g^{-1}, for compact coil or flexible chains, to 20 dL g^{-1}, for extended chains (Alves et al., 2010). On the other hand, viscometry studies with concentrated polymer solutions assess the viscoelastic behaviour (Newtonian or non-Newtonian) and properties of a polymer.

Previously, FucoPol's intrinsic and apparent viscosities were reported to present a quite low variation under a wide range of pH (3.5–8.0) and ionic strength (0.05–0.50 M NaCl) values for the polymer produced from glycerol. Moreover, the polymer produced viscous solutions with a shear-thinning behaviour at different polymer concentrations (0.2–1.2 wt.%) (Torres et al., 2015).

Another important physical property of polysaccharides is the ability to form and/or stabilize emulsions. Emulsions consist of mixtures of at least two immiscible, or poorly miscible, liquids, dispersed one into the other, in the form of small droplets (Bouyer et al., 2012; Klinkesorn, 2013). For emulsion formation, either synthetic (e.g., Tweens and Spans) or natural (e.g., gelatin, caseinate and lecithin) emulsifiers are commonly used (McClements and Gumus, 2016). Their role is to rapidly adsorb to the surface of the recently formed fine droplets, reduce the interfacial tension and protect the newly formed droplets from flocculation or coalescence, forming a protective interfacial layer around them (Bouyer et al., 2012; Klinkesorn, 2013). Emulsifiers are used in bioremediation and various industries, such as textile, paper, polymers, plastics, cosmetics, pharmaceuticals, food, petrochemical and machinery manufacture (Martínez-Checa et al., 2008; Mnif and Ghribi, 2015). To improve the long-term stability of an emulsion stabilizers, such as silica particles and sodium dodecyl sulphate (SDS), are added to prevent the instability of the emulsions by opposing close contact between the droplets. Stabilizers may also act as texture modifiers that increase the viscosity of the continuous phase of the emulsions, slowing down the gravitational separation of the droplets (Bouyer et al., 2012; Klinkesorn, 2013).

Although synthetic emulsifiers (e.g., Triton X-100) are among the most produced compounds in the world, they are extremely toxic to the environment and living organisms (Cserháti et al., 2002; McClements and Gumus, 2016). Besides, they are inherently toxic due to their easy accumulation in living organisms and may interfere on drugs distribution and elimination, causing irritant skins reactions and toxic symptoms in animals and humans (Bouyer et al., 2012; Mnif and Ghribi, 2015). Furthermore, due to extensive use of surfactants, high amounts are released into

the environment, causing problems in wastewater and sludge treatment systems and severe pollution on soil and water (rivers, lakes and sea) (Cserháti et al., 2002; Mnif and Ghribi, 2015).

In alternative, over the last years, natural emulsifiers/stabilizers came to answer the consumer demand for sustainable and environmental friendly commercial products. Usually, the natural emulsifiers employed are biosurfactants (e.g., saponins), phospholipids (e.g., lecithin), random coil biopolymers (e.g., polysaccharides, flexible proteins), compact biopolymers (e.g. globular proteins) and colloidal particles (e.g. starch granules, chitin crystals) (McClements and Gumus, 2016). These compounds, among other roles, help to solubilize hydrophobic substrates due to their hydrophilic and hydrophobic moieties, having high biodegradability, biological compatibility and environmental safety (Bouyer et al., 2012; Mnif and Ghribi, 2015; Satpute et al., 2010). Among them, polymeric bioemulsifiers have attracted a great attention for biotechnological applications. They are mostly amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins, fatty acids or complex mixtures of these biopolymers (Mnif and Ghribi, 2015). The most studied polymeric bioemulsifiers are emulsan, alasan, liposan, mannoprotein and other polysaccharide-protein complexes (Mnif and Ghribi, 2015; Panilaitis et al., 2007).

To improve emulsion stability, polymeric bioemulsifiers, besides their overall interfacial tension lowering capacity, also induce steric or electrostatic interactions, changes in the interface viscosity or elasticity, or changes in the bulk viscosity of the system (Bouyer et al., 2012). Polysaccharide-stabilized emulsions are known for their water-holding capacity and thickening properties and tend to form and stabilize emulsions due to their hydrophilic character and high molecular weight (Bouyer et al., 2012). Some examples of polysaccharides proposed as emulsion forming or stabilizing compounds include xanthan gum, alginates, carrageenans, hyaluronan, chitosan, gum arabic, hydroxypropylmethylcellulose (HPMC), galactomannans and pectin (Bouyer et al., 2012; Klinkesorn, 2013; Ngouémazong et al., 2015; Petri, 2015). Usually, they are odorless, colourless, tasteless, and have low energy value and digestibility (Paraskevopoulou et al., 2005). Their major advantage over other types of natural emulsifiers is the high stability of polysaccharide-coated lipids droplets to environmental stresses (pH and ionic strength) due to the strong steric repulsion between them (McClements and Gumus, 2016). Moreover, from an application perspective, another interest of polysaccharides is that, thanks to their viscosifying properties, they simultaneously stabilize emulsions and control their texture and they can be produced from renewable and low-cost sources (Bouyer et al., 2012; Freitas et al., 2011b). Thus, the use of polysaccharides produced by microorganisms (bacteria, yeast and fungi) as emulsifiers/stabilizers reveal the possibility to formulate stable “clean labelled” emulsions in numerous industries.

The EPS synthesized by the bacterium *Enterobacter* A47 using glycerol (Alves et al., 2010; Freitas et al., 2011a; Reis et al., 2011; Torres et al., 2011, 2012, 2014), glucose or xylose

(Freitas et al., 2014), as sole carbon sources demonstrated interesting emulsifying/stabilizing capacity. The EPS produced using glycerol (EPS-s) demonstrated to have higher emulsification indexes (60 ± 4%) than xanthan gum, alginate and pectin (20 – 56%) for a ratio of sunflower oil/water of 2:3 (*v*/*v*), a good emulsion stabilizing capacity at low temperatures (72%) and after freezing/thawing cycles retained about one third of the initial emulsification index (*EI*) (Freitas et al., 2011a; Freitas et al., 2014). In contrast, EPS produced using glucose (EPS-g) has shown a higher capacity for stabilization of emulsions at high temperatures (up to 90 ºC) and the emulsions formed with the EPS synthetized using xylose (EPS-x) broken within a few hours after being prepared (Freitas et al., 2014).

Many polysaccharides besides being used as hydrocolloids, also possess film-forming properties which make them suitable for the preparation of membranes with distinct characteristics (Freitas et al., 2014). The polysaccharides more often used to produce membranes are extracted from animal sources (e.g., gelatin, chitin), plants (e.g., starch, cellulose) and algae (e.g., alginates, carrageenan) (Ferreira et al., 2016; Freitas et al., 2014; Song and Zheng, 2014).

Polysaccharide membranes have a hydrogen-bonded dense polymer matrix which provides excellent gas (oxygen and carbon dioxide) and aroma barrier properties at low and intermediate relative humidity (Freitas et al., 2014). However, their applications are restricted due to their sensitivity to moisture, limited mechanical properties and high water vapour permeability (Castro-Rosas et al., 2016; Fabra et al., 2013; Freitas et al., 2014). The mechanical strength and barrier properties of polysaccharide-based membranes are dependent of film-forming agent (composition and structure), plasticizer additives, cross-linking agents and solvents (type and amount), manufacturing process, final thickness and storage conditions (Freitas et al., 2014; Preis et al., 2014). For example, the addition of plasticizers (e.g., glycerol or sorbitol) prevents film brittleness, conferring flexibility and ability to elongate, by reducing the intermolecular forces and increasing the mobility between polymer chains (Castro-Rosas et al., 2016; Preis et al., 2014).

Pullulan, gellan gum, kefiran, levan, bacterial cellulose, and bacterial alginates are some examples of microbial extracellular polysaccharides with film-forming capacity commercially used in food packaging and preservation as edible films or coatings, pharmaceutical industries for controlled release of active compounds and medicine as wound dressings (Ferreira et al., 2016; Vijayendra and Shamala, 2014).

The film-forming capacity of EPS produced by *Enterobacter* A47 using glycerol, glucose or xylose as sole carbon sources have been developed and characterized previously (Ferreira et al., 2014 and 2016; Freitas et al., 2014; Meireles et al., 2015). The membranes formed were transparent, poor barriers to water vapour, but good barriers to gases (O_2 and CO_2). The mechanical

tests revealed they were malleable films, not mechanically resistant enough to be used as stand-alone films, but instead, as hydrophilic layers in multi-layered films (Ferreira et al., 2014; Freitas et al., 2014; Meireles et al., 2015).

Taking into account the distinct chemical composition and molecular weight of the extracellular polysaccharides produced by *Enterobacter* A47 using cheese whey (EPS_{CW}) (Chapter 2), out-of-specification tomato paste (EPS_{TP}) (Chapter 3) and a glucose/xylose mixture (EPS_{GX}) (Chapter 4) as substrates, in this chapter their solution properties, as well as their emulsifying and film-forming capacity, were studied.

5.3. Materials and Methods

5.3.1. Solution properties

Intrinsic viscosity

The efflux times of the dilute polymers solutions (0.025 – 0.1 g d L^{-1}, in 0.01 M NaCl) were measured using an automatic viscosity measuring unit AVS 450 (Schott-Gerate, Germany), with an Ubbelhode capillary viscometer (Ref. 53013/Ic, Schott–Gerate, Germany) at the temperature of 25 °C, controlled with a thermostatic bath. Three independent measurements were made for each solution. The measured efflux times were converted into relative and specific viscosities (η_{rel} and η_{sp}, respectively), according to the following equations:

$$\eta_{rel} = \frac{t}{t_0} \quad (2)$$

$$\eta_{sp} = \eta_{rel} - 1 = \frac{(t - t_0)}{t_0} \quad (3)$$

where t (s) is the efflux time of the solution, and t_0 the efflux time of the solvent. Therefore, the intrinsic viscosity of the purified polymer was determined by the average of double extrapolation to zero concentration of the Huggins and Kraemer equations:

$$\frac{\eta_{sp}}{C} = [\eta] + k_H[\eta]^2 C \quad (4)$$

$$\frac{\ln(\eta_{rel})}{C} = [\eta] + k_K[\eta]^2 C \quad (5)$$

where [η] (dL g^{-1}) is intrinsic viscosity, k_H is the Huggins coefficient, k_K is the Kraemer coefficient and C (g dL^{-1}) is the polymer concentration.

Apparent viscosity and viscoelastic properties

Aqueous EPS solutions (1 %, w/w) were loaded in the cone and plate geometry (diameter 3.5 cm, angle 2 degrees) of a controlled stress rheometer (Haake Mars III, Thermo Scientific, Germany) and the shearing geometry was covered with paraffin oil in order to prevent sample dehydration. Flow curves were determined using a steady state flow ramp in the range of shear rate from 0.3 s^{-1} to 700 s^{-1}. Small amplitude oscillatory tests were conducted in order to obtain the viscoelastic properties. Stress sweeps were performed at constant frequency (1 Hz) for a stress range from 0.1 to 1000 Pa, in order to identify the linear viscoelastic region. Frequency sweeps were carried out for a frequency range from 0.01 to 10 Hz, at a constant stress within the linear viscoelastic region. All tests were performed at 25 °C.

5.3.2. Emulsion forming and stabilizing capacity

Emulsification assays were carried out following the method described by Cooper and Goldenberg (Cooper and Goldenberg, 1987). Aqueous solutions (0.5 wt.%) of EPS_{CW}, EPS_{TP} and EPS_{GX} were prepared and used for the tests. In the cylinder test tube (D 16 ×H 100 mm), each EPS aqueous solution was mixed with each hydrophobic compound to be tested (Table 5.1), vortexed (24 000 rpm, 2 min) and left to stand at room temperature (~21 °C) for 24 hours.

Table 5.1: Hydrophobic compounds used at the emulsification tests.

Compound	Source	Applications	References
Corn Oil	Maize	Food industry	Corn Refiners Association, 2006; Dupont et al., 1990; Gunstone, 2011
Sunflower Oil	Sunflower seeds	Food and cosmetic industries	Gunstone, 2011; Raß et al., 2008; Thomas et al., 2015
Olive Oil	Olives	Food industry	Gunstone, 2011; Paraskevopoulou et al., 2005; Thomas, 2002;
Soybean oil	Soybean seeds	Food and ink industries	Gunstone, 2011; Cahoon, 2003; Thomas, 2002

Peanut Oil	Peanuts	Food industry	Gunstone, 2011; Thomas, 2002
Cedarwood Oil	Conifers foliage	Cosmetic and furniture industries	Adams, 1991; faostat.fao.org
Almond Oil	Dried kernel of sweet almonds	Pharmaceutical and cosmetic industries	Ahmad, 2010; Canellas and Saura-Calixto, 1988
Paraffin Oil	Petrochemical	Pharmaceutical and cosmetic industries	JML, 2015; Liu et al., 2006
Benzene	Petrochemical	Petrochemical industry	Aburto-Medina and Ball, 2015; pubchem.ncbi.nlm.nih.gov
Toluene	Petrochemical	Paint and petrochemical industries	Frazer et al., 1995; pubchem.ncbi.nlm.nih.gov
Hexane	Petrochemical	Petrochemical and textile industries	pubchem.ncbi.nlm.nih.gov; Saien et al., 2014

The emulsification index (*EI*, %) was determined using the following equation:

$$EI = \frac{h_e}{h_t} \times 100$$

where h_e (mm) is the height of the emulsion layer and h_t (mm) is the overall height of the liquid column (Freitas et al., 2009, 2014; Satpute et al., 2010). E_{24} (%) is the *EI* determined 24 h after emulsion preparation. The emulsions were left at room temperature and their stability was evaluated over a period of 9 weeks, by periodically measuring the *EI*. The hydrophobic compounds tested included oils (corn oil, sunflower oil, olive oil, soybean oil, peanut oil, cedarwood oil, almond oil and paraffin oil, purchased at the local supermarket), and hydrocarbons (benzene, Riedel de Haën; toluene, Sigma; hexane, Sigma). For a total volume of 5 mL in the test tube (16 mm of diameter), different O/W were tested, namely, 4:1, 3:2, 2:3 and 1:4 (*v/v*), by varying the proportion of aqueous and organic phases.

5.3.3. Film-forming capacity

Films preparation

EPS_{CW}, EPS_{TP} and EPS_{GX} solutions (1.25 wt%) were prepared in deionised water. Glycerol (30 wt%, dry basis) was added as plasticiser. The solutions were left under stirring overnight at room temperature to assure complete dissolution. Next, a mass of 20 g of each mixture was transferred to plastic Petri dishes (diameter of 6.5 cm) and the solvent was allowed to evaporate at 30 °C. The formed films were lifted from the dishes' surface and conditioned at a controlled relative humidity of 45 % before testing.

Films Mechanical Properties

Tensile and puncture tests were performed using a TA-Xtplus texture analyser (Stable Micro Systems, Surrey, England) performed at temperature (T) = 22.0 ± 2.0 °C. The thickness of the tested film stripes/squares was measured using a digital micrometer (Mitutoyo, UK).

For the tensile tests, three film strips of each EPS (20 mm × 50 mm) were attached to tensile grips A/TG and stretched at 0.5 mm/s in tension mode. The tensile strength (stress) at break (TS, MPa) was calculated as the ratio of the maximum force to the films' initial cross-sectional area. The elongation (strain) at break (EB, %) was determined as the ratio of the extension of the sample upon rupture by the initial gage length. The Elastic Modulus (EM, MPa) was calculate through the slope of initial linear region of the stress-strain curve.

Puncture tests were performed by immobilizing three test film squares (20 mm × 20 mm) of each EPS on a specially designed base with a hole of 10 mm in diameter. The samples were compressed at a speed of 1.0 mm/s and punctured through the hole with a stainless steel cylindrical probe (2 mm diameter). The puncture strength (stress) at break (PS, kPa) was expressed as the ratio of the puncture strength (force, N) by the probe contact area (mm^2). The deformation (strain, %) at break was determined as the ratio of the deformation of the sample upon rupture by the initial gage length.

5.4. Results and Discussion

The EPS produced by *Enterobacter* A47 using cheese whey, EPS_{CW} (Chapter 2), out-of-specification tomato paste, EPS_{TP} (Chapter 3), and a glucose/xylose mixture, EPS_{GX} (Chapter 4) (Table 5.2), as substrates were subjected to a preliminary assessment of their solution properties in diluted and concentrated aqueous solutions, their potential to be used as emulsion forming or stabilizer agents and their ability to form films.

Table 5.2: Sugar composition, acyl groups, protein and inorganic salts content, average molecular weight (Mw) and polydispersity index (PDI) of EPS_{CW}, EPS_{TP} and EPS_{GX} produced by *Enterobacter* A47 (Fuc: fucose; Gal: galactose; Glc: glucose; GlcA: glucuronic acid).

EPS	Sugar composition (mol%)				Acyl groups content (wt.%)	Protein content (wt.%)	Inorganic salts content (wt.%)	Mw ($\times 10^6$ Da)	PDI
	Fuc	Gal	Glc	GlcA					
EPS_{CW}	29	21	21	29	32	22	3	1.8	1.2
EPS_{TP}*	37	27	23	12	13	7	4	4.4	1.2
EPS_{GX}	36	22	24	17	19	18	6	3.6	1.1

* Contained a pectin-like polysaccharide (12 wt.%) from the tomato paste feedstock.

5.4.1. Solution properties

Intrinsic viscosity

The intrinsic viscosity of EPS_{CW}, EPS_{TP} and EPS_{GX} was determined in 0.01 M NaCl. A good accuracy and linearity in the extrapolations to zero concentration was achieved for all the samples, in the Huggins and Kraemer plots (Figure 5.1). The values of the relative viscosity for each biopolymer's dilute solution (0.025 to 0.1 g dL^{-1}) were between 1.2 and 2.0.

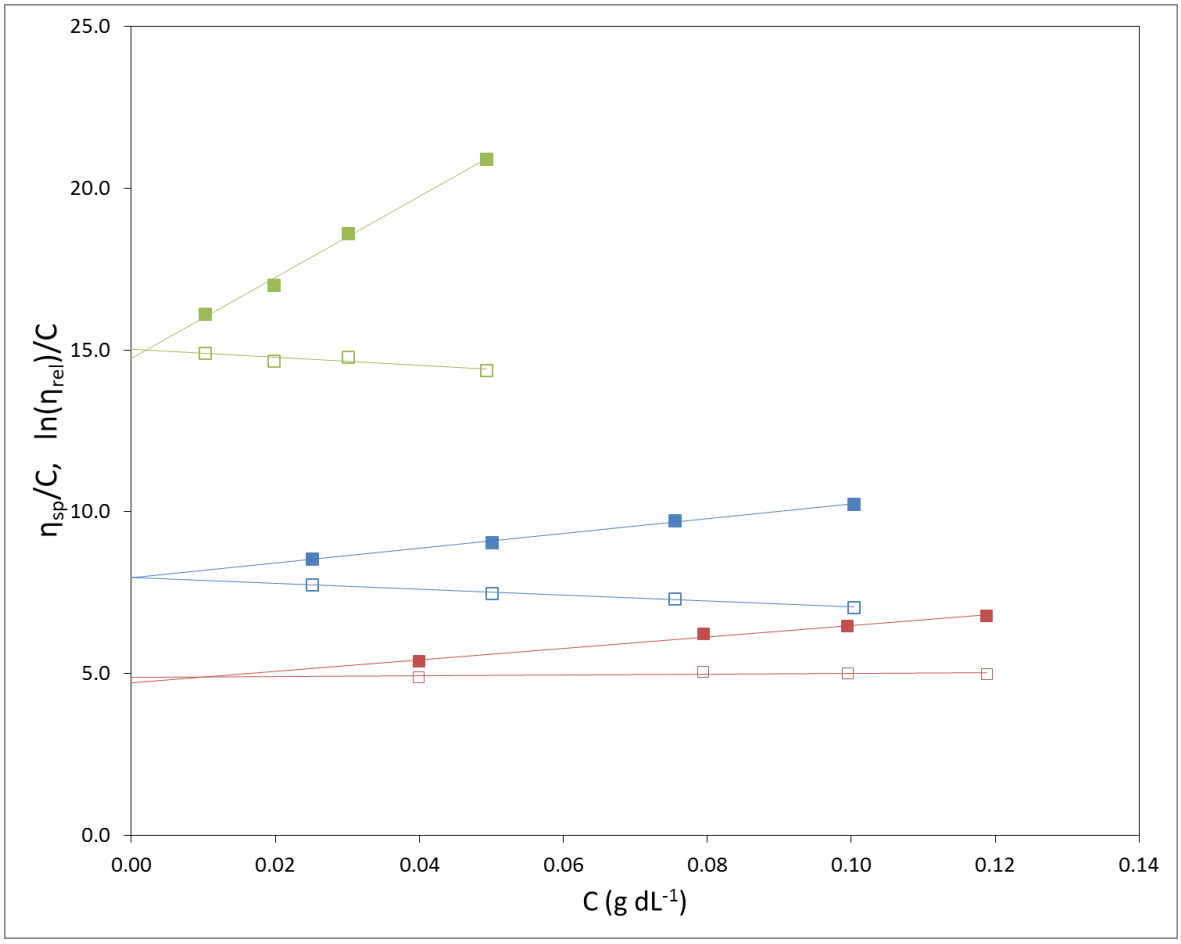

Figure 5.1: Determination of EPS_{CW} (blue), EPS_{TP} (red) and EPS_{GX} (green) intrinsic viscosity, in 0.01 M NaCl, using the Huggins (full symbols) and Kraemer (open symbols) equations.

Comparing the three polysaccharides, EPS_{TP} demonstrated the lowest intrinsic viscosity value (4.7 dL g^{-1}), while EPS_{GX} obtained the highest one (14.7 dL g^{-1}). These values mean that a single molecule of EPS_{GX} occupy more hydrodynamic volume at the given solvent than one molecule of EPS_{TP} (Freitas et al., 2011a). EPS_{CW} demonstrated to have an intrinsic viscosity value (8.0 dL g^{-1}) similar to that of other EPS synthesized by *Enterobacter* A47 using different carbon sources (6.2-7.5 dL g^{-1}) (Freitas et al., 2014). Nevertheless, all values are within the range of those reported for other commercial polysaccharides, such as xanthan and guar gum (5–50 dL g^{-1}) (Freitas et al., 2011a).

The Huggins constant, a parameter highlighting polymer–solvent interactions, was 0.36 for EPS_{CW}, 0.79 for EPS_{TP} and 0.60 for EPS_{GX}. Reminding that Huggins constant values from 0.25 to 0.5 are assigned to good solvents, whilst values above 0.5 to 1.0 are representative of poor solvents (Delpech et al., 2002), the actual Huggins constant for EPS_{CW} supports the hypothesis that this polymer should not be aggregated in the solvent system used. On the other hand, for

EPS_{TP} and EPS_{GX}, the Huggins constant values support the hypothesis that those two polymers might have formed aggregates and, hence, more adequate solvents should be investigated.

Apparent viscosity and viscoelastic properties

The apparent viscosity (η_a, Pa·s) of the different EPS solutions tested are presented in Figure 5.2A. All the flow curves (1.0 wt.%, in 0.01 M NaCl) show a shear thinning behavior. It can be observed that the polysaccharides produced using cheese whey (EPS_{CW}) and tomato paste (EPS_{TP}) have similar values of apparent viscosity (0.1 and 0.3 Pa·s, respectively, at a shear rate of 4 s^{-1}) but EPS_{GX} demonstrated a higher zero shear viscosity (η_0) approaching 15 Pa·s. For the range of shear rates studied, a well-defined first Newtonian plateau is observed for EPS_{CW}, while for the others EPS only an approaching of that plateau is perceived. A similar thickening capacity was observed for the EPS synthesized by *Enterobacter* A47 from xylose (η_0 = 0.08 Pa·s). For the same polymer concentration, ionic strength and pH values, the EPS produced from glycerol or glucose by the same bacterial strain imparted an apparent viscosity (0.27-0.34 Pa·s) similar to the EPS_{TP} (0.3 Pa·s) (Freitas et al., 2014). The high apparent viscosity observed for EPS using a mixture of glucose/xylose (15 Pa·s) is in line with the high intrinsic viscosity achieved for the same polymer.

Interestingly enough, the mechanical spectrum of 1.0 wt.% EPS solutions (Figure 5.2B) shows a high dependence of storage (*G*') and loss moduli (*G*'') with the frequency. For almost all frequency range of EPS_{CW} and EPS_{GX} mechanical spectra, the *G*'' values were higher than those of *G*', except at higher frequencies, where a cross-over is perceived at an angular frequency of about 6.3 and 3.2 Hz, respectively. These results are indicative of a liquid-like behaviour generally observed for entangled polymer chains solutions. The same behaviour was observed for the EPS produced by *Enterobacter* A47 from glycerol, glucose and xylose (Freitas et al., 2014). On the other hand, *G*' and *G*'' cross-over for EPS_{TP} was at lower frequencies (0.18 Hz), which could be related with the lower intrinsic viscosity obtained for this polymer.

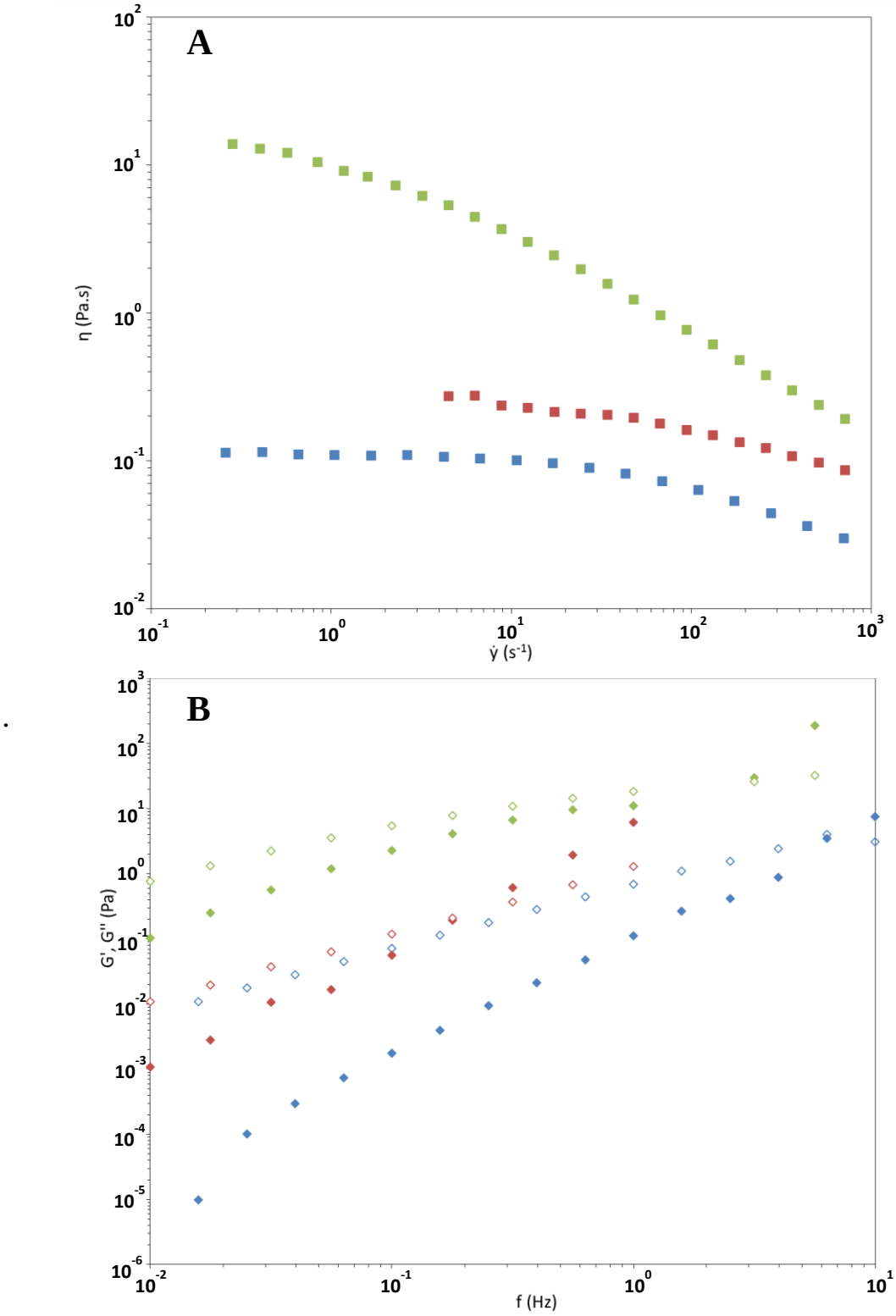

Figure 5.2: Flow curve (A) and mechanical spectrum (B) of 1.0 wt.% EPScw (blue), EPSTP (red) and EPSGX (green) solutions, ((full symbols - G', open symbols - G'').

Solution properties: Overall Perception

The resume table below (Table 5.3) gives an overall overview of the relevant parameters determine for each EPS to study their solutions properties.

Table 5.3: Resume table of the relevant parameters determined to study the EPS's solution properties.

EPS	[η] (dL g^{-1})	k_H	η_0 (Pa·s)	*f* of G' and G'' cross over (Hz)
EPS_{CW}	8.0	0.36	0.1	6.3
EPS_{TP}	4.7	0.79	0.3	0.18
EPS_{GX}	14.7	0.6	15	3.2

Though EPS_{CW}, EPS_{TP} and EPS_{GX} reveal different behaviour in aqueous solutions, they all show great potential to be used in applications as thickening agent in diverse aqueous formulations such as oil drilling fluids, paints, pharmaceuticals, cosmetics and food products.

5.4.2. Emulsion-Stabilizing Capacity

The EPS produced by *Enterobacter* A47 using cheese whey, EPS_{CW} (Chapter 2), out-of-specification tomato paste, EPS_{TP} (Chapter 3), and a glucose/xylose mixture, EPS_{GX} (Chapter 4) (Table 5.2), as substrates were subjected to a preliminary assessment of their potential to be used as emulsion forming or stabilizer agents.

Eleven hydrophobic compounds (Table 5.1) were selected to assess the polymers' ability to form emulsions at different oil/water ratios (*v/v*). The selected hydrophobic compounds included oils and hydrocarbons, representative of compounds used in different industrial and commercial emulsion applications. Vegetable oils, namely, corn oil, sunflower oil, olive oil, soybean oil and peanut oil, are normally used in food products and food processing. For example, olive oil is used in mayonnaise or vinaigrette manufacture (Gunstone, 2011; Thomas, 2002).

Some vegetable oils, such as sunflower oil and soybean oil, are also used in the cosmetic and ink industries, respectively (Gunstone, 2011; Thomas, 2002) (Table 5.1). Almond and cedarwood oils are used in cosmetic industry (Adams, 1991; Ahmad, 2010; Canellas and Saura-Calixto, 1988; faostat.fao.org), while paraffin oil is a mineral oil used in creams and pastes of pharmaceutical and cosmetic industries (Liu et al., 2006; J M L, 2002). Benzene, toluene and hexane are hydrocarbons used, for example, as primary components for glues and paints manufacture

(Aburto-Medina and Ball, 2015; Frazer et al., 1995; pubchem.ncbi.nlm.nih.gov; Saien et al., 2014).

The emulsion's forming and stabilizing capacity of the polymers was evaluated by determining the E_{24} for each hydrophobic compound, at different O/W ratios (*v/v*). Moreover, the stability of the obtained emulsions was evaluated by determining the *EI* over a period of 9 weeks.

EPS_{CW}

EPS_{CW} was able to stabilize the emulsions prepared with all the tested oils, although with differing efficiencies, which was also affected by the O/W ratio (Figure 5.3). According to Willumsen and Karlson (1996), a criterion for determining the emulsion-stabilizing capacity of an emulsifier consists on having an E_{24} of at least 50%. Considering that criterion, only some of the tested conditions resulted in good emulsification.

Specifically, EPS_{CW} was shown to have a good emulsification capacity for olive oil at the 4:1 (*v/v*) O/W ratio (E_{24} = 70 ± 2%), but the E_{24} decreased for lower O/W ratios (Figure 5.3). A similar E_{24} value (69%) was reported for emulsions formed with olive oil and the plant polysaccharide gum arabic, for an O/W of 2:3 (*v/v*) (Hifney et al., 2016) (Table 5.4). Lower values (59-65%) were reported for Fucoidan (Hifney et al., 2016), guar gum (Han et al., 2015) and CMC (Hifney et al., 2016), as well as for the bacterial polysaccharide GalactoPol (Freitas et al., 2009) and the EPS of *B. amyloliquefaciens* (Han et al., 2015) (Table 5.4).

For cedarwood and paraffin oils, on the other hand, the highest E_{24} (69 ± 0% and 72 ± 1%, respectively) were achieved for 3:2 (*v/v*) O/W ratio, decreasing to below 50% for lower O/W ratios (Figure 5.3). Similar profiles were shown for sunflower and peanut oils, with rather good emulsions ($E_{24} \cong 50\%$) being obtained only at the 3:2 (*v/v*) O/W ratio. For all other tested oils (corn, soybean and almond oils) and O/W ratios, the obtained emulsions were weak ($E_{24} < 50\%$). Cedarwood oil emulsions with EPS_{CW} had an E_{24} lower than those prepared with Fucoidan (78%) or gum arabic (84%). However, EPS_{CW} performed considerably better than FucoPol (20%), GalactoPol (30%), xanthan gum (30%), alginate (10%) or pectin (40%) (Table 5.4). The E_{24} obtained for paraffin oil emulsions with EPS_{CW} was similar to that of an EPS produced by the cyanobacterium *Nostoc flagelliforme* (72%) (Han et al., 2014) and for xanthan gum (64%) (Table 5.4).

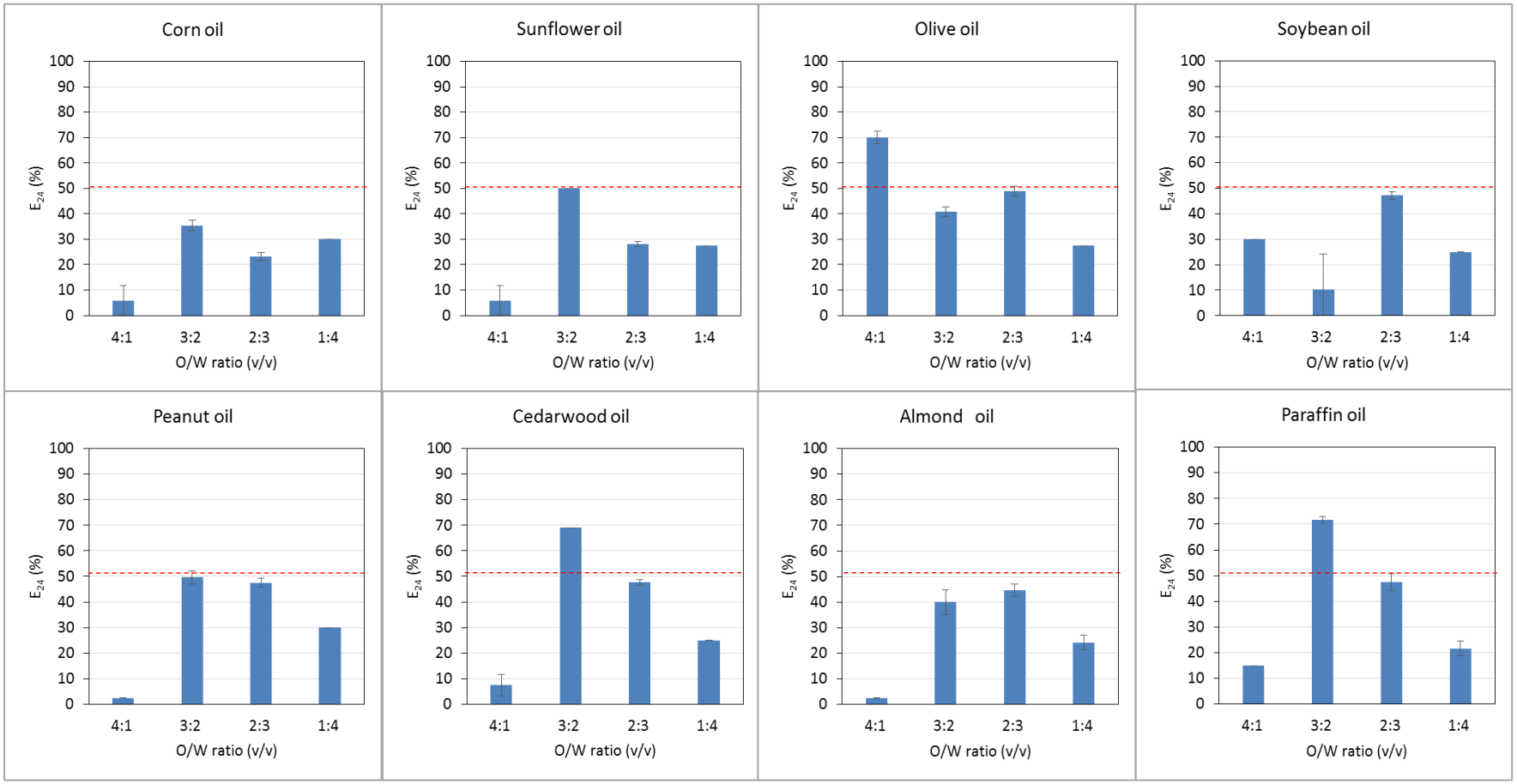

Figure 5.3: E_{24} for emulsions prepared with EPS_{CW} and different oils, at different O/W ratios (*v/v*).

Table 5.4: E_{24} comparison for emulsions prepared with several emulsifiers and different hydrophobic compounds.

Emulsifiers	Concentration (wt.%)	O/W ratio (*v/v*)	E_{24} (%)										
			Corn oil	Sunflower oil	Olive oil	Soybean oil	Peanut oil	Cedarwood oil	Paraffin oil	Benzene	Toluene	Hexane	References
EPS_{CW}	0.5	2:3	23	28	49	47	48	48	48	47	50	46	This work
		3:2	35	50	41	10	50	69	72	63	65	51	
EPS_{TP}		2:3	42	47	52	2	49	49	36	39	31	24	
		3:2	55	50	70	4	58	64	64	43	45	34	
EPS_{GX}		2:3	60	<10	64	96	62	81	64	67	73	25	
		3:2	<10	<10	<10	<10	61	80	79	32	53	24	
FucoPol (EPS-s)	0.5	2:3	64	60				20				30	Freitas et al., 2011a
		3:2		73									Freitas et al., 2014
EPS-g				69									
GalactoPol	0.8	3:2	<10	20	65			30	30	60	65	75	Freitas et al., 2009
EPS (*Nostoc flagelli-forme*)	0.45		82	80		43	74		72			27	Han et al., 2014
EPS 1 *(Bacillus amylo-liquefaciens LPL061)*	1.0			68	59		61						Han et al., 2015
EPS 2 *(Bacillus amylo-liquefaciens LPL061)*				70	60		66						
Xanthan gum	0.5	2:3	90	40				30				40	Freitas et al., 2011a
	0.45	3:2				30	16		64			24	Han et al., 2014
Alginate	0.5	2:3	40	56				10				10	Freitas et al., 2011a
Fucoidan			50	59	62			78		22	47		Hifney et al., 2016
Gum arabic	0.5	2:3				30	16		64			24	
Pectin			60	20				40				30	Freitas et al., 2011a
Guar gum	1.0	3:2		66	59		60						Han et al., 2015
Carboxymethyl cellu-lose (CMC)	0.5	2:3	62	59	59			0		0	0		Hifney et al., 2016
Triton X-100			82	80				80				70	Freitas et al., 2011a

Regarding the hydrocarbons tested, namely benzene, toluene and hexane, their emulsification capacity as a function of the O/W ratio had similar trends: the highest E_{24} values were achieved for the 3:2 (*v/v*) O/W ratios (63 ± 1%, 65 ± 1% and 51 ± 1%, respectively), decreasing to below 50% for lower or higher O/W ratios (Figure 5.4).

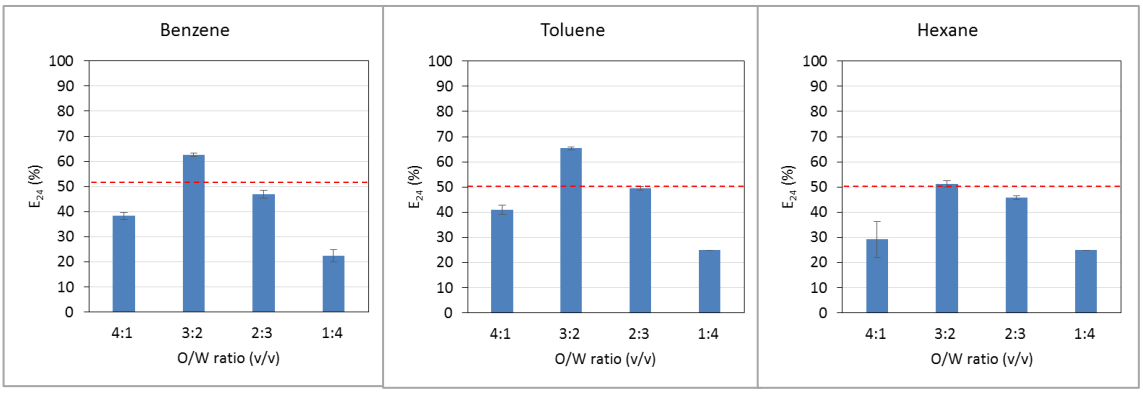

Figure 5.4: E_{24} for emulsions prepared with EPS_{CW} and different hydrocarbons, at different O/W ratios (*v/v*).

The results reveal that EPS_{CW} might replace some other emulsifiers due to its better performance with the tested hydrocarbons (Table 5.4). For example, the E_{24} obtained for benzene and toluene emulsions was considerably higher than that reported for Fucoidan, 22 and 47%, respectively (Hifney et al., 2016).

The *EI* was measured periodically during 9 weeks to infer about the emulsions' stability overtime, which is an important parameter for many applications. As expected, EPS_{CW} stabilizing capacity depended on the O/W ratio and was specific for some of the tested oils and hydrocarbons (Figure 5.5). The most stable emulsions were the ones prepared with EPS_{CW} and olive oil and cedarwood oil (Figure 5.5). For olive oil, the E_{24} at 4:1 (*v/v*) O/W ratio was high but the emulsion was stable for less than a week, while for the other ratios the emulsions were very stable, maintaining at least 50% of the initial *EI* during all the 9 weeks tested. EPS_{CW} maintained an almost constant *EI* for the emulsions prepared with cedarwood oil, at O/W ratios of 3:2 and 2:3 (*v/v*), during the entire test period. At the same O/W ratios, emulsions with paraffin oil demonstrated a good stability by maintaining an *EI* of over 50% of the E_{24} during at least 4 weeks.

Hydrocarbons emulsions demonstrated to be much less stable comparing with oils emulsions. Benzene and toluene emulsions were only stable for one week at high oil/emulsifier ratios and hexane emulsions were stable for less than a week at all ratios (Figure 5.5).

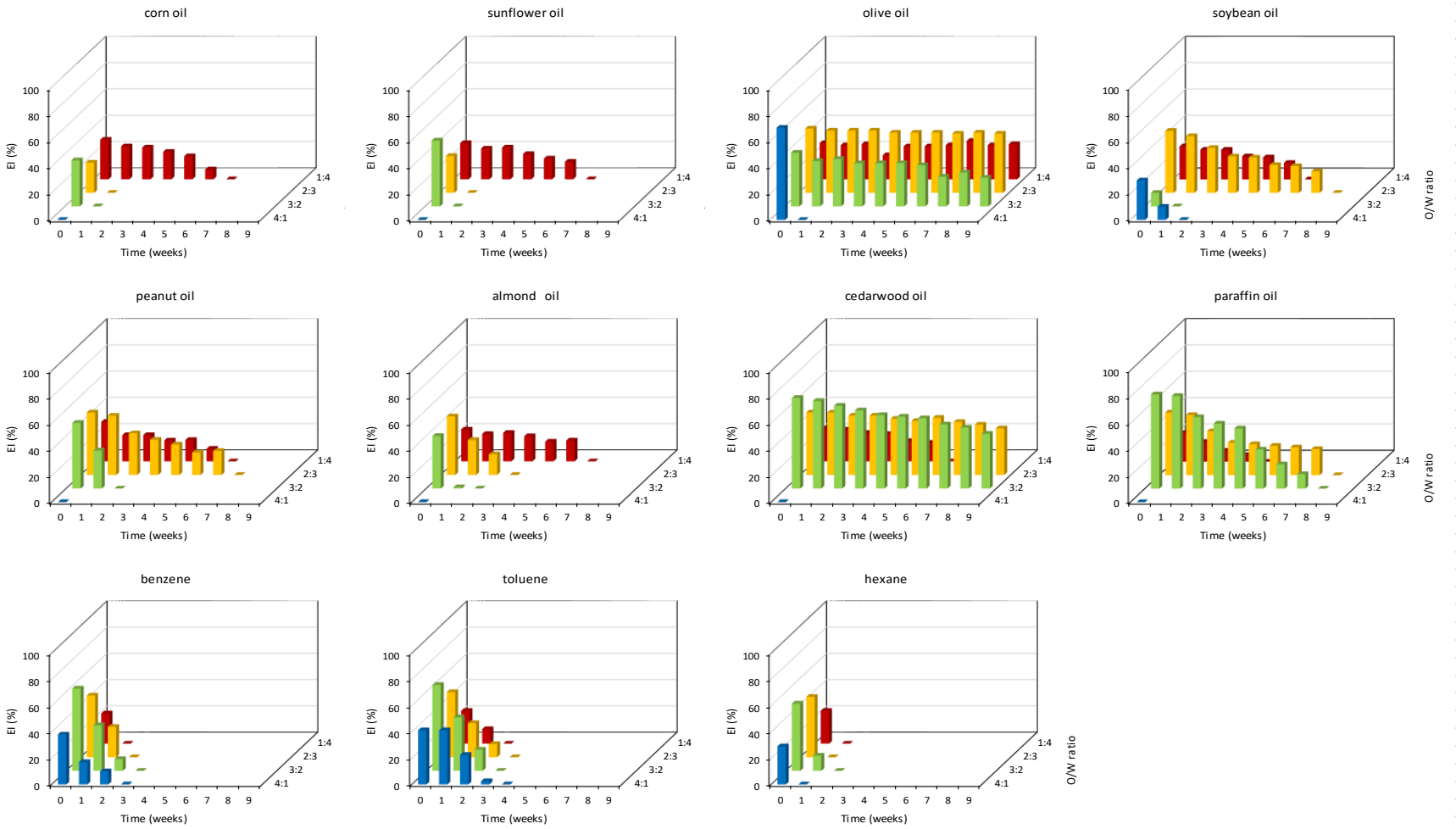

Figure 5.5: *EI* overtime for emulsions prepared with EPS_{CW} and different hydrophobic compounds, at different O/W ratios (*v/v*).

The distinct stability behaviour of the polymer for the different tested hydrophobic compounds might be related to the physico-chemical processes involved in emulsion formation. At lower oil/emulsifier ratios, the emulsion formation and stabilization is not so dependent on emulsifier concentration, but mainly controlled by the hydrodynamic processes of droplet disruption and subsequent emulsifier capacity of prevent coalescence, flocculation and/or Ostwald ripening phenomena (Calero et al., 2013; Dickinson, 2009). The emulsion formation and stabilizing capacity depends not only of environmental and process conditions, but also of emulsifier molecular and chemical properties (Chabrand et al., 2008; Dickinson, 2003, 2009). The good emulsification capacity of EPScw might be related to the presence of hydrophobic groups, such deoxysugars (e.g., fucose), uronic acids (e.g., glucuronic acid), acyl groups or the residual proteins (Han et al., 2014; Neu et al., 1992; Shepherd et al., 1995).

Comparing the chemical composition of the polysaccharides listed in Table 5.4, EPS_{CW} had the higher content in glucuronic acid (29 mol%), acyl groups (32 wt.%) and proteinaceous fraction (22 wt.%). Additionally, polysaccharides with higher molecular weight have better stabilizing capacity because contribute to form more viscous continuous phase (Maalej et al., 2016; Rossi and De Philippis, 2015).

In some industrial applications, such as beverages, pharmaceutics, paints, protective coatings, waxes and polishes, emulsions are required to be stable for extended periods of time. Therefore, a good emulsion stabilizer is necessary to guaranty a high shelf life and endurance to environmental stresses of a commercial product (Bouyer et al., 2012; Kosaric, 2001; Piorkowski and McClements, 2014). Thus, EPS_{CW} could be an alternative for emulsions using cedarwood and paraffin oils at 3:2 (*v/v*) O/W ratio, since the results demonstrated good emulsifying and stabilizing capacity (Figures 5.3 and 5.5).

On the other hand, some polymeric bioemulsifiers are able to break down the formed emulsion and are recognized as de-emulsifiers (Mnif and Ghribi, 2015). Therefore, less stable emulsions may be suitable for use in de-emulsification processes. For example, during the extraction of vegetable oils from theirs sources through aqueous extraction processing, the majority of the oil extracted is emulsified, being necessary a subsequent de-emulsification agent to increase the oil recovery (Chabrand et al., 2008). De-emulsification capacity is also needed before the transport and refining of crude oil, to reduce water content. This process prevents corrosion, scale formation and sludge accumulation in storage tanks (Mnif and Ghribi, 2015). For benzene and toluene emulsions at 3:2 (*v/v*) O/W ratio, EPS_{CW} could be the de-emulsification agent for this kind of application since had demonstrated good E_{24} but low stability over time (Figures 5.4 and 5.5).

EPS_{TP}

The EPS produced by *Enterobacter* A47 using out-of-specification tomato paste as substrate, named EPS_{TP}, demonstrated, in general, the ability to form strong emulsions with some of the tested oils (Figure 5.6). However, it demonstrated to have low emulsification capacity for the tested hydrocarbons (Figure 5.7).

EPS_{TP} demonstrated higher E_{24} for the emulsions prepared with peanut (74 ± 2%), and almond (68 ± 0%) oils comparing with EPS_{CW} which had values below 50% for the same oils. Similar performance between both polymers were achieved with corn, sunflower and paraffin oils, namely, the highest E_{24} values were attained at 3:2 (*v/v*) O/W (55 ± 0%, 50 ± 1% and 64 ± 1%, respectively) (Figures 5.3 and 5.6; Table 5.4). A high E_{24} value (70 ± 3%) was obtained for the olive oil emulsions but a lower EPS_{TP} emulsifier content was necessary compared to that of EPS_{cw} (E_{24} = 70 ± 2% for 4:1 (*v/v*) O/W ratio), (Figure 5.3 and 5.6). These values are higher than those obtained for emulsions prepared with GalactoPol (65%) (Freitas et al., 2009), EPS1 and EPS2 (*Bacillus amyloliquefaciens* LPL061) (59 and 60%, respectively) (Han et al., 2015) and guar gum (59%) (Han et al., 2015) for a 3:2 (*v/v*) O/W ratio.

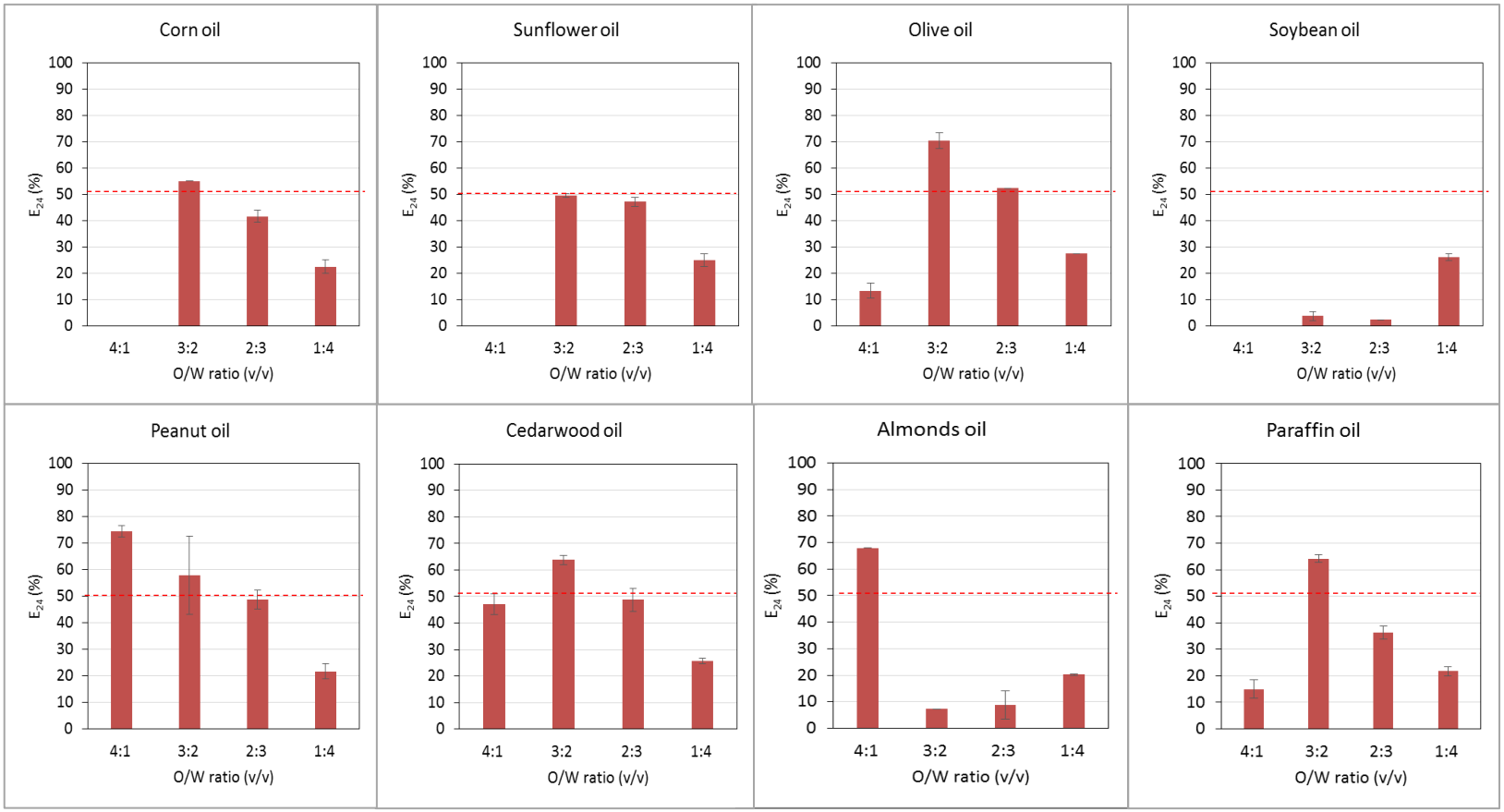

Figure 5.6: E_{24} for emulsions prepared with EPS_{TP} and different oils, at different O/W ratios (*v/v*).

For the hydrocarbons tested, EPS_{TP} demonstrated low emulsification capacity because the E_{24} was below 50% for all O/W ratios and all hydrocarbons (Figure 5.7).

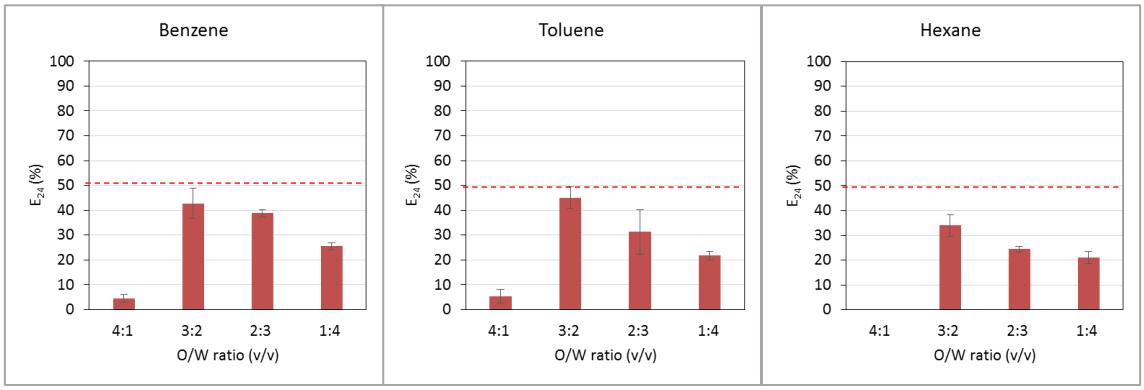

Figure 5.7: E_{24} for emulsions prepared with EPS_{TP} and different hydrocarbons, at different O/W ratios (*v/v*).

Similarly to EPS_{CW}, the most stable EPS_{TP} emulsions were the ones formed with olive oil and cedarwood oil, which maintained their *EI* practically unchanged during the 9 weeks of the test (Figure 5.8). Most of the other emulsions were broken within 1 week or less.

These results are probably related to the physico-chemical properties of EPS_{TP}, which has a pectin-like polysaccharide content of 12 wt.% (Table 5.3). The presence of the pectin-like polysaccharide has probably influenced the ability of the mixture to form and stabilize emulsions. Pectin, a plant cell wall polysaccharide, is increasingly being studied and gradually accepted as emulsifier/stabilizer in food applications (Dickinson, 2003; Ngouémazong et al., 2015). The emulsifying activity of pectin, as for other polysaccharides, is mostly related with its protein, feruloyl, and acetyl groups content, whereas the stabilizing capacity is predominantly attributed to the physico-chemical properties of its carbohydrate portion (Ngouémazong et al., 2015).

Comparing the E_{24} values obtained for EPS_{TP} emulsions prepared with sunflower (47±2%) and cedarwood (49±4%) oils with those using pectin (20 and 40%, respectively) (Freitas et al., 2011a) alone, at 2:3 (*v/v*) O/W ratio, we may conclude that EPS_{TP} performed better. The relatively lower results achieved with corn oil and hexane emulsions were possibly due to an obstruction of neutral sugar side chains to the accessibility of hydrophobic species to the oil/water interface (Ngouémazong et al., 2015). Furthermore, EPS_{TP} had the highest fucose content (37 mol%) among the tested EPS secreted by *Enterobacter* A47, and also the highest average molecular weight (4.4×10^6). However, it had the lowest intrinsic viscosity and glucuronic acid, acyl groups

and proteins content, which may have hampered the emulsion formation and subsequent stabilization through the increase of interface viscosity of the emulsion (Han et al., 2014; Maalej et al., 2016; Rossi and De Philippis, 2015)

Thus, EPS_{TP} could be used as natural emulsifier and stabilizer in food and cosmetic industries, since it stabilized emulsions with olive and cedarwood oils that revealed high stability during more than two months. On the other hand, EPS_{TP} could be used in applications using peanut and almond oils where is needed lower emulsions stability, such as oils extraction, since at 4:1 (*v/v*) O/W ratio the E_{24} were high but the emulsions break down a few hours later (Figure 5.8). Observing the results obtained using benzene, toluene and hexane, could be stated that the EPS_{TP} does not seem to have good emulsification/stabilizing abilities for applications using this type of hydrocarbons.

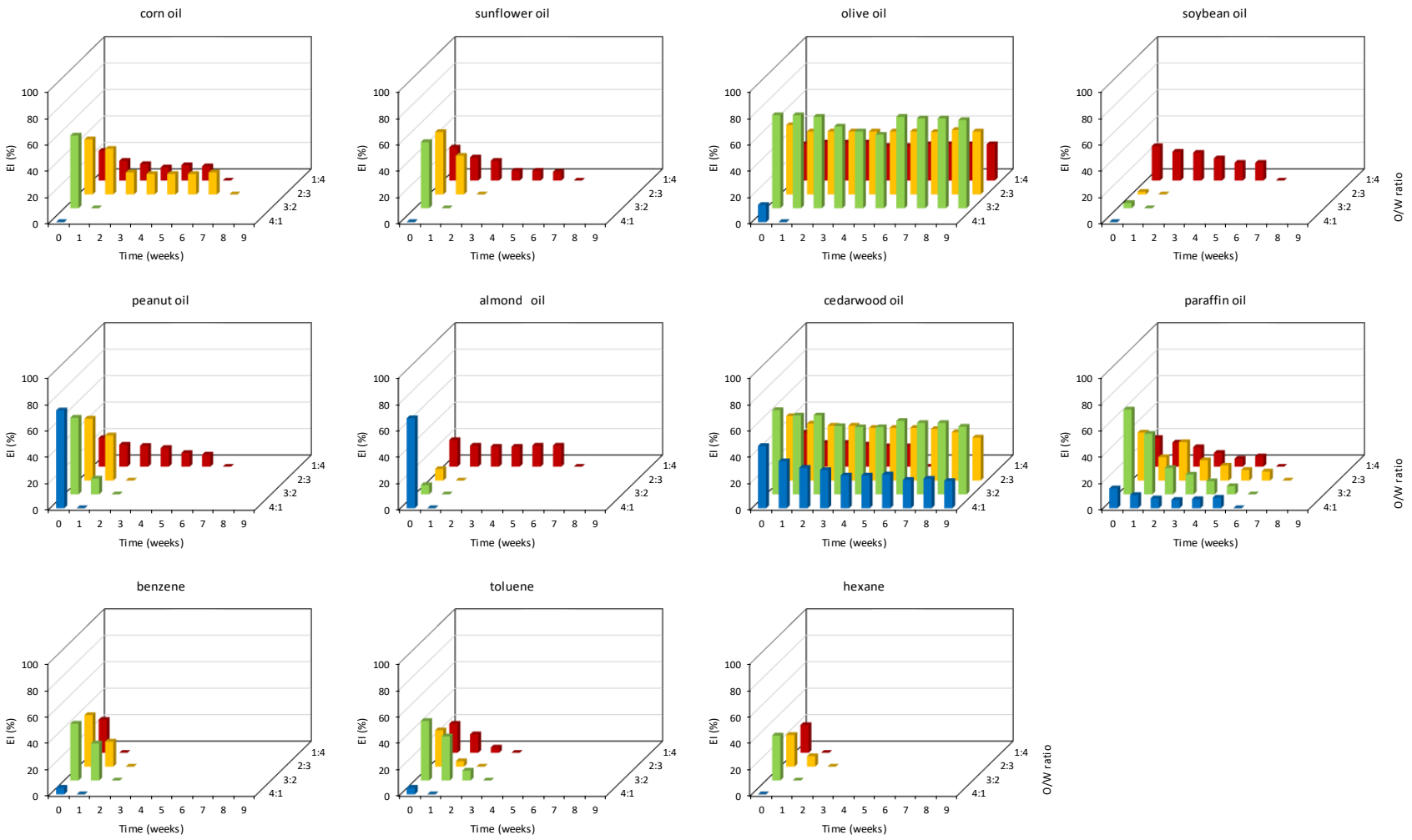

Figure 5.8: *EI* overtime for emulsions prepared with EPS_{TP} and different hydrophobic compounds, at different O/W ratios (*v/v*).

EPS_{GX}

The exopolysaccharide produced by *Enterobacter* A47 using glucose and xylose as substrates (EPS_{GX}) had an interesting behaviour, distinct from that of EPS_{CW} and EPS_{TP}. Indeed, EPS_{GX} demonstrated to have very strong emulsification capacity (E_{24} > 90%) with most of the tested oils, at low O/W ratios. The only exceptions were the emulsions formed with paraffin oil, for which the best E_{24} value was obtained at the 3:2 (*v/v*) O/W ratio (79 ± 0%), decreasing for lower ratios (Figure 5.9). For almost all the oils tested, the E_{24} value was higher than 95%, at 1:4 (*v/v*) O/W ratio, decreasing for higher ratios. For peanut oil, at all the ratios tested, EPS_{GX} formed very good emulsions (E_{24} > 61%). In the case of cedarwood oil emulsions, except for the 4:1 O/W ratio that formed a weak emulsion, all other tested O/W ratios resulted in very good emulsions with E_{24} values above 80%. The fact that only at lower O/W ratios were obtained higher E_{24} values could be related with the insufficient emulsifier concentration which may originated emulsion coalescence, flocculation and/or Ostwald ripening phenomena (Calero et al., 2013; Dickinson, 2009).

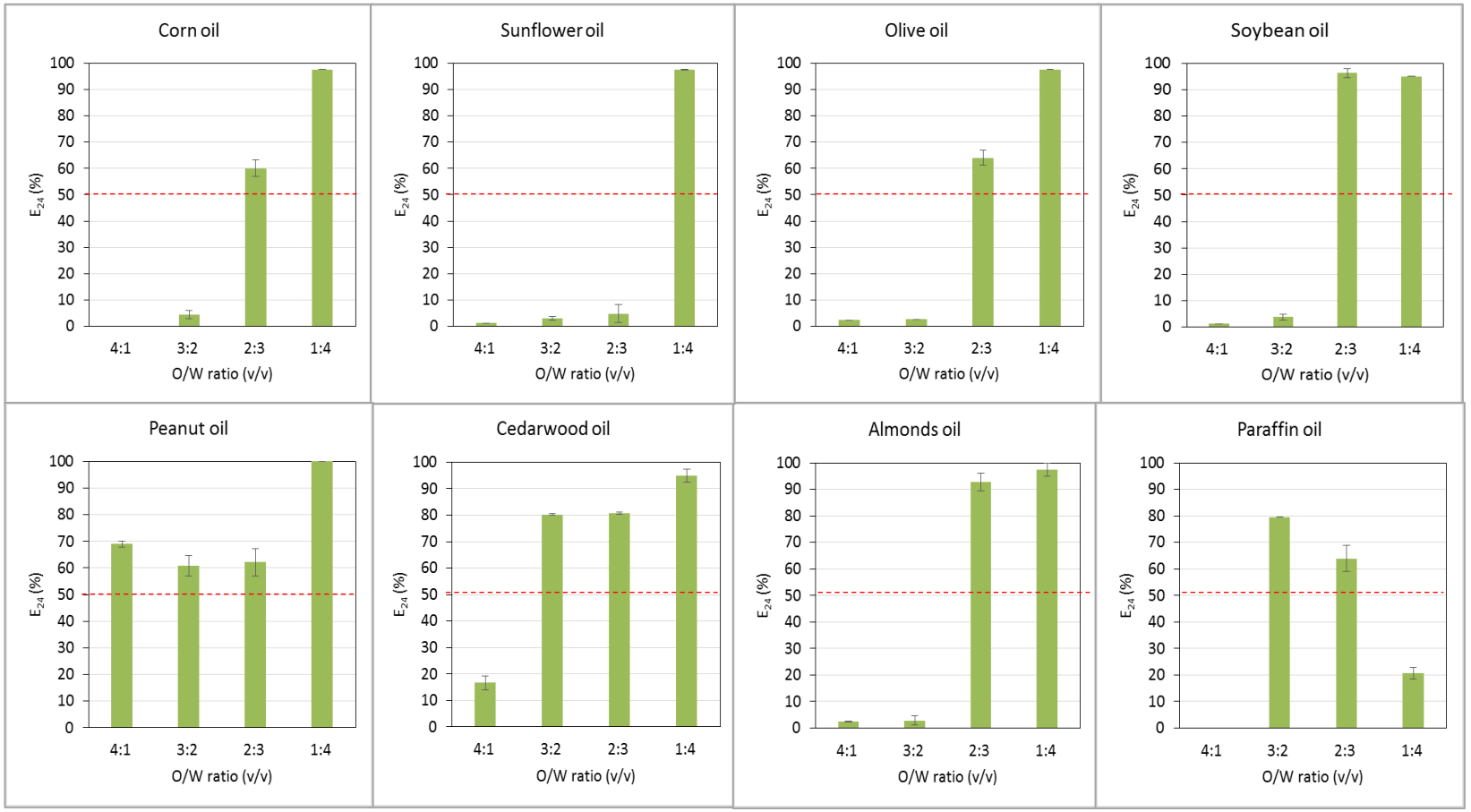

Figure 5.9: E_{24} for emulsions prepared with EPS_{GX} and different oils, at different O/W ratios (*v/v*).

Considering the tested hydrocarbons, EPS_{GX} only formed good emulsions with benzene, at a 2:3 (*v/v*) O/W ratio (E_{24} = 67 ± 6%), and with toluene, at the 2:3 and 3:2 (*v/v*) O/W ratios (E_{24} = 73 ± 4% and 53 ± 4%, respectively) (Figure 5.10).

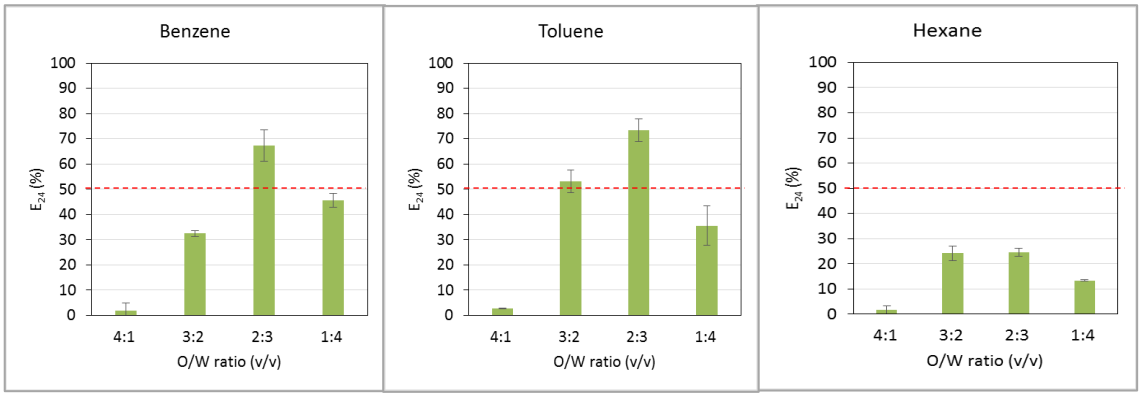

Figure 5.10: E_{24} for emulsions prepared with EPS_{GX} and different hydrocarbons, at different O/W ratios (*v/v*).

As shown in Table 5.4, the E_{24} values obtained for EPS_{GX} using olive oil, soybean oil, peanut oil, cedarwood oil, paraffin oil, benzene and toluene at 2:3 (*v/v*) O/W ratio were higher than the other studied *Enterobacter* A47 EPS and also than some emulsifiers at the same emulsion conditions (FucoPol, GalactoPol, xanthan gum, alginate, Fucoidan, gum arabic, pectin, guar gum and CMC). For example, the obtained E_{24} for cedarwood oil emulsions was so high (81±7%) that EPS_{GX} might replace the synthetic emulsifier Triton X-100 (80%). Also, noteworthy the emulsification capacity of EPS_{GX} for paraffin oil (79±0%) that were higher than that of xanthan gum (64%) (Han et al., 2014), GalactoPol (30%) (Freitas et al., 2009) and the polysaccharide synthesized by Cyanobacteria (72%) (Han et al., 2014). Moreover, EPS_{GX} demonstrated higher emulsification capacity for benzene and toluene with E_{24} values higher than Fucoidan (22%) and CMC (0%) (Hifney et al., 2016). The higher intrinsic viscosity (14.7 dL g^{-1}) and high protein content may have impacted on the E_{24} high values (Table 5.1), since, as explained above for EPS_{CW}, these two properties could improve the physico-chemical processes involved in emulsion formation (Dickinson, 2009; Maalej et al., 2016).

Despite the high E_{24} values observed for the EPS_{GX} emulsions, they were not very stable for a long period of time, since most of them broke within 1 week or less (Figure 5.11). The exceptions were olive and cedarwood oils, whose emulsions (at the O/W ratios of 2:3 and 3:2 (*v/v*), respectively) were stable (i.e., kept at least half their E_{24} value) for the entire test period (9 weeks). According that criterion, other stable emulsions were the ones formed with cedarwood oil (at the 3:2 (*v/v*) O/W ratio) and with paraffin oil (at the 3:2 and 2:3 (*v/v*) O/W ratios), which were stable for around 3 weeks (Figure 5.11).

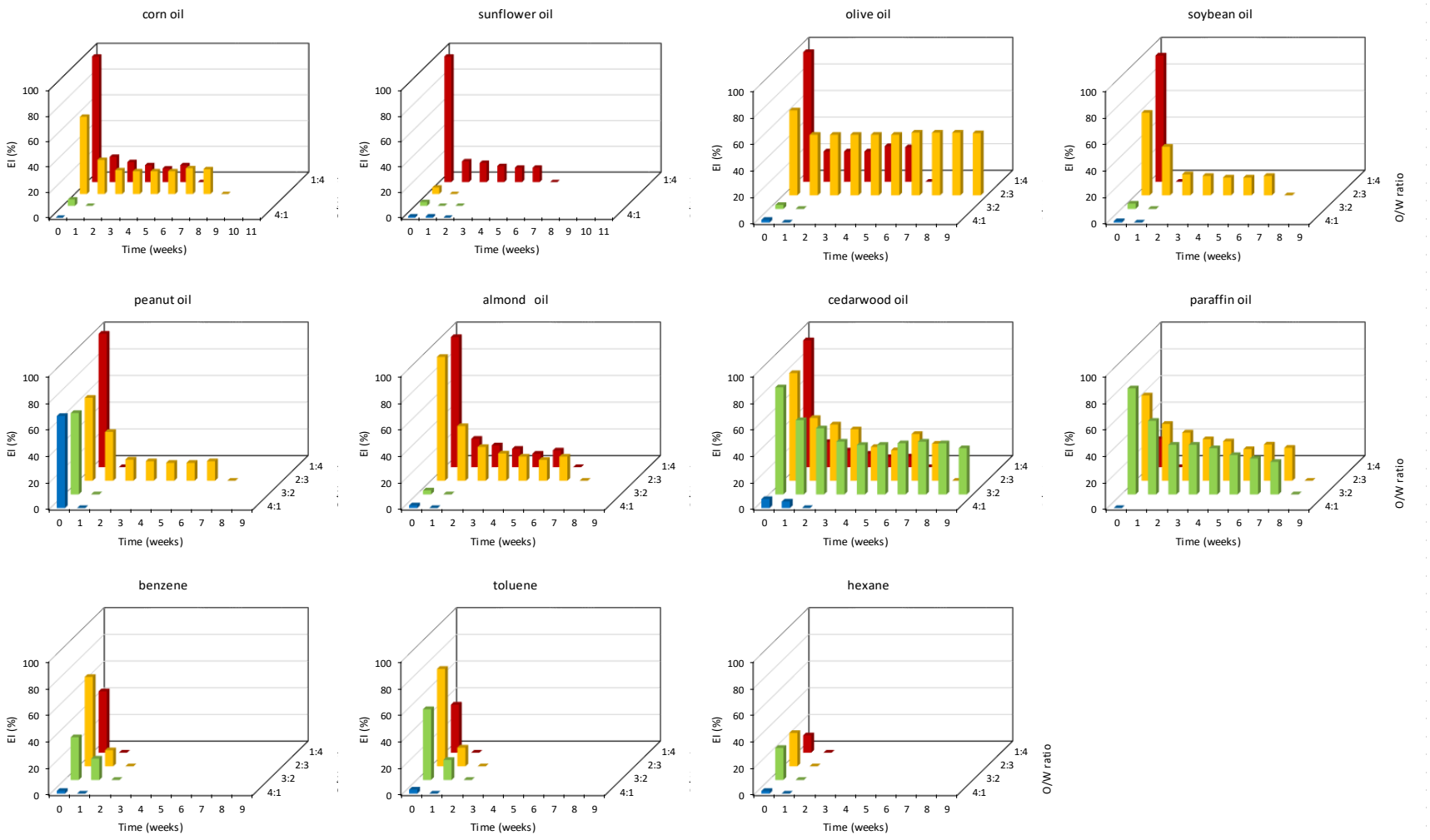

Figure 5.11: *EI* overtime for emulsions prepared with EPS_{GX} and different hydrophobic compounds, at different O/W ratios (*v/v*).

Regarding the strong hydrocarbon emulsions formed but with lower stabilization overtime, EPS_{GX} could be applied wherein it is desired that after their intended use, the emulsions are broken down into oil and water (de-emulsification), within a relatively short period of time (e.g. petroleum, bioremediation or oil extraction technologies) (Chabrand et al., 2008).

Emulsion-Stabilizing Capacity: Overall Perception

The resume table below (Table 5.5) gives an overall analysis of the ability of each EPS to form and stabilize emulsion with the different tested hydrophobic compounds.

The results reveal that EPS_{CW} might replace other emulsifiers that stabilize cedarwood and paraffin oils emulsions and could be a de-emulsification agent for processes using sunflower oil, olive oil, benzene and toluene emulsions since had demonstrated good E_{24} but low stability over time. EPS_{TP}, could be used as natural emulsifier and stabilizer in food and cosmetic industries, since it revealed high stability with olive and cedarwood oils emulsions and as a de-emulsification agent in applications using peanut and almond oils. Since EPS_{GX} demonstrates to be a great emulsifier for all the oils tested but a weak stabilizer, it could be used in petroleum industries, oil extraction technologies and bioremediation processes.

Table 5.5: Resume table classifying emulsions according to emulsifying index after 24h (E_{24}) and stability overtime of the EPS studied at different O/W ratios and different hydrophobic compounds. $E_{24} \geq 80\%$ ( ), 50 - 79% ( ), 20 - 49% ( ), < 20% ( ), stable 9 weeks (**VS**), stable 4 to 8 weeks (**S**), stable 3 to 1 week (**LS**) and stable less than a week (**UNS**).

EPS	O/W ratio (v/v)	Corn Oil	Sunflower Oil	Olive Oil	Soybean Oil	Peanut Oil	Cedarwood Oil	Almond Oil	Paraffin Oil	Benzene	Toluene	Hexane
EPS_{CW}	4:1	UNS	UNS	UNS	UNS	UNS	UNS	UNS	UNS	UNS	LS	UNS
	3:2	UNS	UNS	VS	UNS	LS	VS	UNS	S	LS	LS	UNS
	2:3	UNS	UNS	VS	S	S	VS	LS	S	LS	LS	UNS
	1:4	S	S	VS	S	S	S	S	LS	UNS	UNS	UNS
EPS_{TP}	4:1	UNS	UNS	UNS	UNS	UNS	VS	UNS	S	UNS	UNS	UNS
	3:2	UNS	UNS	VS	UNS	UNS	VS	UNS	LS	LS	LS	UNS
	2:3	LS	LS	VS	UNS	LS	VS	UNS	LS	LS	UNS	UNS
	1:4	LS	LS	VS	S	S	S	S	LS	UNS	LS	UNS
EPS_{GX}	4:1	UNS	UNS	UNS	UNS	UNS	LS	UNS	UNS	UNS	UNS	UNS
	3:2	UNS	UNS	UNS	UNS	UNS	S	UNS	S	LS	UNS	UNS
	2:3	LS	UNS	VS	LS	LS	LS	LS	LS	UNS	UNS	UNS
	1:4	UNS	UNS	UNS	UNS	UNS	UNS	UNS	UNS	UNS	UNS	UNS

5.4.3. Film-forming Capacity

In this Chapter, films prepared with EPS_{CW}, EPS_{TP} and EPS_{GX} were characterized. The formed films were transparent with a brownish tone and exhibited hygroscopic behaviour (Figure 5.12). Further investigation regarding their mechanical properties was developed through tensile and puncture tests.

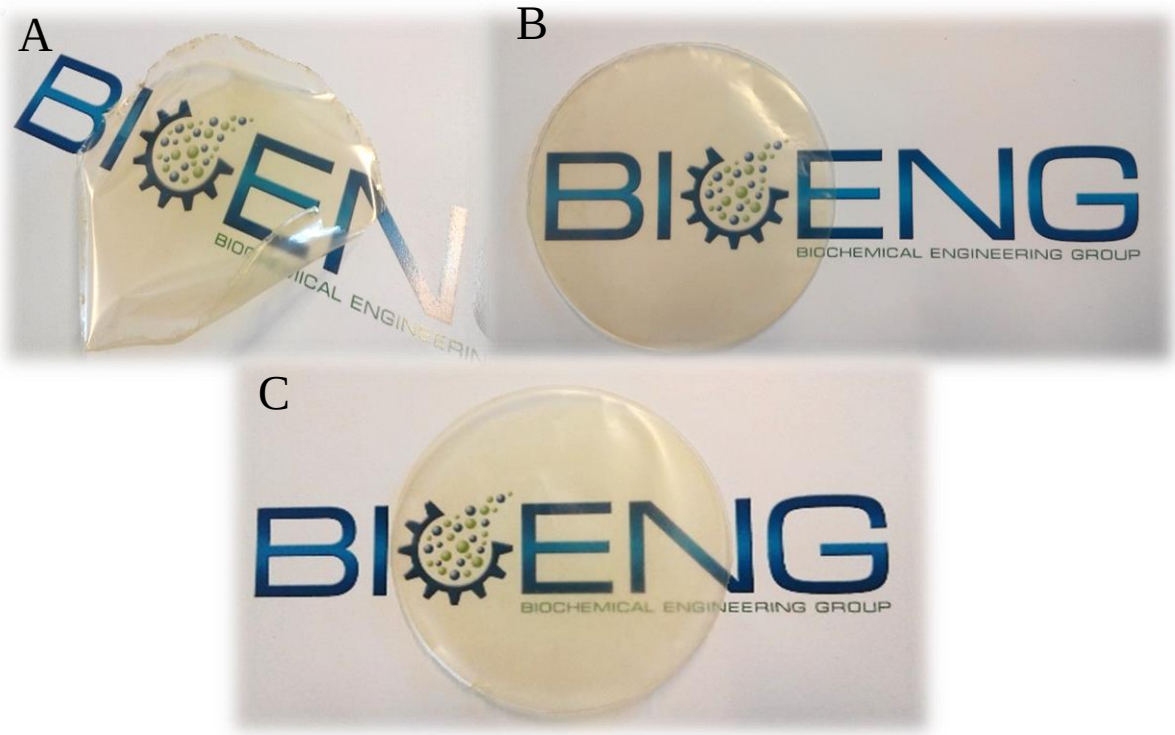

Figure 5.12: Photos of typical films from EPS_{CW} (A), EPS_{TP} (B) and EPS_{GX} (C).

Mechanical properties

The films prepared with EPS_{CW}, EPS_{TP} and EPS_{GX} were subjected to tensile and puncture tests, after being equilibrated at relative humidity values of 45% at 20 ± 2 °C. Characteristic stress–strain curve of tensile (A) and puncture tests (B) are presented in Figure 5.13 and the calculated parameters are shown in Table 5.5 and Table 5.6, respectively.

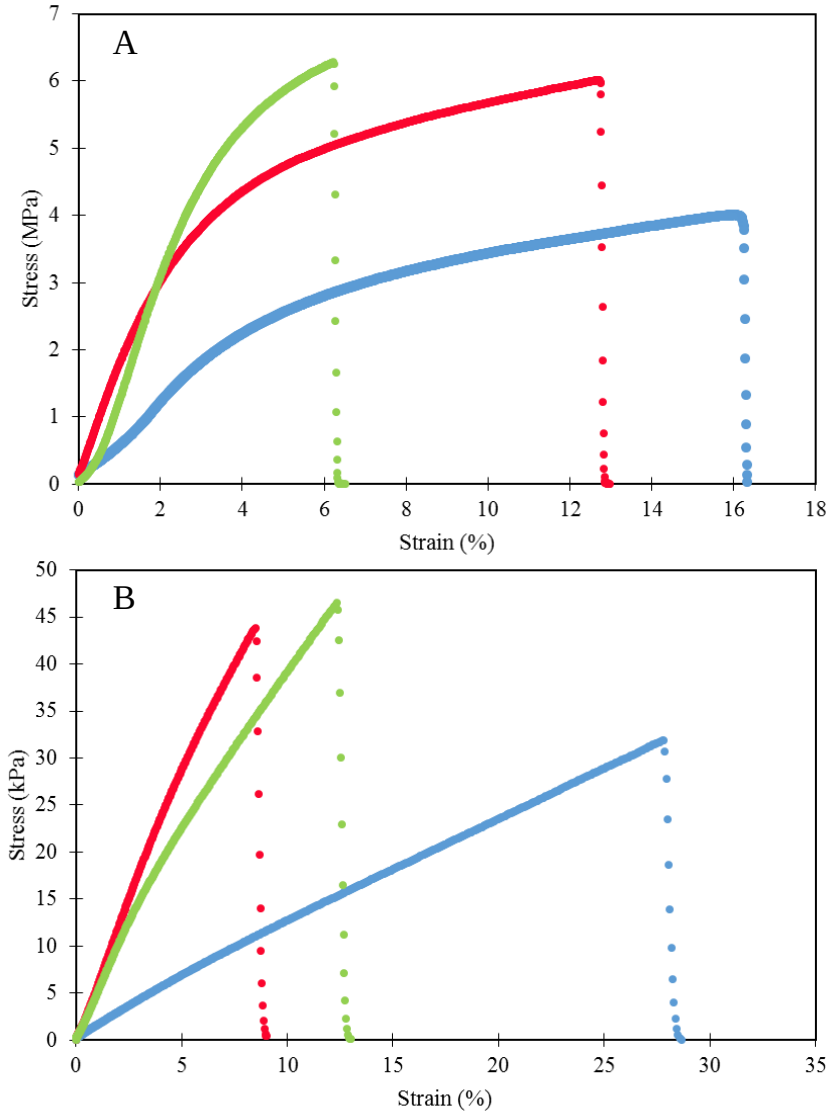

Figure 5.13: Typical stress–strain curves of the EPSCW (blue), EPSTP (red) and EPSGX (green) film samples for tensile tests (A) and puncture tests (B).

In the respective table, alongside with experimental results from tensile (Table 5.6) and puncture tests (Table 5.7), are the values referred in the literature for other polysaccharides' films from natural resources.

Table 5.6: Tensile tests results of films prepared with EPS synthesised by *Enterobacter* A47 with the different carbon sources and of other microbial polysaccharides referred in the literature (n.a. data not available).

Film	Additives	Tensile strength at break, TS (MPa)	Elongation at break, EB (%)	Elastic modulus, EM (MPa)	References
EPS_{CW}	0.3 g glycerol/g polymer	3.6 ± 0.4	18.6 ± 2.5	50.8 ± 1.0	This study
EPS_{TP}	0.3 g glycerol/g polymer	7.2 ± 1.2	12.0 ± 0.7	159.5 ± 2.1	
EPS_{GX}	0.3 g glycerol/g polymer	7.6 ± 1.2	9.8 ± 3.7	184.6 ± 28.4	
FucoPol	0.3 g glycerol/g polymer	9.8 ± 0.7	5.4 ± 0.6	322.0 ± 44.7	Freitas et al., 2014
EPS-g	0.3 g glycerol/g polymer	15.5 ± 0.3	8.1 ± 1.0	457.8 ± 32.3	
EPS-x	0.3 g glycerol/g polymer	3.8 ± 0.2	22.1 ± 4.3	14.5 ± 4.7	
FucoPol	0.5 g citric acid/g polymer	7.6 ± 2.2	6.6 ± 2.6	237.5 ± 43.7	Ferreira et al., 2016
Bilayer (FucoPol/Chitosan)	0.5 g citric acid/g FucoPol + (0.5 g citric acid/g Chitosan + 0.5 g glycerol/g Chitosan)	11.9 ± 6.2	38.4 ± 11.3	137.0 ± 36.8	
d/FucoPol-PES	0.67 g Genipin/g polymer	4.1 ± 0.0	4.5 ± 0.2	157.2 ± 14.0	Meireles et al., 2015
DU/FucoPol-PES	0.67 g Genipin/g polymer	3.1 ± 0.3	2.8 ± 0.4	155.6 ± 51.1	
GalactoPol	no	28.7 ± 2.6	2.4 ± 0.3	1275 ± 150	Meireles et al., 2013
d/GalactoPol-PES	0.67 g Genipin/g polymer	3.5 ± 0.4	18.9 ± 4.4	59 ± 3	
DU/GalactoPol-PES	0.67 g Genipin/g polymer	6.8 ± 1.6	3.5 ± 1.3	254 ± 20	
Pectin	0.3 g glycerol/g polymer	10	4	n.a.	Esposito et al., 2016
Gelatin	0.3 g glycerol/g polymer	20	70	n.a.	Rivero et al., 2010
Pullulan	0.3 g glycerol/g polymer	1	675	1	Trovatti et al., 2012
Corn starch	0.3 g glycerol/g polymer	16.1 ± 5.3	20.9 ± 7.1	n.a.	Jost and Stramm, 2015
Alginate	0.3 g glycerol/g polymer	15.6 ± 1.0	29.1 ± 2.3	n.a.	
κ-Carrageenan	0.3 g glycerol/g polymer	24.4 ± 1.8	15.6 ± 3.9	n.a.	Cerqueira et al., 2014
Chitosan	0.33 g glycerol/g polymer	7.7 ± 0.8	71.8 ± 4.3	n.a.	Cerqueira et al., 2012
Galactomannan	0.33 g glycerol/g polymer	13.2 ± 0.7	11.9 ± 0.5	n.a.	

Table 5.7: Puncture tests results of films prepared with EPS synthesised by *Enterobacter* A47 with the different carbon sources and of other microbial polysaccharides referred in the literature (n.a. data not available).

Film	Additives	Test probe	Puncture strength at break (kPa)	Deformation at break (%)	Force at break (N)	Distance at break (mm)	References
EPS_{CW}	0.3 g glycerol/g polymer	cylindrical (2 mm of diameter)	29.5 ± 4.8	28.4 ± 0.6	2.3 ± 0.4	3.8 ± 0.2	This study
EPS_{TP}			42.2 ± 1.6	9.4 ± 0.9	3.2 ± 0.2	2.1 ± 0.2	
EPS_{GX}			52.0 ± 3.2	13.6 ± 2.0	4.1 ± 0.2	2.7 ± 0.2	
GalactoPol	no	cylindrical (2 mm of diameter)	2959 ± 261	3.8 ± 0.6	n.a.	n.a.	Alves et al., 2011
Calcium alginate	no	hemispherical tip (3.5 mm of diameter)	25	2.5	n.a.	n.a.	Crossingham et al., 2014
Cellulose acetate	no	cylindrical (5 mm of diameter)	n.a.	n.a.	23.4 ± 1.2	2.7 ± 0.8	Santos et al., 2016
Chitosan lactate	0.6 g sorbitol/g polymer	hemispherical tip (2 mm of diameter)	1.4 ± 0.2	n.a.	n.a.	n.a.	Kowalczyk et al., 2015
Oxidized potato starch	0.6 g sorbitol/g polymer		4.2 ± 0.2	n.a.	n.a.	n.a.	
Gelatin	0.6 g sorbitol/g polymer		14.3 ± 0.3	n.a.	n.a.	n.a.	
Gellan	0.67 g glycerol/g polymer	cylindrical (8 mm of diameter)	n.a.	n.a.	34.3	7.3	Yang and Paulson, 2000

The results show that comparing the three different polymers, EPS_{CW} films elongate more (EB=18.6 ± 2.5%) but have lower tension at break (3.6 ± 0.4 MPa), which results in a lower EM (50.8 ± 1.0 MPa). On the other hand, EPS_{TP} and EPS_{GX} films presented a slightly higher TS (7.2 ± 1.2 and 7.6 ± 1.2 MPa, respectively) and EM (159.5 ± 2.1 and 184.6 ± 28.4 MPa) but EPS_{GX} films were slightly stiffer (EB=9.8 ± 3.7 MPa). In terms of puncture tests, the behaviour was similar for all tested films, although EPS_{TP} demonstrated to be slightly more rigid because it showed the lowest deformation at break (9.4 ± 0.9%).

These differences in terms of the films' mechanical properties might be related to the biopolymers chemical composition, as EPS_{CW} had higher glucuronic acid (29 mol%) and acyl groups content (32 wt.%) (Table 5.2). Moreover, EPS_{CW} and EPS-x have similar mechanical properties results (TS and EB) envisaged to be also correlated to the similar lower average molecular weight of both polymers, and to possible differences on the polymer inter-chain interactions within the films matrices (Tables 5.2 and 5.6).

It is difficult to compare the mechanical properties of the obtained films and the ones already described in other works because they are strongly dependent on the addition and concentration of plasticizers, cross-linking agents and other additives upon film formation. Thus, for a similar plasticizer concentration (0.3 g glycerol/g polymer), the films developed in this study, demonstrated results of the same order of magnitude as those referred for pectin and Galactomannan (Table 5.6). On the other hand, gelatin, Pullulan, Corn starch, Alginate, κ-Carrageenan and Chitosan demonstrate higher elongation at break (EB) (Table 5.6). Comparing with films prepared with different plasticizers, it can be noticed that the GalactoPol-PES film with 0.67 g of Genipin/g polymer reveal mechanical properties very similar to the EPX_{CW} film, demonstrating to be more malleable films. Moreover, EPS_{GX} revealed tensile properties similar to FucoPol with 0.5 g citric acid/g polymer as cross-linking and plasticizer agent but with lower EM (Table 5.6). For a comparison of puncture test results from the EPS films formed using EPS from *Enterobacter* A47 with other polysaccharides, is notice a similarity with the value obtained of puncture strength at break for gelatin film with 0.6g sorbitol/g polymer as plasticizer (14.3 ± 0.3 kPa).

In conclusion, the mechanical properties of EPS films produced from EPS_{CW}, EPS_{TP} and EPS_{GX}, reveal a polymeric structure that is not mechanically resistant enough to be used as a stand-alone film. However, there is the potential to be used as a hydrophilic layer incorporated in a multi-layered material.

5.5. Conclusions

The EPS produced by *Enterobacter* A47 using cheese whey (EPS_{CW}), out-of-specification tomato paste (EPS_{TP}) and a mixture of glucose/xylose (EPS_{GX}) as substrates, have revealed interesting aqueous solutions properties, the ability to form and stabilize emulsions, and to form films with good mechanical characteristics. In conclusion, the EPS produced by *Enterobacter* A47 using industrial wastes/by-products as substrate, may be a sustainable substitute of synthetic and natural thickening agents, emulsifiers/stabilizers in several industries processes and final products, such textile, paper, polymers, plastics, cosmetics, pharmaceuticals, food and petrochemicals. Although the mechanical tests shown that the films produced are not mechanically resistant enough to be used as a stand-alone film, there is the potential to be used as a hydrophilic layer incorporated in a multi-layered material.

CHAPTER 6

General Conclusions and Future Work

6.1. General Conclusions

The studies performed during this Ph.D. thesis evaluated the capacity of *Enterobacter* A47 to growth using a wide range of industrial by-products/wastes as substrates, the production of EPS and their physico-chemical properties.

In particular, cheese whey was converted into a novel fucose- and glucuronic acid-rich polysaccharide by the bacterium *Enterobacter* A47. One interesting feature of this bioprocess is the use of a culture that is able to directly use lactose, without any pretreatment, as substrate. The EPS produced, EPS_{CW}, is a novel value-added polysaccharide with two bioactive sugar monomers, fucose and glucuronic acid, which confers it a huge potential for application in different areas, including cosmetics, pharmaceuticals and food products. Also, it was the first time that out-of-specification tomato paste was used as the sole substrate for the production of a microbial polymer. Tomato paste proved to be an adequate source of nutrients, including sugars, ammonium and phosphate, for *Enterobacter* A47 growth and exopolysaccharide synthesis. The best bioprocess performance, in terms of polymer production and volumetric productivity, was higher for the continuous mode operation, which guarantied non limiting availability of carbon and nutrients. Further, *Enterobacter* A47 demonstrated the capacity to use as substrate a mixture of glucose/xylose sugars. The preliminary results of the cultivation of *Enterobacter* A47 using for the first time a hydrolysate from an abundant lignocellulosic by-product, BSG, as substrate, indicates that the culture as the ability to growth and produce EPS.

Regarding EPS's physico-chemical properties of the EPS produced by *Enterobacter* A47 using cheese whey (EPS_{CW}), out-of-specification tomato paste (EPS_{TP}) and a mixture of glucose/xylose (EPS_{GX}) as substrates, have revealed interesting properties, the ability to form and stabilize emulsions, and to form films with good mechanical characteristics and they may be a sustainable substitute of synthetic and natural thickening agents, emulsifiers/stabilizers in several industries processes and final products, such textile, paper, polymers, plastics, cosmetics, pharmaceuticals, food and petrochemicals. Although the mechanical tests shown that the films produced are not mechanically resistant enough to be used as a stand-alone film, there is the potential to be used as a hydrophilic layer incorporated in a multi-layered material.

Taking into account the versatile properties presented, combined to the prospective lower production costs from using a low-cost carbon source, The EPS has an enormous potential to be used on several industrial applications. Furthermore, the use of food industrial wastes/by-products to produce value-added EPS could constitute a new step for implementation of a more sustainable industrial production, which EPS could be applicable on the final product and/or packaging.

6.2. Future Work

Based on the work developed and on the results obtained, the following suggestions for future work can be proposed:

- Further investigation of the EPS production using the tested industrial by-products as substrate, in terms metabolic pathway in order to better understand the EPS synthesis process and optimize stoichiometric and kinetic parameters;
- Study of more cost-effective saccharification methods for BSG, such as, enzymatic hydrolysis with cellulases and hemicellulases that also could generate less inhibitory compounds for cell growth.
- Improvement of EPS recovery methods, to increase the purity and yield of the *Enterobacter* A47 EPS, while taking into consideration possible industrial applications. For example, the use of membranes with higher cut-off than the contaminant polysaccharide's Mw.
- Further studies of the EPS properties to better understand the best application for each EPS produced, such as, thermal properties, solubility and stability in several solvents, the influence of temperature, pH and ionic strength on the viscosity and viscoelasticity of EPS solutions, gel forming capacity, flocculating and/or suspending agents.
- The biocompatibility of the EPS should be assessed by cytotoxic assays. The biological activity may also be evaluated, as well as the antioxidant properties, anti-inflammatory effect and antimicrobial activity.

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