Cristiana Mateus Tavares
Bachelor's degree in Biology

Bioproduction of high-value products from novel soil and plant-associated bacteria

Dissertation for Master's degree in Biotechnology

Supervisor: Doctor Cristiana Andreia Vieira Torres,
UCIBIO-REQUIMTE, FCT-UNL

Co-supervisor: Doctor Francisco Xavier Inês Nascimento,
Postdoc researcher, FCT-UNL

Jury

President: Doctor Isabel Maria Godinho de Sá Nogueira

Examiner: Doctor Alexandra Peregrina Lavín

Vogal: Doctor Cristiana Andreia Vieira Torres

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Abstract

Nowadays, there is a great demand for natural, renewable and sustainable products that could replace those on the market, which are responsible for harming the environment. To this end, efforts have been made to find these substitutes and, at the same time, to make them economically viable. A good alternative are bacteria, as they are able to produce different substances with unique characteristics, are easy to manipulate allowing to obtain tailor-made products and allow an easy control of their production conditions.

This work was focused on the study of five new marine bacteria (*Marinomonas* sp. A3A, *Pantoea cypripedi* NE1, *Pseudomonas arsenicoxydans* ACM1, *Pseudomonas mandellii* ACM7 and *Pseudomonas* sp. REST10) isolated from different sites and environments, with a possible potential for the production of value-added products, namely polyhydroxyalkanoates (PHA) and exopolysaccharides (EPS).

The main objective was to study and maximize the growth and production of biopolymers by the different bacteria. To this end, tests were conducted in Erlenmeyer flasks to determine the best environmental conditions and composition of the medium.

In these assays, two rich media (TSB and LB) and one simple medium (E media) were evaluated. Different temperatures, salt and glucose concentrations were tested. All bacteria demonstrated the ability to grow and produce EPS and PHAs. TSB medium seemed the most promising to proceed in the reactor tests. The bacteria with the best results, namely those of the genus *Pseudomonas*, were selected for cultivation in batch mode reactors.

The study showed that *Pseudomonas arsenicoxydans* ACM1 grown on TSB medium achieved the highest EPS value (5.33 g/L) and *Pseudomonas Mandellii* ACM7 presented the highest CDW value (10.7 g/L) when grown on the same medium at 15ºC. The bacteria were able to produce PHAs and exopolysaccharides with rare sugars in their composition, showing potential to reach the market.

Keywords

Pseudomonas Mandellii ACM7; *Pseudomonas arsenicoxydans* ACM1; *Marinomonas* sp. A3A; *Pantoea cypripedi* NE1; *Pseudomonas* sp. REST10; Exopolysaccharides.

Resumo

Atualmente, existe uma grande procura por produtos naturais, renováveis e sustentáveis que substituam os existentes no mercado, responsáveis por prejudicar o ambiente. Para tal, têm sido realizados esforços para conseguir encontrar estes substitutos e que, ao mesmo tempo, consigam ser economicamente viáveis. Uma boa alternativa são as bactérias, capazes de produzir diferentes substâncias com características únicas e fáceis de manipular, permitindo que os produtos obtidos satisfaçam as necessidades desejadas, além de possibilitarem um fácil controlo das suas condições de produção.

Com este intuito, o trabalho centrou-se no estudo de cinco novas bactérias marinhas (*Marinomonas* sp. A3A, *Pantoea cypripedi* NE1, *Pseudomonas arsenicoxydans* ACM1, *Pseudomonas mandellii* ACM7 e *Pseudomonas* sp. REST10) isoladas de diferentes locais e ambientes com potencialidade para a produção de produtos de valor acrescentado, nomeadamente, polihidroxialcanoatos (PHA) e exopolissacáridos (EPS).

O principal objetivo foi estudar e maximizar o crescimento e a produção dos biopolímeros pelas diferentes bactérias. Para tal, foram realizados ensaios em Erlenmeyer para determinar quais as melhores condições ambientais e composição do meio.

Nestes ensaios, testaram-se dois meios ricos (TSB e LB) e um meio simples (meio E). Foram testadas diferentes temperaturas, concentrações de sal e de glucose. Todas as bactérias demonstraram capacidade para crescer e produzir EPS e PHAs. O meio TSB pareceu o mais promissor para prosseguir os ensaios em reator. De seguida, selecionaram-se as bactérias com os melhores resultados, nomeadamente as do género *Pseudomonas,* para serem cultivadas em reator em modo batch.

O estudo mostrou que a bactéria *Pseudomonas arsenicoxydans* ACM1 cultivada em meio TSB obteve o maior valor de EPS (5.33 g/L) e a *Pseudomonas Mandellii* ACM7 apresentou o maior valor de CDW (10.7 g/L) quando cultivada com o mesmo meio a 15ºC. As bactérias foram capazes de produzir PHAs e exopolissacáridos com açúcares raros na sua composição, demonstrando ter potencial para entrar no mercado.

Palavras chave

Pseudomonas Mandellii ACM7; *Pseudomonas arsenicoxydans* ACM1; *Marinomonas* sp. A3A; *Pantoea cypripedi* NE1; *Pseudomonas* sp. REST10; Exopolissacarídos.

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CDW – Cell Dry Weight

DO – Dissolved Oxygen

EPS – Exopolysaccharide

FF – Feast and Famine

GC – Gas Chromatography

HB - Hydroxybutyrate

HD – Hydroxydecanoate

HDd –Hydroxydodecanoate

HHx – Hydroxyhexanoate

HO – Hydroxyoctanoate

HPLC – High Performance Liquid Chromatography

HTd – Hydroxytetradecanoate

HV – Hydroxyvalerate

LAB – Lactic Acid Bacteria

LB - Luria-Bertani

Mcl-PHA – Medium-chain-length Polyhydroxyalkanoates

MMC –Mixed Microbial Culture

N - Nitrogen

OD –Optical Density

PHA – Polyhydroxyalkanoates

PHB - Polyhydroxybutyrate

RI – Refractive Index

Scl-PHAs – Short-chain-length Polyhydroxyalkanoates

SLPM – Standard Liters Per Minute

TSB - Tryptic Soy Broth

Wt % - Weight Percent

Chapter 1 :
Introduction and Motivation

1.1. Microbial diversity and its importance

Bacteria are the most diverse and abundant microbial lifeforms on Earth. These microorganisms are able to adapt to most stable environments on Earth, due to their unique strategies to deal with the changes and adverse conditions that may occur in their habitat (Tsiamis et al., 2014; Heinrich, M. (Ames Research Centre, California, 1978); Brooks et al., 2011; Ahmad et al., 2008).

Nowadays, with the increasing demand from the consumers for more sustainable products and processes, the food, cosmetic, pharmaceutical and environmental industries are striving to obtain natural products that can replace the pollutant synthetic additives currently found on the market (Rosenberg et al., 2013; Andhare et al., 2014). This is one of the reasons for the recent interest in the study of bacteria, which hold great potential as new sources of bioactive compounds and metabolic activities for a wide range of industrial applications (Tsiamis et al., 2014).

The application of microorganisms in industrial biotechnology processes appears to be a promising area, however, obtaining suitable microorganisms, that can present both high yields in the synthesis/production of novel compounds and the ability to resist to industrial conditions is still a challenge. One way to overcome this, may reside in the isolation and characterization of diverse/new bacteria that thrive in different habitats (e.g. soil, plants, cold regions) (Pessôa et al., 2019).

For example, marine bacteria are known to produce bioactive compounds with unusual properties, different from the ones extracted in terrestrial sources, in fact, they are often considered taxonomically unique (Asha Devi et al., 2011; Debnath et al., 2007; Debbab et al., 2010). This occurs because marine environments present a variety of differential conditions of temperature, light, chemistry and pressure that caused microorganisms to develop numerous special adaptations to survive to this extreme and stressful conditions (Debnath et al., 2007; Debbab et al., 2010; Heinrich, M. (Ames Research Centre, California, 1978); Torsvik & Øvreås, 2008). Therefore, marine bacteria produce several bioactive compounds with interesting characteristics, that leads to an enormous set of chemicals that can be used as raw material for industrial, agricultural, environmental, pharmaceutical, and medical uses. Moreover, some marine bacteria are salt-resistant, a feature that can be important in industrial processes (Debnath et al., 2007).

Soil, namely, the rhizosphere (the portion of soil surrounded and influenced by the root of vascular plants) also harbors a diverse and rich microbial community. Bacteria are those that are found in a larger number. Normally, 1 gram of rhizosphere soil contains about 10^8 –10^{12} bacterial cells. Of these, the most prevalent and most efficient at colonizing the roots are the gram-negative bacteria of the genus *Pseudomonas*. This high number is due to the symbiotic relationships between

plants and bacteria: the plant root exudes nutrients (carbon sources) obtained from photosynthesis that in most soils are extremely low, other exudates can also be sugars, amino acids, vitamins, tannins, alkaloids, phosphatides, and other substances such as growth factors. In return, the bacteria facilitate the acquisition of other nutrients to plants (e.g. iron, phosphate, ammonia) (Kennedy & de Luna, 2005).

Therefore, microorganisms can have an impact in the plant development, growth, health, and environmental adaptation, either in a beneficial, neutral or deleterious way (Ahmad et al., 2008). For example, microorganisms have been used for many years in agriculture to increase the resistance of plants to adverse and stressful conditions, such as droughts, salts, nutrient deficiency, and heavy metal contamination (Ahmad et al., 2008). Bacteria can perform various metabolic functions that impact the plant and soil health, some of which are nutrient cycling, organic matter formation and decomposition, soil structure formation, plant growth formation and nitrogen fixation, improving their mineral nutrition. (Dastager et al., 2011; Heinrich, M. (Ames Research Centre, California, 1978); Jacoby et al., 2017).

Plant-associated bacteria have numerous applications, one of which is the improvement of plant capabilities for the phytoremediation of contaminated environments, decreasing or removing pollutants that are harming the environment (Barea, 2015).

Ultimately, obtaining and studying novel bacteria capable of producing interesting compounds with applications relevant to the industry, namely biopolymers such as polyhydroxyalkanoates and exopolysaccharides was the goal of this work.

Exopolysaccharides (EPS) are high molecular weight polymers produced by some microorganisms as a protective barrier against harmful conditions. They present interesting and diverse physicochemical properties with commercial interest in different biotechnological areas (Roca et al., 2015; Roca et al., 2016). Polyhydroxyalkanoates (PHAs) are biodegradable polyesters of hydroxy fatty acids, used as intracellular carbon and energy storage by many different bacteria. PHAs are versatile materials, considered as bioplastics, presenting great potential as substitutes for synthetic plastics (Kourmentza et al., 2009; Johnson, 2010; Serafim et al., 2008b; Wen et al., 2010).

Five bacteria were studied, namely *Pseudomonas arsenicoxydans* ACM1, isolated from the rhizosphere of a moss (*Sanionia uncinata*) on the Collins glacier, Antarctica. It produces exopolysaccharides, oxidizes metals, degrades phenols and other aromatics; *Pseudomonas mandellii* ACM7, also isolated from the rhizosphere of the moss (*Sanionia uncinata*) on the Collins glacier, Antarctica. It produces PHAs and degrades various aromatic compounds. *Pseudomonas sp.* REST10, isolated from the interior (endophyte) of a marine and halophyte plant (*Spartina*

alternifolia) in Florianópolis, Brazil. It produces alginate and surfactants. *Marinomonas* sp. A3A, was isolated from a macroalgae (*Gelidium*) from S. Martinho do Porto, Portugal. Produces ectoin. Lastly, *Pantoea cypripedi* NE1, was isolated from the interior of a legume nodule (*Sesbania*) in Florianópolis, Brazil. Produces exopolysaccharides (equivalent to amylovoran) and fixes nitrogen.

With this work, it is intended to verify if the bacteria in question produce the added-value products detected in the genetic analysis and to evaluate their productivity in producing them.

1.2. Polyhydroxyalkanoates (PHAs)

Plastic is a material present in our daily lives that helped us to improve our life quality and added more comfort to it. Plastics are one of the most used materials and play a big role in almost all industries due to their desirable properties, namely its strength, lightness, durability, cheapness, transparency and being water and degradation resistant (Khanna & Srivastava, 2005; Johnson, 2010).

The non-biodegradability of synthetic plastics has a huge negative impact in nature, due to its accumulation in the environment, becoming a worldwide problem (Możejko-Ciesielska & Kiewisz, 2016). All the solutions available to deal with the management of plastic waste (e.g. incineration, recycling and biodegradation) have problems associated to them. Therefore, there is an increasing interest to look for better alternatives to substitute conventional plastics (Kaur et al., 2017).

One of the most promising options are the polyhydroxyalkanoates (PHAs) (Kaur et al., 2017). PHAs are biocompatible and biodegradable polyesters with properties similar to synthetic plastics (Kessler et al., 2001; Możejko-Ciesielska & Kiewisz, 2016). As they are biobased polymers, they have the potential to compete with them, being considered a potential alternative as renewable and biodegradable plastics, besides being able to be produced by various microorganisms. (Kaur et al., 2017; Możejko-Ciesielska & Kiewisz, 2016)

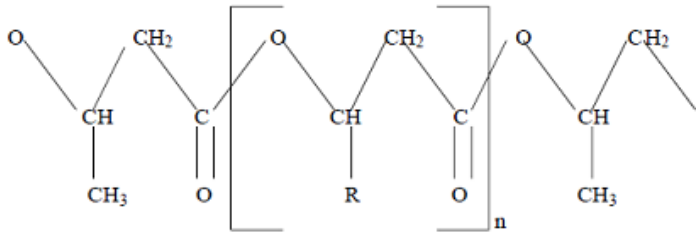
PHAs are considered an attractive substitute to synthetic plastics, since they can exhibit several interesting properties capable of overcoming them, such as thermoplasticity, elastomericity, non-toxicity, biocompatibility and being made from renewable resources (Kourmentza et al., 2017; Możejko-Ciesielska & Kiewisz, 2016; van der Walle et al., 2001; Johnson, 2010; Kim & Lenz, 2001). PHAs can be totally degraded by microorganisms in soil, sea, lake water or sewage under

aerobic and anaerobic conditions; in the first condition by water and carbon dioxide and in the later by methane. (Khanna & Srivastava, 2005).

Besides been biodegradable, PHAs have another important feature, when compared with their biodegradable competitors (e.g. starch and proteins in moisture resistance), which is their hydrophobicity (van der Walle et al., 2001).

PHA is a polyester consisting of several R-hydroxyalkanoic acids (HA) monomers (Figure 1.1), produced within the cell cytoplasm as discrete granules under environmental stress conditions (Johnson, 2010; G. Chen, 2010; Kourmentza et al., 2009; Babel et al., 2001; Możejko-Ciesielska & Kiewisz, 2016).

Many different bacteria are able to produce PHAs and accumulate them as intracellular energy and carbon compounds (Wen et al., 2010; Johnson, 2010). Usually, PHA accumulation occurs when an essential nutrient for the bacterial growth, such as nitrogen or phosphorus, is limited and the carbon source is in excess (Kourmentza et al., 2017; Khanna & Srivastava, 2005). Some bacteria can accumulate PHAs up to 70% to almost 90 wt% of their cellular dry weight, normally varying between 30% to 80% (Johnson, 2010; Kim & Lenz, 2001).



n varies from 600 to 35000

R= hydrogen	Poly(3-hydroxypropionate)
R=methyl	Poly(3-Hydroxybutyrate)
R=ethyl	Poly(3-hydroxyvalerate)
R=propyl	Poly(3-hydroxyhexanoate)
R=pentyl	Poly(3-hydroxyoctanoate)
R=nonyl	Poly(3-hydroxydodecanoate)

Figure 1.1 - General structure of polyhydroxyalkanoates (Khanna & Srivastava, 2005).

1.2.1. Fermentation strategies: PHA production from pure and mixed cultures

PHA can be produced by pure or mixed microbial cultures (MMC) (Fradinho et al., 2019). The PHA production by mixed cultures do not require reactor and culture medium sterilization, as much equipment and control requirements, and are able to use low-cost substrates, including industrial and agricultural wastes (Shalin et al., 2014; Johnson, 2010). However, MMC are operated under a process designated as Feast and Famine (FF), where the culture growth is restricted due to the alternate substrate availability, selecting the organisms able to use the carbon source to

accumulate polymer. Therefore, MMC needs at least two bioreactors, one for selecting the microorganisms able to accumulate PHA and the other to accumulate PHA (Fradinho et al., 2016, 2019).

On the other hand, pure cultures are able to grow and produce PHA in a single bioreactor in batch or fed-batch mode, taking around 38 to 72 hours for fermentation. The process involves two-stages. In the first stage (growth), an inoculum of bacteria is introduced into a sterile solution containing trace metal nutrients, a nitrogen and a suitable carbon source. In the second stage, for improving PHA storage growth limiting conditions are imposed to the process, i.e. an essential nutrient (such as N, P or O_2) is purposely limited (Laycock et al., 2013).

Pure cultures allow to obtain higher PHA content (up to 80%) and higher productivity leading to moderate downstream costs. When working with these cultures, a single bacteria strain is being employed resulting in a well-defined polymer composition, the same would not happen with MMC where the result would be a mixture of polymers (Agustín Martinez et al., 2015). Further, the use of agro-industrial wastes or by-products by pure cultures for PHA production has been successfully achieved (e.g., fruit pulp, used cooking oil, spent coffee grounds oil, sugar cane, bagasse, whey) overcoming the need for expensive pure carbon sources (Cruz et al., 2016; Elain et al., 2016).

The properties of PHAs are determined essentially by their chemical structure and monomer composition. The variation of these properties leads to a set of different potential applications (van der Walle et al., 2001). The monomer composition of PHAs is dependent on the nature of the carbon source, the bacterial host of the polymer and the fermentation conditions used in their production. (Możejko-Ciesielska & Kiewisz, 2016; van der Walle et al., 2001). By changing these conditions, it is possible to have some control over the monomeric composition of PHAs, allowing the tailoring of the polymer material properties to achieve the desired demands for a specific application, e.g., flexibility can be changed by varying the length of the fatty acid chain; (van der Walle et al., 2001; Kessler et al., 2001; Khanna & Srivastava, 2005) allowing PHAs to stand out from its existing competitors in the market who do not have this possibility to adapt to the desired characteristics (Kessler et al., 2001).

1.2.2. PHA classification

PHAs are largely classified into two groups based on the number of carbon atoms that comprise their monomeric unit: scl-PHAs (short chain length PHAs) and mcl-PHAs (medium chain length PHAs), where scl-PHAs are composed of 3-5 carbon atoms, and mcl-PHAs by 6-14 carbon atoms.

(Możejko-Ciesielska & Kiewisz, 2016; B; Kim & Lenz, 2001; van der Walle et al., 2001; Khanna & Srivastava, 2005). They exhibit very different properties from each other (Kim & Lenz, 2001).

Scl-PHAs are composed only by 3HB (3-hydroxybutyrate) and 3HV (3-hydroxyvalerate) units, while the mcl-PHAs are usually composed by monomers, such as 3-hydroxyhexanoate (3HHx), 3- hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDd), 3- hydroxytetradecanoate (3HTd). Mcl-PHAs are produced by several strains, mainly of the *Pseudomonas* genera, for example *Pseudomonas putida, Pseudomonas chlororaphis, Pseudomonas. citronellolis* and *Pseudomonas oleovarans*. (Kim & Lenz, 2001; Możejko-Ciesielska & Kiewisz, 2016; Rebocho et al., 2019)).

From all PHAs, PHB (Polyhydroxybutyrate) was the first PHA being discovered by Lemoinge, in 1926. It is the most widely studied and best characterized PHA. PHB hold great interest due to their similar properties to conventional plastics like polypropylene or polyethylene, and have the potential to replace them in several applications, such as, in packaging films (Kourmentza et al., 2009; Khanna & Srivastava, 2005).

However, they are difficult materials to be processed, since they are brittle and stiff materials with high degree of crystallinity, and melting temperature (180ºC) that is close to their degradation temperature (200ºC) (Kourmentza et al., 2017; Kim & Lenz, 2001). Nevertheless, by incorporating HV units (units with a relatively low molecular weight and melting point), the polymer gets a lower melting temperature, a different morphology and a much higher flexibility, resulting in the co-polymer poly (3-hydroxybutyrate-co-3-hydroxyvalerates) [poly(3HB-co-HV)]. The co-polymer is marketed under the name Biopol. (Kim & Lenz, 2001; Kaur et al., 2017).

The mcl-PHAs are less crystalline and are more flexible and elastic materials when compared to the scl-PHAs. Duo to the presence of monomers, such as 3HO, 3HD and 3HDd, mcl-PHAs present mechanical properties with improved elastic and flexibility features in contrast to those of scl-PHAs (Możejko-Ciesielska & Kiewisz, 2016). Therefore, materials based on mcl-PHAs are considered as suitable candidates for a variety of applications, such as rubbers, smart latexes, adhesives and glues (Muhr et al., 2013).

1.2.3. Applications of PHAs

Due to their properties (biodegradability, water resistance, oxygen impermeability and biocompatibility), PHAs can be used for many different types of applications (Lin et al., 2015).

Such applications include packaging (e.g., shampoos, cosmetic containers, containers and cups for food products) hygienic (e.g., razors, diapers, feminine hygiene products), agricultural (e.g.,

mulch films) and biomedical products (e.g., suture thread, wound dressings, tweezers). It is also used as a substitute for non-biodegradable plastics, as single-use items. They can also be used in the form of an aqueous latex for paper coatings (Khanna & Srivastava, 2005; van der Walle et al., 2001).

PHAs can also serve as biodegradable carriers for long-term dosage of drugs (Khanna & Srivastava, 2005).

PHB is biocompatible with mammalian tissue and is reabsorbed at a slow rate; it is also used as osteosynthetic materials in the stimulation of bone growth due to their piezoeletectric properties, being very attractive for biomedical purposes (Khanna & Srivastava, 2005).

1.3. Exopolysaccharides (EPS)

Polysaccharides are high molecular weight biopolymers (10^4- 10^7), with a very diverse composition and chemical structure (Andhare et al., 2014; Roca et al., 2015). They are mainly composed of carbohydrates (with monosaccharides as repeating units), such as neutral sugars (namely arabinose, ribose, xylose, glucose, galactose, mannose, rhamnose, fucose), amino sugars (e.g., D-Glucosamine and D-Galactosamine) and uronic acids (e.g., glucuronic and galacturonic acids) (Rosenberg et al., 2013; Andhare et al., 2014; Freitas et al., 2011; Ates, 2015; Potter, 2003). Organic and inorganic components such as sulfate, phosphate, acetic acid, succinic acid and pyruvic can also be found in some EPS. (Andhare et al., 2014; Freitas et al., 2011; Potter, 2003) This wide variety of sugars/acids/inorganic components translates into a wide variety of molecular structures (Freitas et al., 2011).

Polysaccharides can be divided in homopolysaccharides (only composed of a single monomer) or heteropolysaccharides (comprised in two or more monomeric units) based on their monomeric composition, where the repeating units are linked by a glycosidic bond. (Rosenberg et al., 2013; Andhare et al., 2014; Bajaj et al., 2007). Besides that, in some cases, single polysaccharide chains may associate with each other through intra or intermolecular non-covalent bonds, conferring to the macromolecule a certain geometry and rigidity, which will determine the polymer´s properties both in solid state and in solution (Kumar et al., 2007; Reis et al., 2011).

In Nature, polysaccharides can be obtained from plants (e.g. cellulose, pectin, starch), algae (e.g. agar, alginate,carrageenan) and be produced by microorganisms (e.g. alginate, dextran, gellan, xanthan gum) (Kumar Singha, 2012; Cruz et al., 2011).

They present diverse physiological roles, functioning as structural elements, maintaining mechanical shape and rigidity of the living cells (e.g. cellulose and pectin in plant cell wall; chitin in arthropod exoskeletons and yeast and fungi cell walls), as energy reserve substances (e.g. starch or inulin in plants; glycogen in animals and microorganisms), or as adhesion and protective barriers (microorganisms) (Kaplan, 1998; Kumar et al., 2007; Elnashar, 2011).

From their location in the cell, microbial polysaccharides can be divided into: i) intracellular polysaccharides, ii) structural polysaccharides and iii) extracellular polysaccharides or exopolysaccharides (EPS). Intracellular polysaccharides and structural polysaccharides are integral parts of the cell, while EPS are synthesized inside the cell and exported outside either as soluble or insoluble polymers, with some exceptions such as levans, dextrans, mutans and alternans that are produced outside the cell by the action of secreted enzymes that are capable of transforming the substrate into EPS (Rosenberg et al., 2013; Andhare et al., 2014; Freitas et al., 2011; Kumar et al., 2007; Ates, 2015).

EPS-producing microorganisms can be found in several ecological niches and can be isolated from several sources, including terrestrial and aquatic environments (Rosenberg et al., 2013; Kumar et al., 2007; Kumar Singha, 2012). EPS-producing microorganisms can be found in environments with high carbon / nitrogen ratio, for example, effluents from the sugar, paper or food industries and from wastewater plants (Andhare et al., 2014; Kumar et al., 2007; Kumar Singha, 2012). Most EPS-producing marine microorganisms are gram-negative bacteria, such as *Pseudomonas, Acinetobacter, Vibrio* and *Alteromonas* (Rosenberg et al., 2013). Lactic acid bacteria (LAB) are the known EPS-producing mesophilic group (Andhare et al., 2014; Kumar Singha, 2012).

Most of the EPS functions for the cells are of protective nature against the harsh external environmental conditions (e.g. predation, competition, desiccation, temperature, salinity, radiation, light intensity, pH, toxins and antibiotics), by serving as a protective layer to the cells and providing them stability (Rosenberg et al., 2013, Andhare et al., 2014; Kumar et al., 2007; Kumar Singha, 2012; Ates, 2015). Moreover, EPS also provides bacterial aggregation, surface attachment, microbial-plant symbiosis and initiation of flocculation (Ates, 2015; Rosenberg et al., 2013; Kumar Singha, 2012; Kumar et al., 2007). Thus, microorganisms capable of producing this biopolymer have an advantage in certain situations of environmental stress, in comparison with those that do not have these protection mechanisms (Ates, 2015).

In recent years, there has been an increased demand for natural polymers for various industrial and biotechnological applications, explaining the great deal of interest in EPS produced by microorganisms, due to their unique properties (Kumar Singha, 2012; Kumar et al., 2007; Rosenberg

et al., 2013). This has led to a greater effort in isolating and identifying new microorganisms capable of producing it, with the aim of discovering biopolymers with new and improved characteristics (Ates, 2015; Kumar et al., 2007; Rosenberg et al., 2013).

Recent research has been directed to replace the EPS produced by plants and algae with their bacterial counterparts, as they present higher growth rates, faster and reproducible high-throughput production processes, improved physical properties, an easier way to manipulate and control the production conditions, in addition to not being influenced by climate changes or seasonality (Kumar Singha, 2012; Cruz et al., 2011; Freitas et al., 2011; Kumar et al., 2007; Andhare et al., 2014; Ates, 2015; Roca et al., 2015). Bacterial polymers also compete with oil-based non-renewable polymers, however, they present several advantages to these, namely, the fact that they are sustainable since they are produced from renewable sources, their biodegradability, biocompatibility, lack of toxicity, and both environmental and human compatibility (Rehm, 2010; Ates, 2015; Andhare et al., 2014).

However, for new microbial polysaccharides to conquer the market as commodity products, it is crucial to lower their production costs, which can be performed by: (i) using low cost substrates, (ii) improving product yield by optimizing fermentation conditions, (iii) developing higher yielding strains (e.g. by mutagenesis or genetic manipulation), and (iv) optimizing downstream processing. The great opportunity to bacterial EPS to be developed at industrial scale and commercialized relies on high value market niches (e.g. cosmetics, pharmaceuticals and biomedicine), since microbial polysaccharides may present the desired degree of purity and functional properties that the traditional polysaccharides obtained from plants and algae may not have. In this case, the potential designed characteristics and quality of the product will exceed production costs and product yield issues (Freitas et al., 2011).

1.3.1. Production of EPS

Bacterial EPS are produced industrially by single strain bacteria (Roca et al., 2015). They can be produced during the different phases of growth depending on the microorganism used (Rosenberg et al., 2013; Potter, 2003). The composition and amount of EPS synthesized is genetically determined, however, several factors may have an influence, namely, media composition (e.g. carbon and nitrogen source) and cultivation conditions (e.g. pH and temperature), leading to different functional properties of EPS in the end (Roca et al., 2015; Freitas et al., 2011; Kumar Singha, 2012; Ates, 2015; Potter, 2003). In this way, it is possible to manipulate, to some extent, the characteristics of EPS to meet a given application of interest, by changing its growth conditions

(Roca et al., 2015). EPS production is favored when there is a high carbon/nitrogen ratio, that is, when the carbon source is in excess and we have an essential nutrient in limited quantities, such as nitrogen or phosphate (Kumar Singha, 2012; Freitas et al., 2011). Most bacterial EPS are produced under aerobic conditions (Freitas et al., 2011).

The most used carbon source for its production are sugars (e.g. glucose, sucrose, fructose) (Freitas et al., 2011). However, these substrates are very expensive, and are usually only used for high-value applications, where high purity and product quality are required (Freitas et al., 2011; Potter, 2003).

The substrates used on an industrial or laboratory scale are chosen with different purposes (Potter, 2003). On a laboratory scale, the greatest interest is to understand which substrates are used and what is the biopolymers yield value obtained when different physiological conditions are tested (Potter, 2003). Thus, for this purpose, pure substrates are usually used (Potter, 2003). On an industrial scale, the parameter that is most taken into account is the cost, because if it is not profitable, there is no production, however, the yield and the final use of the product are also very important factors to be considered (Potter, 2003). Thus, other cheaper alternatives are sought, namely, agro-food or industrial wastes and byproducts (e.g. molasses, cheese whey or glycerol byproduct), in order to reduce the overall production costs (Freitas et al., 2011; Potter, 2003; Roca et al., 2015). Besides these appear to be a promising solution, they could present some problems, since different metabolic pathways can be followed, leading to a possible production of different polymers and unwanted byproducts (Freitas et al., 2011). Also, some non-reacted components may accumulate in the medium, and possible become inhibitors, affecting the product yield and can be carried-over to the final product, requiring a greater investment in downstream processes (Freitas et al., 2011; Roca et al., 2015). Moreover, is difficult to guarantee their supply in terms of quantity and quality and they may need costly treatments before they can be used (Roca et al., 2015).

Another problem associated with the production of EPS is the alteration of the rheology of the fermentation medium (Freitas et al., 2011). At first, it behaves like a Newtonian fluid, where the viscosity is similar to that of water, however, with the production of EPS, it changes to a very viscous fluid, making it very difficult to guarantee proper agitation, oxygenation and other important parameters of the bioreactor (Freitas et al., 2011; Potter, 2003). A possible solution to this problem would be to increase the stirring rate or improve the mechanical mixing using different configuration paddles (Freitas et al., 2011).

The production of EPS is either by batch or fed-batch fermentation. In batch cultures, the production of EPS occurs when some nutrient has already been depleted in the medium and is usually maximum when there is a high carbon/ nitrogen ratio (Kumar et al., 2007).

One more expensive factor to consider is the recovery of EPS (concentration, isolation and purification), due to its low concentration, presence of impurities (e.g. cells) and the high viscosity of the broth (Kumar et al., 2007).

1.3.2. Applications of EPS

Due to their unique characteristics (chemical structure, physicochemical and rheological properties), EPS have a wide range of applications covering several areas, including food, feed, packaging, chemical, textile, bioremediation, waste water treatment, oil drilling, cosmetics and pharmaceutical industry, agriculture, and medicine (Ates, 2015; Andhare et al., 2014; Bajaj et al., 2007; Cruz et al., 2011; Freitas et al., 2011; Kumar et al., 2007; Kumar Singha, 2012; Roca et al., 2015). In these areas they can have several functions as they can be used as adhesives, binding and flocculating agents, absorbents, stabilizers, lubricants, gelling agents , drug delivery vehicles, coagulants, high-strength materials, emulsifiers, film formers, viscosifiers and thickening and suspending agents (Ates, 2015; Kumar Singha, 2012; Bajaj et al., 2007; Freitas et al., 2011). Table 1.1 shows some examples of microbial EPS that are commonly used in industry.

Table 1.1 - Microbial EPS and their possible applications (Rosenberg et al., 2013).

Biopolymers	Possible Applications
Acetan	Viscosifier and gelling agent
Alginate	Immobilization and microencapsulation
Cellulose	Temporary artificial skin, natural nondigestable fibers, hollow fibers or membranes, and acoustic membranes in audiovisual equipment
Curdlan	Gelling agent
Cyclosophorans	Encapsulation of drugs and food component
Dextran	Blood plasma extender or blood flow improving agent, cholesterol lowering agent, and microcarrier in tissue/cell culture
Emulsan	Emulsification and immobilization

Gellan	Solidification/gelling agent
Hyaluronic acid	Moisturization and synovial fluid replica
Kefiran	Gelatination and viscoelasticity
Levan and Alternan	Similar as dextran
Succinoglycan	Gelling agent and immobilization
Welan	Stabilizer and viscosifier
Xanthan	Emulsification and gelatination

1.4. Motivation

Nowadays, there is a great demand for more sustainable, renewable and environmentally friendly polymers to replace those that have been commercialized until now based on fossil fuels.

A very interesting possibility are the microbial polymers since they are capable of producing many different substances with unique properties, namely exopolysaccharides and polyhydroxyalkanoates.

Microbial diversity has only started to be explored more recently so, lots of research needs to be done in this regard. Thus, there might be many potential sources still available, that may contain microorganisms able of producing compounds that can have interesting characteristics with commercial power.

Therefore, it makes sense to isolate and characterize microorganisms from several different sites, to see if they have potential to be used in new applications.

Marine environments are a rich natural source of microbial EPS, that presents a very diverse set of conditions that bacteria need to adapt in order to be able to survive.

The aim of this work was to study various bacteria isolated from different locations and environments (e.g. Antarctica, S. Martinho do Porto, Brazilian plant roots) to find out if they are capable of producing interesting value-added products, such as exopolysaccharides and polyhydroxyalkanoates.

Setting the standard cultivation process by studding the effect of environmental conditions (temperature) and medium composition aiming at maximizing cellular growth and polymer production. The polymers were characterized, in terms of chemical composition.

Chapter 2 :
Material and Methods

2.1. Bacterial strain and culture media

The bacterial strains used in this work were *Pseudomonas* sp. REST10, *Marinomonas* sp. A3A, *Pseudomonas mandellii* ACM7, *Pseudomonas arsenicoxydans* ACM1 and *Pantoea cypripedi* NE1. All the bacterial strains were cryopreserved, using glycerol (20% v/v) as a cryoprotectant, at -80 °C.

The following culture media were used:

Table 2.1 TSB, LB and E medium for 1L of solution

TSB medium		LB medium		E medium	
tryptone	17.0 g	tryptone	10.0 g	K_2HPO_4	5.8 g
soy peptone	3.0 g	yeast extract	5.0 g	KH_2PO_4	3.7 g
K_2HPO_4	2.5 g	NaCl	10.0 g	$(NH_4)2HPO_4$	1.1 g
Glucose	2.5 g			$MgSO_4$ solution	10.0 mg
				Glucose solution	20 mL
				Mineral solution diluted (1:10) containing:	10 mL
				$FeSO_4.7H_2O$	2.78 g
				$MnCl_2.4H_2O$	1.98 g
				$CoSO_4.7H_2O$	2.81 g
				$CaCl_2.2H_2O$	1.67 g
				$CuCl_2.2H_2O$	0.17 g
				$ZnSO_4.7H_2O$	0.29 g

Medium E, $MgSO_4$ solution, mineral solution and glucose solution were autoclaved separately, at 121 °C for 20 minutes, and mixed after cooling down to avoid salts precipitation.

2.2. Inoculum preparation

To prepare the inoculum, it was performed the reactivation of the cultures by plating a sample of the cryopreserved vials in TSB-agar plates and incubating them at 30ºC, during 24h. Afterward,

a single colony of each culture was inoculated into 50 mL of TSB medium in 100 mL Erlenmeyer flasks, and incubated at 200 rpm and 30ºC or at 150 rpm and 16ºC, during 24 h. These cultures served as inoculum.

20 mL of the inoculum were transferred to 200mL cultivation assays (in 500 mL baffled shake flasks).

2.3. Shake flasks assays

Shake flasks cultivations were performed with a working volume of 200 mL using baffled shake flask of 500 mL.

The assays run for 72-144h and culture broth samples were taken periodically for measurements of the optical density at 600 nm. At the end 40 ml sample were centrifuged (8000 rpm, 15 min, 4 °C; Sigma 4-16KS), the cell-free supernatant was stored at -20 ºC for EPS and glucose quantification, and the pellet was lyophilized for biomass and PHA quantification.

The experiments were performed in orbital shakers at 30 °C (200 rpm) and 16ºC (150 rpm), to evaluate the effect of temperature.

TSB medium was tested with its glucose formula concentration (2.5 g/L) and after, for the assays with better results TSB medium were tested with 10 g/L glucose.

The effect of the salt concentration was also evaluated. The assays were performed with a salt concentration similar to that found in sea water (30g/L) and with the salt-free medium.

LB medium was tested with its salt concentration (10g/L), with a salt concentration similar to sea water (30g/L) and without added salt. This medium was also tested with the addition of glucose (10 g/L).

In E medium it was also studied the effect of salt, and tests were performed without and with 30g/L of added salt.

2.4. Bioreactor assays

2.4.1. Bacterial Strains

The bacterial strains used in the bioreactor assays were *Pseudomonas* sp. REST10, *Pseudomonas mandellii* ACM7 and *Pseudomonas arsenicoxydans* ACM1. The bacteria were reactivated by inoculation in TSB-agar plates with a sample of the cryopreserved bacteria and incubated at 30ºC.

2.4.2. Inoculum and Cultivation Medium

A pre-inoculum was prepared by inoculating a single colony isolated from the TSB-agar plates into 50 mL of the medium being tested in the bioreactor in 100 mL erlenmeyers flasks, and incubated in an orbital shaker at 200 rpm and 30 ºC, for 24 h, except for the bioreactor performed with *Pseudomonas sp.* REST10 which was for 48 h. Such cultures served as pre-inocula for the bioreactor experiments. The inoculum was prepared by transferring 20 mL of the pre-inoculum to 500 mL baffled shake flasks with 200 mL of the medium being tested in the bioreactor. The baffled flasks were incubated at 30 °C, 200 rpm, for 24 h, and 48h in the case of the *Pseudomonas sp*. REST10 bioreactor.

2.4.3. Batch Cultivation

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

The temperature was kept at 30.0 ± 0.1 °C and the pH was automatically controlled at 7.0 ± 0.05, in the assays with marine bacteria, by the automatic addition of NaOH (5 M) and HCl (2 M). A silicon based antifoam (Sigma-Aldrich, Germany) was used in order to prevent the formation of foam throughout the process. The air flow rate was maintained at 2 SLPM (standard liters per minute) during the cultivation and the dissolved oxygen concentration (DO) was controlled at 30% of the air saturation by automatic adjustment of the stirrer speed (300-800 rpm) provided by two 6-blade impellers.

Samples were periodically taken from the bioreactor for quantification of cell dry weight, PHA, EPS, ammonia, protein, nitrogen and glucose. Culture broth samples (20 mL) taken periodically during the cultivation runs were centrifuged (8000 rpm, 15 min, 4 °C; Sigma 4-16KS), the cell-free supernatant was stored at -20 ºC for EPS, ammonia, protein, nitrogen and glucose quantification, and the pellet was lyophilized for biomass and PHA quantification.

The effect of different conditions, such as temperature and cultivation medium were tested for each bacterium (Table 2.1)

Table 2.2 - Different conditions tested for each bacteria in the batch cultivations.

Bacteria	Medium	Glucose (g/L)	Temperature (ºC)
Pseudomonas sp. REST10	TSB	2.5	22

***Pseudomonas mandellii* ACM7**	TSB	20	30
	TSB	20	16
***Pseudomonas arsenicoxydans* ACM1**	E	20	30
	TSB	20	30

2.5. Analytical techniques

2.5.1 Cellular Growth

Cellular growth was monitored during the experiments by measuring the optical density of the cultivation broth at 600 nm (OD600nm). Samples were diluted with deionized water whenever necessary, in order to have the OD600nm value below 0.4. Deionized water was used as zero reference.

2.5.2. Biomass Quantification

The cell dry weight (CDW) was determined by gravimetry. The samples were centrifuged (8000 rpm, 15 min, 4 ºC) and the cell pellets were washed once by resuspension in deionized water (6mL) and centrifuged again (8000 rpm, 15 min, 4 ºC). After centrifugation, the supernatant was discarded and the washed pellets were lyophilized for 48 hours at -108 °C. The CDW was obtained by weighing the lyophilized cell pellets. All samples were done in duplicate.

2.5.3. Nile Blue Staining

In an Eppendorf tube, 0.5 µL of Nile Blue was added to 0.5 mL cultivation broth sample and placed in an oven at 70 ° C for 10 minutes. After this time, slides were prepared which were observed under the microscope (Olympus BX51 epifluorescence) under contrast light and fluorescent light, with a magnification of 100x.

2.5.4. Glucose quantification

The glucose quantification in the supernatant samples was obtained by high performance liquid chromatography (HPLC), using a VARIAN Metacarb 87H column coupled to a refractive index (RI) detector. The analysis was performed at 50 °C using H2SO4 0.01 N, as eluent with a flow rate of 0.6 mL/min. The samples were prepared by diluting the cell-free supernatant, in the eluent

(H_2SO_4, 0.01 N), in a proportion according to the glucose concentration tested in the medium. All samples were filtered using VWR centrifuge filters (0.2 μm). A standard cultivation curve was constructed using glucose solutions as standards, in a concentration ranging from 0.01 to 1g/L.

2.5.5. Total nitrogen quantification

For the determination of total nitrogen, a kit (LCK 388, LATON®) was used with a detection range of 20-100 mg/L. The sample (0.2 mL) was placed into a digestion flask, and a 2.3 ml of solution A and 1 tablet B were added. The flasks were put in a HT 200S (HACH® - LANGE) digester for 15 min at 170 ºC. After cooling to room temperature, the digestion flasks were inverted a few times and a 0.5 mL of the digested solution was transferred to a new vial, which was later added, 0.2 mL of solution D (Photometric Detection with 2,6-Dimethylphenol) and quickly stirred. After 15 min the absorbance was read in a DR2800 tm spectrophotometer (HACH®).

2.5.6. Ammonium and phosphorus quantification

The ammonium and phosphates content was determined by a colorimetric method, using a flow segmented analyzer (Skalar 5100, Skalar Analytical, The Netherlands). The standard solutions were prepared using phosphorus (KH_2PO_4) and ammonia (NH_4Cl) in milli-Q water in concentration of 4 – 20 ppm. The cell-free supernatant was diluted in deionized water and analysed.

2.5.7. Protein content in EPS and supernatant

For the determination of the protein content, 25 mg of EPS and 0.5 mL of supernatant were mixed with 1 mL 20% NaOH and placed at 100 ºC, for 5 min. After cooling on ice, 170 µL of $CuSO_4 .5H_2O$ (25% w/v) were mixed. The samples were centrifuged (3500×g, for 5 min) and the absorbance was measured at 560 nm. Albumin (Sigma-Aldrich) solutions (0.05 – 3.0 g.L-1) were prepared and used as standards (calibration curve in appendix 7.2).

2.5.8. EPS quantification

EPS production across cultivation was evaluated by extraction of the polymer from the cell-free supernatant by dialysis. For this process, dialysis membranes (ZelluTrans Carl Roth, MWCO 12000 - 14000) with 6 mL of supernatant, were placed in a 5 L beaker with deionized water, under constant stirring. Sodium azide (5mL) was added to the water to prevent biological contamination and sample degradation. The water was changed 3 to 4 times a day. Before every water change,

a sample was collected for conductivity measurement. Dialysis was finished after three days, when the dialysis water conductivity was the closest to deionized water conductivity (after approximately 72 hours when conductivity was lower than 10 μS/cm).

All samples were collected, frozen at -80 °C and lyophilized for 48 hours at -108 °C. After that, they were weighed for EPS gravimetric quantification.

2.5.9. EPS sugar monomer composition

The EPS was characterized in terms of its sugar monomers composition. The samples were prepared by dissolving 5 mg of the lyophilized sample in deionised water (5 mL) and hydrolysing them with trifluoracetic acid (TFA) (0.1 mL, 99%), in a dry bath for 2 hours at 120°C.

Samples sugar monomers composition was determined by HPLC using a CarboPac PA10 250x4 column (Dionex) coupled with an AminoTrap 50x4 column (Dionex). The analysis was performed at 25 ºC with sodium hydroxide (NaOH, 18 mM) as eluent, at a flow rate of 1 mL/min. D-(-)-arabinose (99%, Sigma), L-rhamnose monohydrate (99%, Fluka), D-(+)-galactose (99%, Fluka), D-(+)-glucose anhydrous (99%, Scharlau), Sucrose (99%, Fluka), D-(-)-fructose (99%, Scharlau), D-(+)-mannose (99%, Fluka), D-glucuronic acid (98%, Alfa Aesan), D-(+)-galacturonic acid monohydrate (97%, Fluka), D-(+)-Xylose (99%, SIGMA-ALDRICH) and D-(+)-Fucose (98 %, Scharlau) were used as standards (5 to 50 ppm).

2.5.10. PHA Quantification and Composition

The PHA content in the biomass and its monomer composition were determined by gas chromatography (GC) analysis. 2-4 mg of biomass were hydrolysed in 2 mL of methanol acidic (20% (v/v) sulphuric acid (SIGMA-ALDRICH) in methanol (Fisher Chemical)) and 2 mL of benzoic acid in chloroform (1 g/L) (SIGMA-ALDRICH), on an oil bath at 100˚C, for 4 h. Then, 1 mL of water was added. After separation of the organic and aqueous phases, the organic phase, with the resulting methyl esters, was transferred to vials and analysed by GC (430-GC, Bruker) with a Restek column of 60m, 0.53 mmID, 1 μM df, Crossbond, Stabilwax. The injection volume was 2.0 μL, with a running time of 32 min, a constant pressure of 14.50 psi and helium as carrier gas. The heating ramp was 0 to 3 min, a rate of 20˚C/min, until 100˚C, 3 to 21 min a rate of 3˚C/min until 155˚C and 21 to 32 min a rate of 20˚C/min until 220˚C. Mcl-PHA (VersaMerTM PHAs, PolyFerm Canada) and PHB-HV standards were prepared at 1 g/L and then diluted to give concentrations in the range 0.05 and 1 g/L.

2.6. Calculations

The active biomass (X, g/L) was determined by the following equation:

$$X = CDW_t - PHA_t \quad \text{Eq. 1}$$

where CDW is the cell dry weight concentration (g/L) at a certain time t (h) and PHA is the concentration (g/L) of PHA at that time t (h).

The specific cell growth rate (μ, h^{-1}) was determined by the following equation:

$$\mu = \mathrm{Ln}\left(\frac{\mathrm{Xt}}{\mathrm{X0}}\right) \quad \text{Eq. 2}$$

where Ln is natural logarithm, Xt is active biomass concentration (g/L) at a certain time t (h) and $X0$ is active biomass concentration (g/L) at the beginning of fermentation.

The PHA volumetric productivity (rPHA, g/L·h) and EPS volumetric productivity (rEPS, g/L·h) were determined by the following equations:

$$\text{rp PHA} = \frac{dPHA}{dt} \quad \text{Eq. 3}$$

$$\text{rp EPS} = \frac{dEPS}{dt} \quad \text{Eq. 4}$$

where PHA is the PHA concentration (g/L) produced at time t (hours) and EPS is the EPS concentration (g/L) produced at time t (hours).

The yields of active biomass on substrate (Yx/s, g/g), PHA on substrate (Ypha/s, g/g) and EPS on substrate (Yeps/s, g/g) were determined by the following equations:

$$\mathrm{Y\,x/s} = \frac{\mathrm{X\,f - X\,i}}{\mathrm{Sf - Si}} \qquad \text{Eq. 5}$$

$$\mathrm{Y\,PHA/s} = \frac{\mathrm{PHA\,f - PHA\,i}}{\mathrm{Sf - Si}} \qquad \text{Eq. 6}$$

$$\mathrm{Y\,EPS/s} = \frac{\mathrm{EPS\,f - EPS\,i}}{\mathrm{Sf - Si}} \qquad \text{Eq. 7}$$

where X_f and Xi are the final and initial active biomass concentration, Sf and Si are the final and initial substrate, PHAf and PHAi are the final and initial PHA produced, EPSf and EPSi are the final and initial EPS produced, respectively.

Chapter 3 :
Results and Discussion

3.1. Shake flasks assays

The bacteria chosen to be evaluated in this work were isolated from different habitats and environments, to which a genomic analysis was carried out, where it was possible to verify the existence of genes that code for the presence of exopolysaccharides (EPS) and polyhydroxyalkanoates (PHA). Thus, tests were performed in Erlenmeyer flasks in order to evaluate the production of EPS and PHAs by the different bacteria.

Therefore, the bacteria *Pseudomonas mandellii* ACM7, *Pseudomonas arsenicoxydans* ACM1, *Pseudomonas sp.* REST10, *Marinomonas sp*. A3A and *Pantoea cypripedi* NE1 were tested under different conditions.

3.1.1. Growth in TSB medium

All the 5 bacteria tested showed to be able to grow in the rich medium TSB (Tryptic soy broth) (Table 3.1) at 30ºC. The bacteria that presented the highest cell growth was *Pseudomonas arsenicoxydans* ACM1 reaching 1.91±0.02 g/L of CDW, within 53 hours. *Pseudomonas mandellii* ACM7 attained a very similar value of CDW (1.81±0.02 g/L), which is interesting since both bacteria were isolated from the rhizosphere of a moss (*Sanionia uncinata*) of an Antarctic glaciar. *Pseudomonas* sp. REST10 and *Pantoea cypripedi* NE1 presented the lowest cellular growth, namely 1.31±0.03 and 1.13±0.01 g/L, respectively. On the other hand, *Marinomonas* sp. A3A was not able to grow. This bacterium was isolated from marine algae, hence the salt concentration in TSB medium (0.5 % w/v) was very low to enable the culture cellular growth.

Table 3.1 - Cell dry weight (g/L), EPS production (g/L) and PHA detection, after 53 h shake flask cultivations on TSB medium at 30ºC.

Bacteria	CDW (g/L)	EPS (g/L)	PHA*
Marinomonas sp. A3A	0	0	--
Pseudomonas sp. REST10	1.31 ± 0.03	2.59 ± 0.08	++
Pseudomonas arsenicoxydans ACM1	1.91 ± 0.02	2.20 ± 0.01	+++
Pseudomonas mandellii ACM7	1.81± 0.02	1.03 ± 0.24	+
Pantoea cypripedi NE1	1.13 ± 0.01	2.32 ± 0.04	++

*(+ + +) intense fluorescence; (+ +) medium fluorescence; (+) light fluorescence.

Concerning biopolymer production all the bacteria tested were able to produce both EPS and PHA.

Pseudomonas sp. REST10 produced the highest concentration of EPS (2.59±0.08 g/L) (Table 3.1), *Pseudomonas arsenicoxydans ACM1* and *Pantoea cypripedi* NE1synthesized similar EPS quantities (2.20±0.01 and 2.32±0.04 g/L, respectively). The culture *Pseudomonas mandellii* ACM7 attained the lower concentration of EPS (1.03±0.24 g/L).

According with the literature, *Pseudomonas aeruginosa* B1, *P. fluorescens* B5, *P. stutzeri* B11 and *P. putida* B15 produced 75 mg. L^{-1}, 63 mg. L^{-1}, 41 mg. L^{-1} and 67 mg. L^{-1} of EPS, respectively, in shake flask assays with a nutrient medium consisting of peptones, yeast extract and beef extract (Onbasli & Aslim, 2009). These values are lower than those obtained in the screening assay (Table 3.1), showing that these bacteria have potential for EPS production, as it is not common to obtain these EPS values in shake flasks assays since the cultivation conditions are not optimized.

3.1.2. Effect of different conditions in shake flasks assays

3.1.2.1. Effect of the presence of salt

In their natural environment, marine bacteria often have to deal with stressful conditions, one being the high osmolarity due to the presence of salt (Elabed et al., 2019; Sleator & Hill, 2002). In order to understand whether the presence of salt has an influence on the growth and production of polymers, the bacteria were grown in TSB medium with an increased salt concentration (3 %w/v) taking into account the concentration present in marine water.

Therefore, *Marinomonas* sp. A3A and *Pseudomonas* sp. REST10, bacteria isolated from a marine environment, were tested in TSB medium with a salt concentration of 3%.

Table 3.2 - Cell dry weight (g/L), EPS production (g/L) and PHA detection, after shake flask cultivations on TSB medium with 0.5 and 3 % w/v of salt at 30ºC.

Bacteria	Salt (% w/v)	CDW (g/L)	EPS (g/L)	PHA*
Marinomonas sp. A3A	3	0.64 ± 0.02	1.15 ± 0.09	+++
Pseudomonas sp. *REST*10	0.5	1.31 ± 0.01	2.59±0.08	+++
Pseudomonas sp. REST10	3	1.09 ± 0.03	1.84 ±0.04	+++

*(+ + +) intense fluorescence; (+ +) medium fluorescence; (+) light fluorescence.

According with literature, most species of *Marinomonas sp.* require the presence of salt to grow (Lucena et al., 2016). In the case of the strain tested in this work, the same behavior was observed,

i.e. the bacteria was not able to grow in low concentration of salt. However, when the salt concentration was increased to 3% w/v the culture was able to grow, achieving a CDW of 0.64 g/L (Table 3.2), being also able to produce EPS in a concentration slightly lower than the other bacteria evaluated. On the other hand, for *Pseudomonas* sp. REST10 the salt concentration increase resulted in a concentration decrease for CDW and more evident for EPS.

3.1.2.2. Effect of temperature

Temperature is one of the main agents responsible for controlling the microbial growth and metabolite synthesis, being considered an essential factor when preparing an assay (Torres et al., 2012). Thus, the bacteria were grown at different temperatures, namely, 16ºC and 30ºC, to understand its impact.

For these assays, all bacteria were cultured in TSB medium with the salt concentration where the best results were obtained depending on the culture.

Table 3.3 - Cell dry weight (g/L), EPS production (g/L) and PHA detection, after shake flask cultivations on TSB medium (0.5 and 3 %w/v) at different temperatures (16ºC and 30ºC).

Bacteria	Salt (% w/v)	T (ºC)	CDW (g/L)	EPS (g/L)	PHA*
Marinomonas sp. A3A	3	30	0.64 ± 0.02	1.15 ± 0.09	+++
Marinomonas sp. A3A	3	16	1.58 ± 0.07	1.51 ± 0.09	+
Pseudomonas sp. REST10	0.5	30	1.31 ± 0.03	2.59±0.08	+++
Pseudomonas sp. REST10	0.5	16	2.28 ± 0.01	2.51 ± 0.08	+++
Pseudomonas arsenicoxydans ACM1	0.5	30	1.91 ± 0.02	2.20 ± 0.05	++
Pseudomonas arsenicoxydans ACM1	0.5	16	1.56 ± 0.03	2.41 ± 0.01	++
Pseudomonas mandellii ACM7	0.5	30	1.81 ± 0.02	1.03 ± 0.24	++
Pseudomonas mandellii ACM7	0.5	16	3.60 ± 0.02	1.15 ± 0.11	++
Pantoea cypripedi NE1	0.5	30	1.13 ± 0.01	2.32 ± 0.04	++
Pantoea cypripedi NE1	0.5	16	1.08 ± 0.01	2.06 ± 0.01	++

*(+ + +) intense fluorescence; (+ +) medium fluorescence; (+) light fluorescence.

Marinomonas sp. A3A, *Pseudomonas* sp. REST10 and *Pseudomonas mandellii* ACM7 achieved the higher cellular growth (1.58 ± 0.07 g/L, 2.28 ± 0.01g/L and 3.60 ± 0.02 g/L, respectively) nearly doubling their cellular growth when cultivated at 16ºC. In the case of *Pantoea cypripedi* NE1 and *Pseudomonas arsenicoxydans* ACM1, the difference in the CDW concentrations achieved was minimal (Table 3.3).

The EPS values produced does not seem to be very influenced by temperature, since the values obtained at both temperatures were similar (Table 3.3). However, these were slightly higher, at 16ºC, for *Marinomonas* sp.A3A, *Pseudomonas arsenicoxydans* ACM1 and *Pseudomonas mandellii* ACM7 (1.51 ± 0.09 g/L, 2.41 ± 0.01 g/L and 1.15 ± 0.11 g/L, respectively) and higher at 30ºC for *Pseudomonas* sp. REST10 (2.59 ± 0.08 g/L) and *Pantoea cypripedi* NE1 (2.32 ± 0.04 g/L). The highest value of EPS still being for *Pseudomonas* sp. REST10 at 30ºC (2.59 ± 0.08 g/L) and also achieved the highest values at 16ºC (2.51 ± 0.08 g/L).

The same was true for the production of PHAs, where the results were also very identical, except in the case of *Marinomonas* sp.A3A, whose presence of PHA was more perceptible at 30ºC. As verified for the EPS value, the greatest presence of PHA was also achieved by *Pseudomonas* sp. REST10, and also for *Marinomonas* sp. A3A at 30ºC (+++), while the least PHA detected was by *Marinomonas sp.*A3A grown at 16ºC.

Considering all the results, can be concluded that *Marinomonas* sp. A3A is the bacteria most affected by temperature, seeming to be able to produce more PHA at higher temperatures, namely at 30ºC, but produce more EPS and achieve greater growth at 16ºC.

In general, it seems that at 16ºC *Marinomonas* sp. A3A, *Pseudomonas* sp. REST10 and *Pseudomonas mandellii* ACM7 may obtain better results, both in terms of cellular growth and production of biopolymers.

Several species of *Marinomonas* have an optimal temperature range for growth of 15–25 ° C, which is consistent with the results obtained (Espinosa et al., 2010).

For a large number of strains of *Pseudomonas* sp. their growth was determined between 4ºC and 41ºC, the majority being characterized as mesophilic (Chakravarty & Gregory, 2015), being able to grow in a wide temperature range, as can be confirmed with this assay.

Little is known about the optimal growth temperature of the genus *Pantoea*, but in literature is reported that *Pantoea agglomerans* can grow between 4ºC to 42ºC, appearing to be a versatile species in terms of temperature (Abbas et al., 2017).

3.1.2.3. Effect of glucose concentration

Another parameter that can influence the cellular growth and product synthesis is the carbon source concentration. Therefore, as these bacteria are new, it was decided to test them with different concentrations of glucose (2.5 g/L and 10 g/L) to understand its impact. Glucose is a carbon source, which is usually present in small amounts in the soil, constituting a limiting resource for microbial activity (Reischke et al., 2014). For fermentation to happen, glucose (carbon source) needs to be in excess.

Table 3.4 - Cell dry weight (g/L), EPS production (g/L) and PHA detection, after shake flask cultivations on TSB medium with different glucose concentrations (2.5g/L and 10g/L).

Bacteria	glucose (g/L)	CDW (g/L)	EPS (g/L)	PHA*
Marinomonas sp. A3A	2.5	0.64 ± 0.02	1.15 ± 0.09	+++
Marinomonas sp. A3A	10	0.74 ± 0.02	1.29 ± 0.16	+
Pseudomonas sp. REST10	2.5	1.09 ± 0.03	2.59 ±0.08	+++
Pseudomonas sp. REST10	10	2.36 ± 0.02	1.46 ± 0.05	++
Pseudomonas arsenicoxydans ACM1	2.5	1.56 ± 0.03	2.41 ± 0.01	+++
Pseudomonas arsenicoxydans ACM1	10	2.61 ± 0.03	2.00 ± 0.09	++
Pseudomonas mandellii ACM7	2.5	1.81 ± 0.02	1.03 ± 0.24	++
Pseudomonas mandellii ACM7	10	2.83 ± 0.01	1.28 ± 0.03	++
Pantoea cypripedi NE1	10	0.59 ± 0.01	1.15 ± 0,02	+

*(+ + +) intense fluorescence; (+ +) medium fluorescence; (+) light fluorescence

The TSB medium in its original formulation is only composed of 2.5 g/L of glucose. As this amount is very low**,** it was decided to test the bacteria with a concentration of 10g /L of glucose to make sure that the carbon source was not depleted, as it needs to be in excess to increase EPS and PHA production.

As expected, the CDW value was higher when cultivated with 10g/L of glucose, except for *Pantoea cypripedi* NE1 were the same was not verified. One possibility is that this bacterium does not need such a large amount of glucose, and so when tested with 10g/L it has an inhibitory effect, which can be corroborated by the low values of the specific growth rate (0.03 h^{-1}) and the polymers produced (Table 3.4).

Concerning the EPS production, was higher when cultivated with 10 g/L of glucose for *Marinomonas* sp. A3A (1.29 ± 0.16 g/L) and *Pseudomonas mandellii* ACM7 (1.28 ± 0.03 g /L). For the remaining bacteria, namely, *Pseudomonas* sp. REST10, *Pseudomonas arsenicoxydans* ACM1 and *Pantoea cypripedi* NE1 the EPS concentration reached was considerably lower than in the cultivations with 2.5 g/L of glucose (Table 3.4).

Regarding the presence of PHA, the results were only maintained for *Pseudomonas mandellii ACM7* in both concentrations tested, while for the remaining bacteria, PHA was less detected when cultured with 10 g /L glucose.

When looking at these results, it was expected that the bacteria were able to produce a larger amount of polymers with a concentration of 10g/L of glucose. These results are probably due to the problems associated with cultivation in shake flasks, namely the aeration conditions and stirring that are not ideal, as well as the non-control of pH which can compromise cell viability and therefore the accumulation of PHA and EPS.

3.1.3. Characterization of PHA and EPS synthesized in TSB medium

Both PHA and EPS obtained in the best studied conditions were evaluated about its composition.

For PHA detection, all the strains were tested with Nile blue, which is a lipophilic dye used to stain colonies and to distinguish between PHA-accumulating and non-accumulating strains (Spiekermann et al., 1999), being possible to observe PHA accumulation by fluorescence microscopy. Figure 3.1 shows that all the bacteria presented fluorescence that indicates accumulation of PHA granules by each strain. However, despite the fluorescence observed at microscope, it was

not possible to quantify the PHA accumulated by each strain through gas chromatography, probably due to the low PHA cellular content. The low content could be related with the low glucose concentration (2.5 g/L) in the TSB medium, since the bacteria need to have carbon available for PHA synthesis (Nikodinovic-Runic et al., 2013).

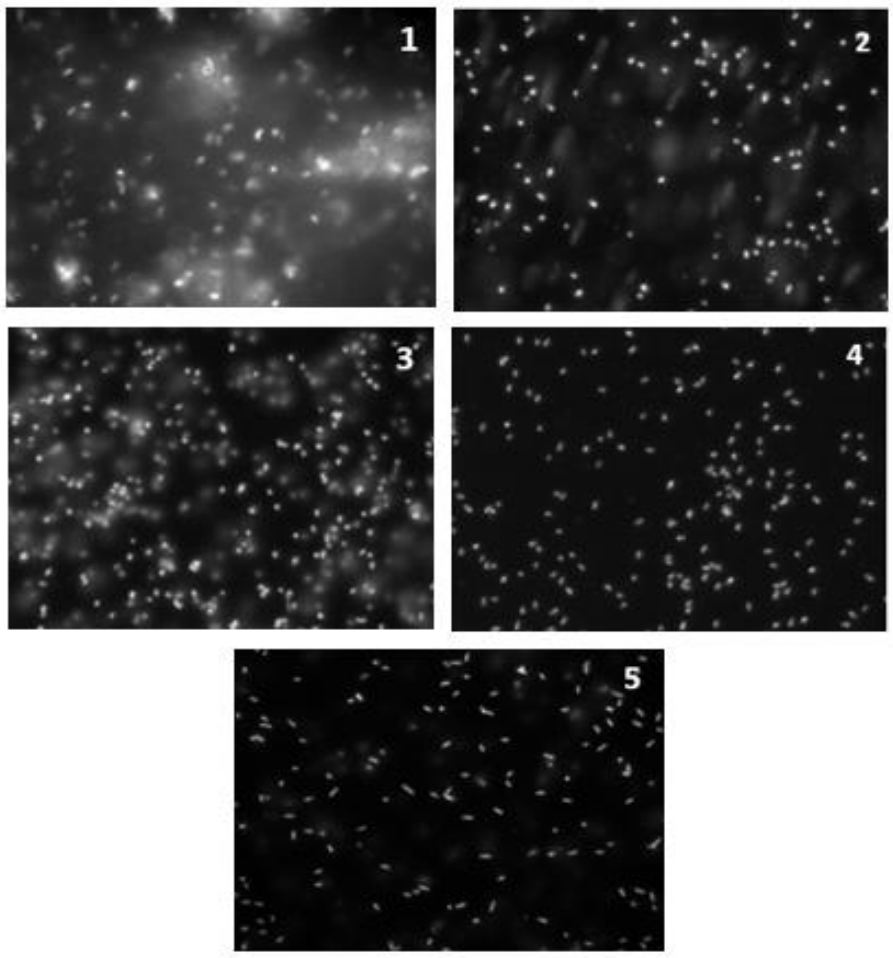


Figure 3.1 - Fresh samples of *Marinomonas* sp. A3A (1), *Pantoea cypripedi* NE1 (2), *Pseudomonas arsenicoxydans* ACM1 (3), *Pseudomonas mandellii* ACM7 (4) and *Pseudomonas* sp. REST10 (5) stained with Nile Blue under the microscope (1000x) after 53 h of cultivation.

The EPS produced by the bacteria were evaluated about sugar composition (Figure 3.2). For all the EPS synthesized by the different bacteria, the sugar in the highest percentage is galactose (24 and 43 % mol). Rhamnose (13 and 26 % mol) and arabinose (18 and 21 % mol) are present in the EPS synthesized by all bacteria with the exception of the EPS produced by *Pseudomonas arsenicoxydans* ACM1, which is only composed by fructose (7 % mol), galactose (47 %mol) and glucose (46 % mol). Beyond the EPS produced by *Pseudomonas arsenicoxydans* ACM1, glucose is also present in the EPS produced by *Marinomonas* sp. A3A, *Pseudomonas mandellii* ACM7 and *Pseudomonas* sp. REST10. Moreover, in the composition of the different EPS were also

detected fructose (*Pseudomonas mandellii* ACM7, *Pseudomonas arsenicoxydans* ACM1 and *Marinomonas* sp. A3A), fucose and mannose (*Marinomonas* sp. A3A and *Pantoea cypripedi NE1*). Acidic sugars, namely galacturonic (*Marinomonas* sp. A3A, *Pantoea cypripedi* NE1, *Pseudomonas mandellii* ACM7 and *Pseudomonas* sp. REST10) and glucuronic acid (*Marinomonas* sp. A3A and *Pantoea cypripedi* NE1) were also detected in the EPS synthesized by the different bacteria.

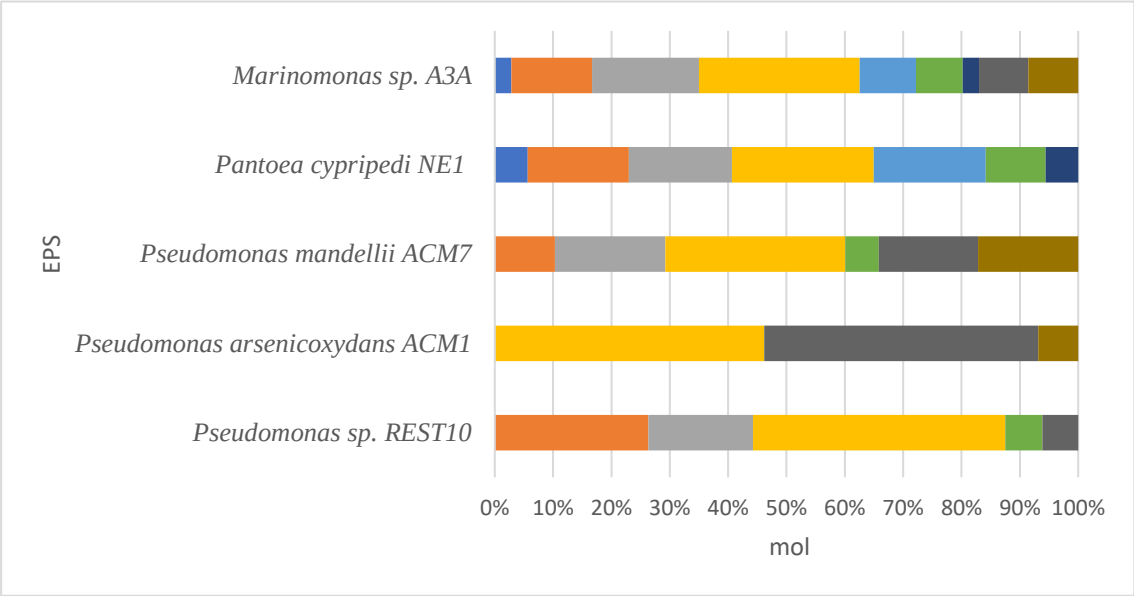


Figure 3.2 - Sugar monomers composition (% mol) profile for the EPS synthesized by the bacteria *Marinomonas sp*. A3A, *Pantoea cypripedi* NE1, *Pseudomonas mandellii* ACM7, *Pseudomonas arsenicoxydans* ACM1 and *Pseudomonas sp.* REST10 grown in TSB medium. Dark blue – fucose; Orange - rhamnose; Grey – arabinose; Yellow – galactose: Light blue – mannose; Dark grey – glucose; Brown – fructose; Green – galacturonic acid; Purple – glucuronic acid.

Some of the sugars presented in the composition of EPS can be considered rare when they are not easily found in nature. Therefore, the scarcity makes them very valuable, and thus bacteria capable of producing those sugars are of great interest. Within the sugars considered rare are fucose, rhamnose and uronic acids. On the other side the most common sugars are glucose, galactose, fructose, xylose, ribose and arabinose (Roca et al., 2015).

The EPS produced by *Marinomonas* sp. A3A and *Pantoea cypripedi* NE1 have an interesting composition, since it is composed by several sugars, namely some rare sugars, such as fucose, rhamnose and uronic acids (galacturonic and glucuronic acids). The EPS produced by *Pseudomonas mandellii* ACM7 and *Pseudomonas* sp. REST10 are the most similar to each other, having in their composition, rhamnose and galacturonic acid, considered rare sugars. As these EPS have

some potential in their composition, it will be interesting to carry out further studies to test the bacteria under different conditions to see if the percentage of rare sugars could be increased.

In the literature *Bacillus licheniformis* B22 grown in TSB medium presented a sugar monomer composition similar to that obtained in this work. The sugar obtained in a higher percentage were galactose, glucose and fructose, the same was found for *Pseudomonas arsenicoxydans* ACM1 and *Pseudomonas mandellii* ACM7 but, in this one, arabinose also reached a higher percentage. For the remaining bacteria it only occurred for galactose (Figure 3.2). Also presented a smaller percentage in mannose, rhamnose and arabinose and it was composed of uronic acids in a very low amount, as found in these bacteria.

3.1.4. Bacteria cultivation on other mediums

Beyond the tests with TSB broth, bacteria were also cultivated on other media to understand their impact on the growth and production of polymers. Thus, the bacteria were then grown in LB medium, a nutritionally rich medium and in medium E, a simple medium, mostly composed of inorganic salts.

3.1.4.1. LB media

The bacteria were first tested with the original formula of the LB medium, except for *Marinomonas* sp. A3A since from previous, the bacterium did not grow on LB medium (data not shown).

Table 3.5 - Cell dry weight (g/L), EPS production (g/L) and PHA detection, after shake flask cultivations on LB medium at 30ºC.

Bacteria	CDW (g/L)	EPS (g/L)	PHA*
Pseudomonas sp. REST10	1.27 ± 0.04	2.18 ± 0.08	+++
Pseudomonas arsenicoxydans ACM1	1.40 ± 0.02	0.96 ± 0.46	++
Pseudomonas mandellii ACM7	1.13 ± 0.05	0.67 ± 0.02	++
Pantoea cypripedi NE1	0.56 ± 0.02	1.06 ± 0.16	+

* (+ + +) intense fluorescence; (+ +) medium fluorescence; (+) light fluorescence.

Table 3.5 shows that the bacterium with a better cellular growth was *Pseudomonas arsenicoxydans* ACM1 attaining 1.4 ± 0.02 g/L of CDW, followed by *Pseudomonas* sp. REST10 (1.27 ± 0.04 g/L) and *Pseudomonas mandellii* ACM7 (1.13 ± 0.05 g/L), respectively. *Pantoea cypripedi* NE1 (0.56 ± 0.02 g/L) was the one that obtained the lowest value.

In the TSB medium, *Pseudomonas arsenicoxydans* ACM1 also obtained the highest CDW value (1.91±0.02 g/L) and *Pantoea cypripedi* NE1 the lowest (1.13 ± 0.01 g/L). However, in this medium the bacterium that obtained the second highest CDW value was *Pseudomonas mandellii* ACM7 (1.81 ± 0.02 g/L) and not *Pseudomonas* sp. REST10 (1.31 ± 0.03 g/L) as in the medium LB. When comparing the values obtained with the LB and TSB medium, the TSB medium allowed to obtain a higher biomass value for all the bacteria.

Regarding the production of EPS and PHA, the one that stood out the most was *Pseudomonas* sp. REST10 with 2.18 ± 0.08 g/L of EPS, as in TSB medium and, was also the one with the higher presence of PHA. All the other bacteria synthesized a much inferior concentration of EPS than in TSB medium (Table 3.1 and 3.5).

After the bacteria were tested with the LB medium in its original formulation, tests were performed with an increased salt concentration (3% w/v). Furthermore, the impact of glucose was also attained by adding glucose to the medium as a new carbon source.

Table 3.6 - Cell dry weight (g/L), EPS production (g/L) and PHA detection, after shake flask cultivations on LB medium with different salt (1% or 3% w/v of NaCl) and glucose concentrations (0 or 10 g/L of glucose).

Bacteria	NaCl (% w/v)	Glucose (g/L)	CDW (g/L)	EPS (g/L)	PHA*
Marinomonas sp. A3A	3	0	0.60 ±0.01	0.74 ± 0.06	++
Marinomonas sp. A3A	3	10	1.39±0.05	1.11 ± 0.03	+
Pseudomonas sp. REST10	3	0	1.24 ± 0.01	0.62± 0.09	++
Pseudomonas sp. REST10	3	10	2.27± 0.02	0.87± 0.1	+++

Pseudomonas sp. REST10	1	10	2.52± 0.01	0.97± 0.06	+++
Pantoea cypripedi NE1	1	10	0.28±0.01	0.90 ± 0.03	+
Pseudomonas mandellii ACM7	1	10	1.90±0.02	0.9 ± 0.03	+
Pseudomonas arsenicoxydans ACM1	1	10	1.77± 0.01	0.53 ± 0.08	+++

*(+ + +) intense fluorescence; (+ +) medium fluorescence; (+) light fluorescence

Marinomonas sp. A3A was grown with a salt concentration similar to that of previous assays with TSB medium, without and with the addition of glucose (10 g/L). Looking at the data, this bacterium was able to obtain a higher CDW value (1.39 ± 0.05 g/L) and EPS production (1.11 ± 0.03 g/L) when grown with glucose, concluding to be a good source of carbon to be applied in the growth of these bacteria. Considering the cellular growth was better than when grown on TSB medium in the same conditions (0.74±0.02 g/L).

The same was tested for *Pseudomonas* sp. REST10 (since it also comes from a marine environment), which achieved a greater growth (2.27 ± 0.02 g/L) and production of polymers (0.87 ± 0.1 g/L of EPS; +++ for PHA) with the addition of glucose. This bacterium was also tested with glucose, but with no extra salt addition (10g/L of NaCl) where the results obtained were even better, obtaining a higher CDW value (2.52 ± 0.01 g/L) and EPS produced (0.97 ± 0.06 g/L). When comparing these results with those obtained without salt and glucose addition (Table 3.5), they were better, except for the EPS concentration (2.18 ± 0.08 g/L), which was very superior in the original formula of LB medium. The detection of PHA was similar in both cases.

Pantoea cypripedi NE1, *Pseudomonas mandellii* ACM7 and *Pseudomonas arsenicoxydans* ACM1 were tested with 10g/L of glucose. *Pantoea cypripedi* NE1 achieved better results when grown with the original LB media without glucose addition (Table 3.5). *Pseudomonas mandellii* ACM7 achieved a higher CDW (1.90 ± 0.02 g/L) and EPS value (0.9 ± 0.03 g/L) with glucose added to the medium (Table 3.6). In the case of *Pseudomonas arsenicoxydans* ACM1, it also managed to obtain a higher CDW (1.77 ± 0.01 g/L) with this supplemented medium, however the EPS value was lower (0.53 ± 0.08 g/L) than with the original medium (0.96 ± 0.46 g/L).

The CDW value that stood out the most was that of *Pseudomonas* sp. REST10 (2.52 ± 0.01 g/L), while the lowest was obtained by *Pantoea cypripedi* NE1 (0.28 ± 0.01 g/L). EPS values were not very different from each other, with the highest value being obtained by *Marinomonas* sp. A3A (1.11 ± 0.03g/L) and the lowest by *Pseudomonas arsenicoxydans* ACM1 (0.53 ± 0.08 g/L).

3.1.4.2. Characterization of EPS in LB medium

The sugar composition of EPS produced by the bacteria is shown in figure 3.3 where can be observed that mannose is the sugar in the highest percentage (30 to 65 % mol), followed by glucose (26 to 28 % mol). Galactose (4 to 29% mol) is present in *Pantoea cypripedi* NE1, *Pseudomonas arsenicoxydans* ACM1 and *Pseudomonas* sp. REST10 and rhamnose (7 to 15 mol%) is found in *Pantoea cypripedi* NE1, *Pseudomonas mandellii* ACM7 and *Pseudomonas* sp. REST10.

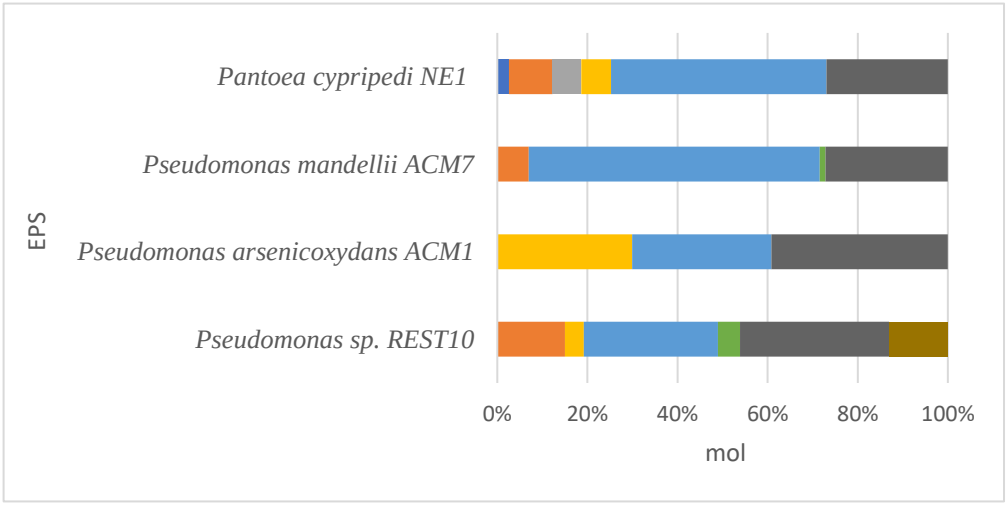


Figure 3.3 - Sugar monomers composition (% mol) profile for the EPS synthesized in LB medium by the bacteria *Pantoea cypripedi* NE1, *Pseudomonas mandellii* ACM7, *Pseudomonas arsenicoxydans* ACM1 and *Pseudomonas* sp. REST10. Dark blue – fucose; Orange - rhamnose; Grey – arabinose; Yellow – galactose; Light blue – mannose; Dark grey – glucose; Green – galacturonic acid; Brown – fructose.

Fructose (13 mol%) is only present in *Pseudomonas* sp. REST10 and this bacterium together with *Pseudomonas mandellii* ACM7 have in their constitution galacturonic acid in a small percentage (5 mol%), a uronic acid with great potential.

Pantoea cypripedi NE1 shows the a great diversity of sugars, being the only one where fucose (3 mol%) and arabinose (6 mol%) where detected, even though in very low concentrations.

From this analysis it can be concluded that *Pantoea cypripedi* NE1 and *Pseudomonas* sp. REST10 produced EPS with a more interesting composition of sugars due to its greater diversity and content in rare sugars. (Figure 3.3). On the other hand, *Pseudomonas mandellii* ACM7 and

Pseudomonas arsenicoxydans ACM1 synthesized EPS with a simpler composition, being composed mainly by rhamnose, mannose and glucose (*P. mandellii* ACM7) and galactose, mannose and glucose *(P. arsenicoxydans* ACM1) which are considered common sugars. When comparing the EPS obtained by the cultures in LB medium with the EPS obtained in the TSB medium (Figure 3.2) it is possible to verify that the sugar monomer composition varied considerably. EPS synthesized with TSB medium are in general richer in rare sugars and in uronic acids. Another difference noted when comparing the results in both media is that in the LB medium the sugar in a higher percentage is mannose, while in the TSB medium it is galactose. The differences obtained can be related with the much inferior EPS synthesis in LB medium for *Pseudomonas mandellii* ACM7 and *Pseudomonas arsenicoxydans* ACM1 moreover, the production of EPS and its monomeric sugar composition depend on the nitrogen and carbon sources and its ratio in the culture medium, which may justify the differences found (Yilmaz et al., 2012).

3.1.4.3. Medium E

Finally, the bacteria were tested in medium E with 10g/L of glucose to compare the differences in cellular growth and polymer production between the different media. Taking into consideration some of the better results achieved with TSB and LB medium only some conditions of salt concentration and temperature were tested.

Table 3.7 - Cell dry weight (g/L), EPS production (g/L) and PHA detection, after shake flask cultivations on E medium with different temperatures (16ºC or 30ºC) and salt concentrations (0 or 30 g/L of NaCl).

Bacteria	T (ºC)	NaCl (%w/v)	CDW (g/L)	EPS (g/L)	PHA*
Marinomonas sp. A3A	30	3	0.83±0.01	0.30±0.02	+++
Pseudomonas sp. REST10	16	0	1.85±0.03	0.11±0.03	+++
Pseudomonas sp. REST10	30	3	1.68±0.01	0.39±0.04	+++
Pseudomonas mandellii ACM7	30	0	2.04±0.01	0.20±0.09	+
Pseudomonas arsenicoxydans ACM1	16	0	2.18±0.01	1.08±0.02	++

Pantoea cypripedi NE1	30	0	0.52±0.01	0.37±008	+

* (+ + +) intense fluorescence; (+ +) medium fluorescence; (+) light fluorescence)

Pantoea cypripedi NE1 when cultivated in medium E, obtained the lowest CDW (0.52 ± 0.01 g/L) and a low EPS value (0.37 ± 0.08 g/L). With the same conditions, *Pseudomonas mandellii* ACM7 was able to obtain a CDW value much higher (2.04 ± 0.01 g/L), however, it managed to obtain an even lower value of EPS (0.20 ± 0, 09 g/L).

Pseudomonas sp. REST10 was cultivated on medium E, under two conditions, at 16ºC and at 30ºC with the addition of 3% (w/v) of NaCl. It obtained a higher CDW value at 16ºC (1.85 ± 0.03 g/L), a higher production of EPS at 30ºC (0.39 ± 0.04 g/L) and a similar PHA presence in both conditions. The values did not show significant differences, appearing to be a versatile bacterium resistant to different conditions, without having a major impact on its metabolism.

Pseudomonas arsenicoxydans ACM1 when cultivated in medium E, obtained the highest CDW (2.18 ± 0.01 g/L) and EPS value (1.08 ± 0.02 g/L), seeming to be the most favored with these media.

Marinomonas sp. A3A was one of the bacteria with the lowest results in this medium, except for the great presence of PHA detected (Figure 3.4). Having obtained better results in the remaining parameters when grown with richer media (LB and TSB), appearing to need a more nutritious broth to obtain greater growth and EPS production.

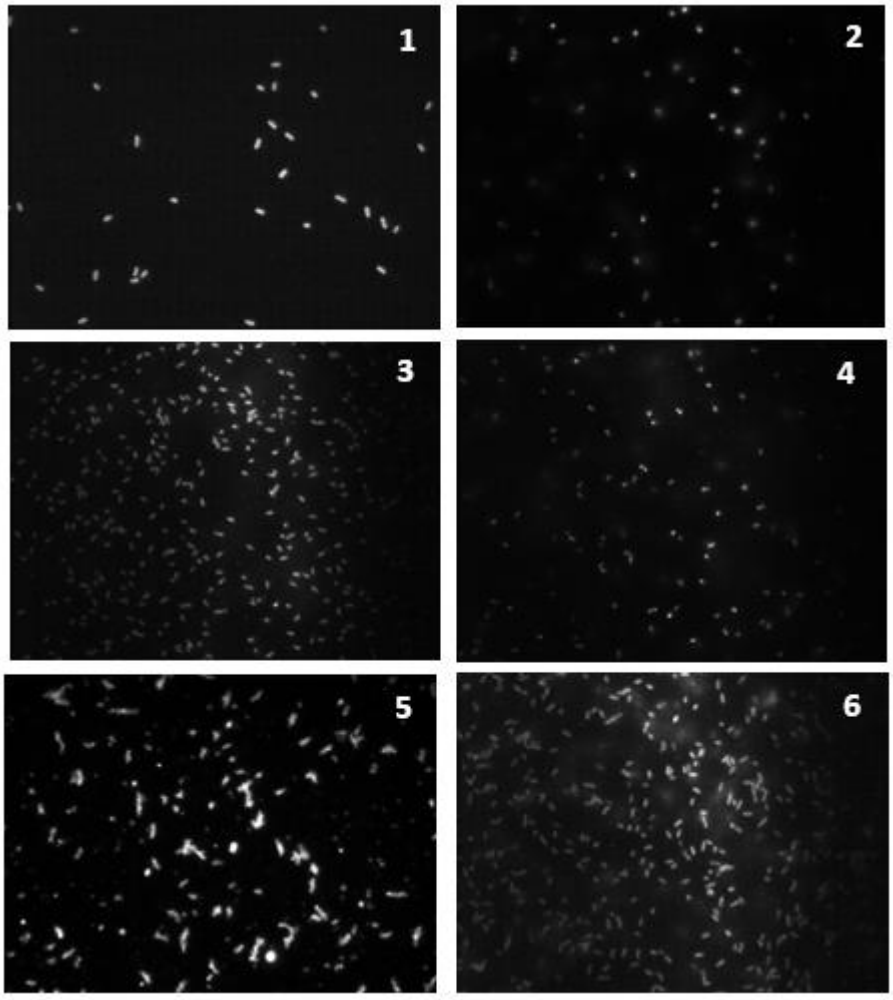


Figure 3.4 - Fresh samples of *Pantoea cypripedi* NE1 (1), *Pseudomonas mandellii* ACM7 (2), *Pseudomonas* sp. REST10 at 16ºC (3), *Pseudomonas arsenicoxydans* ACM1 (4), *Marinomonas* sp. A3A (5) and *Pseudomonas* sp. REST10 at 30ºC 3% (w/v) of NaCl (6) grown in medium E, stained with Nile Blue under the microscope (1000x).

3.1.4.4. Characterization of the EPS synthesized in medium E

The monomeric sugar composition of EPS synthesized by the different cultures cultivated in medium E is presented in figure 3.5.

It is interesting to notice that the EPS synthesized by the bacteria cultivated in medium E are totally different from that cultivated on TSB and LB medium (Figure 3.2 and 3.3). In this case the sugar presented in a higher concentration is galactose (12 to 43% mol) instead of mannose as in the LB medium, that nearly disappears (3% mol in the EPS synthesized by *Pseudomonas mandellii* ACM7).

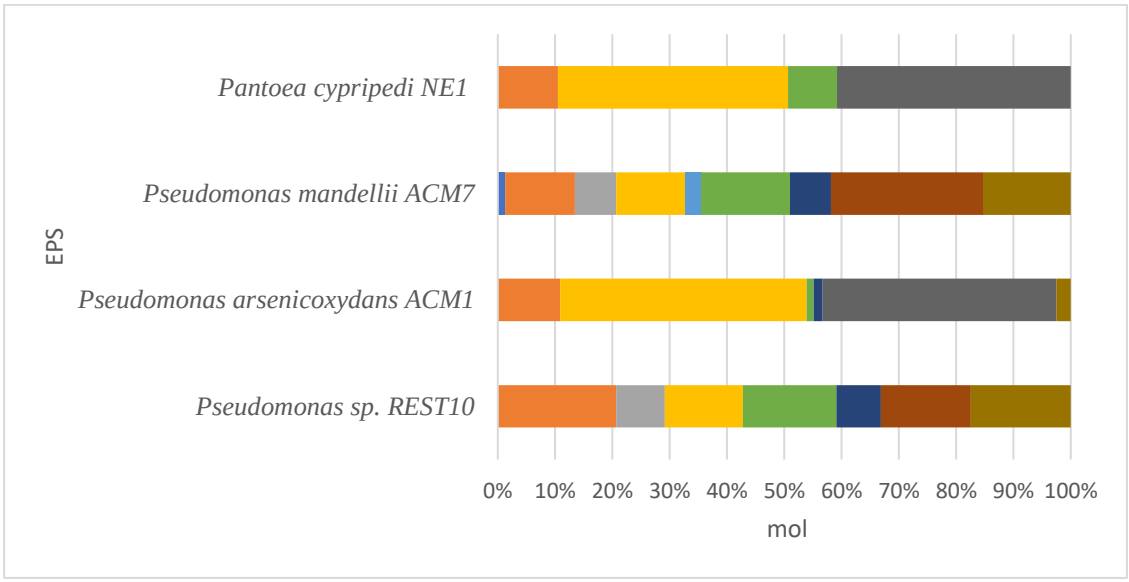


Figure 3.5 - Sugar monomers composition (% mol) profile for the EPS synthesized by the bacteria *Marinomonas* sp. A3A, *Pantoea cypripedi* NE1, *Pseudomonas mandellii* ACM7, *Pseudomonas arsenicoxydans* ACM1 and *Pseudomonas* sp. REST10 cultivated in medium E. Dark blue – fucose; Orange - rhamnose; Grey – arabinose; Yellow – galactose: Light blue – mannose; Dark grey – glucose; Brown – fructose; Green – galacturonic acid; Purple – glucuronic acid; Dark orange – sucrose.

Glucose (41% mol) is also present in a high concentration as in LB medium and in TSB medium with *Pseudomonas arsenicoxydans* ACM1, however is only present in the EPS synthesized by *Pantoea cypripedi* NE1 and *Pseudomonas arsenicoxydans* ACM1. Rhamnose (10 to 19 % mol) and galacturonic acid (1 to 16 % mol) are present in the EPS composition of all bacteria, which was not observed for the EPS synthesized in the other media (LB and TSB).

Pseudomonas sp. REST10, *Pseudomonas arsenicoxydans* ACM1 and *Pseudomonas mandellii* ACM7 also have glucuronic acid (1 to 16% mol) and fructose (2 to 17% mol) in their composition. Arabinose (7 to 8% mol) is only present in *Pseudomonas mandellii* ACM7 and *Pseudomonas* sp. REST10. Fucose, a rare sugar, was only produced by *Pseudomonas mandellii* ACM7 with a very low concentration of approximately 1% mol.

EPS produced by *Pseudomonas mandellii* ACM7 and *Pseudomonas* sp. REST10 obtained similar sugar compositions and have a very interesting composition, with a wide variety of sugars, some of which rare sugars, such as rhamnose and uronic acid with a wide variety of possible applications (e.g. anti-inflammatory substances, antioxidant, building blocks to synthesize the nucleoside analogs which are used as antiviral agents). Some bacterial polysaccharides rich in rare sugars are polymers containing fucose, such as colanic acid, fucogel, and clavan, or rhamnose, such as rhamsan, gellan, diutan, or welan gum, very often in combination with uronic acids (e.g. glucuronic or galacturonic acids). These polymers are being studied for new applications in

pharmaceuticals, cosmetics, food products, and can also be used as sources of rare sugar monosaccharides and precursors for the synthesis of high-value molecules (Roca et al., 2015).

PHAs could not be quantified, but their presence was detected in all bacteria with this medium (figure 3.4).

According to the literature, *Pseudomonas oleovorans* NRRL B-14682 grown in E medium presented four sugar residues, namely galactose, in a higher percentage (71%), followed by mannose (23%), rhamnose (4%) and glucose (3%), respectively. *Pantoea cypripedi* NE1 and *Pseudomonas arsenicoxydans* ACM1 also showed a high percentage of galactose, but also glucose with a similar percentage. All bacteria were reported to have galactose and rhamnose, however glucose was only present in *Pantoea cypripedi* NE1 and *Pseudomonas arsenicoxydans* ACM1 and mannose was only found in *Pseudomonas mandellii* ACM7 in a lower percentage than *Pseudomonas oleovorans* NRRL B-14682. The bacteria of this study showed a more diversified EPS composition and a bigger presence in rare sugars than the bacteria mentioned in the literature, which reveals the potential and interest of these bacteria (Freitas et al., 2009).

3.1.5. Experiments summary

In Table 3.8 the best results obtained for each bacteria in the three cultivation media tested (TSB, LB and E) are summarized. TSB medium demonstrated to be the most suitable medium for cultivation of the tested bacteria, since the higher EPS concentration as well as the best cellular growth were achieved in the cultivations with this medium. It makes sense, since it is the richest, being the one most likely to fulfill the nutritional requirements of bacteria and providing them with the necessary nutrients to achieve better results.

Despite TSB medium showed the best results for EPS production and CDW, the EPS synthesized with medium E presented an interesting sugar monomer composition.

The exception is *Pseudomonas arsenicoxydans* ACM1 that achieved higher CDW when cultivated in medium E.

About temperature the best results were achieved at 16ºC instead of 30ºC, nevertheless in the majority of the studies the difference in cellular growth and EPS production was not very high.

Table 3.8 - Cell dry weight (g/L), EPS production (g/L) and PHA detection, after shake flask cultivations on TSB, LB and E medium.

Bacteria	Medium	T (ºC)	Salt (%w/v)	Glucose (g/l)	CDW (g/L)	EPS (g/L)	PHA*
Marinomonas sp. A3A	TSB	16	3	2.5	**1.58±0.07**	**1.51±0.03**	++
Marinomonas sp. A3A	LB	30	3	10	1.39±0.05	1.11±0.03	++
Marinomonas sp. A3A	E	30	3	10	0.83±0.01	0.3±0.02	+++
Pseudomonas sp. REST10	TSB	16	0.5	2.5	**2.28±0.01**	**2.51±0.08**	++
Pseudomonas sp. REST10	LB	30	1	0	1.27±0.04	2.18±0.08	+++
Pseudomonas sp. REST10	E	30	3	10	1.68±0.01	0.39±0.04	+++
Pseudomonas mandellii ACM7	TSB	16	0.5	2.5	**3.60 ± 0.02**	**1.15±0.11**	+
Pseudomonas mandellii ACM7	LB	30	1	10	1.90±0.02	0.90±0.03	++
Pseudomonas mandellii ACM7	E	30	3	10	2.04±0.01	0.20±0.09	+
Pseudomonas arsenicoxydans ACM1	TSB		0.5	2.5	1.56±0.03	**2.41±0.01**	+++
Pseudomonas arsenicoxydans ACM1	LB	30	1	0	1.40±0.02	0.96±0.46	++
Pseudomonas arsenicoxydans ACM1	E	16	0	10	**2.18±0.01**	1.08±0.02	++
Pantoea cypripedi NE1	TSB	30	0.5	2.5	**1.13±0.01**	**2.32±0.04**	++
Pantoea cypripedi NE1	LB	30	1	0	0.56±0.02	1.06±0.16	+
Pantoea cypripedi NE1	E	30	0	10	0.52±0.01	0.37±0.08	+

* (+++) intense fluorescence; (++) medium fluorescence; (+) light fluorescence

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3.2. Bioreactor assays

3.2.1. Batch bioreactor experiments

Bioreactor experiments were conducted by bacteria of the genus *Pseudomonas*, namely *Pseudomonas arsenicoxydans* ACM1, *Pseudomonas mandellii* ACM7 and *Pseudomonas* sp. REST10.

It was decided to choose these bacteria since the results obtained in the shake flask test were promising for EPS production and PHA production was also detected. In fact different species of the genus *Pseudomonas* are known to be good EPS and PHA producers (Freitas et al., 2009; Rebocho et al., 2019). Moreover, the bacteria evaluated in this study are new species isolated from different locations, being interesting to understand their potential and to compare them with other *Pseudomonas* sp. reported in literature. Taking into consideration previous results, bacteria performance was evaluated in 2 L bioreactor working on a batch mode under controlled conditions of pH (7.0), temperature (16ºC, 22ºC, 30 ºC) and dissolved oxygen concentration (20%) using TSB and E medium.

Figure 3.6 presents the cultivation profiles for *Pseudomonas arsenicoxydans* ACM1 (a) TSB medium and (b) medium E (Figure 3.6 a and b), *Pseudomonas mandellii* ACM7 in TSB medium (c) at 16ºC and (d) at 30ºC (Figure 3.6 c and d) and *Pseudomonas* sp. REST10 in TSB medium at 22ºC (Figure 3.6 e). All the runs had different times duration since as this was the first time that bioreactor cultivations were performed the assays run until the dissolved oxygen started increasing, which usually indicates the exhaustion of carbon source or the decreasing of cellular viability.

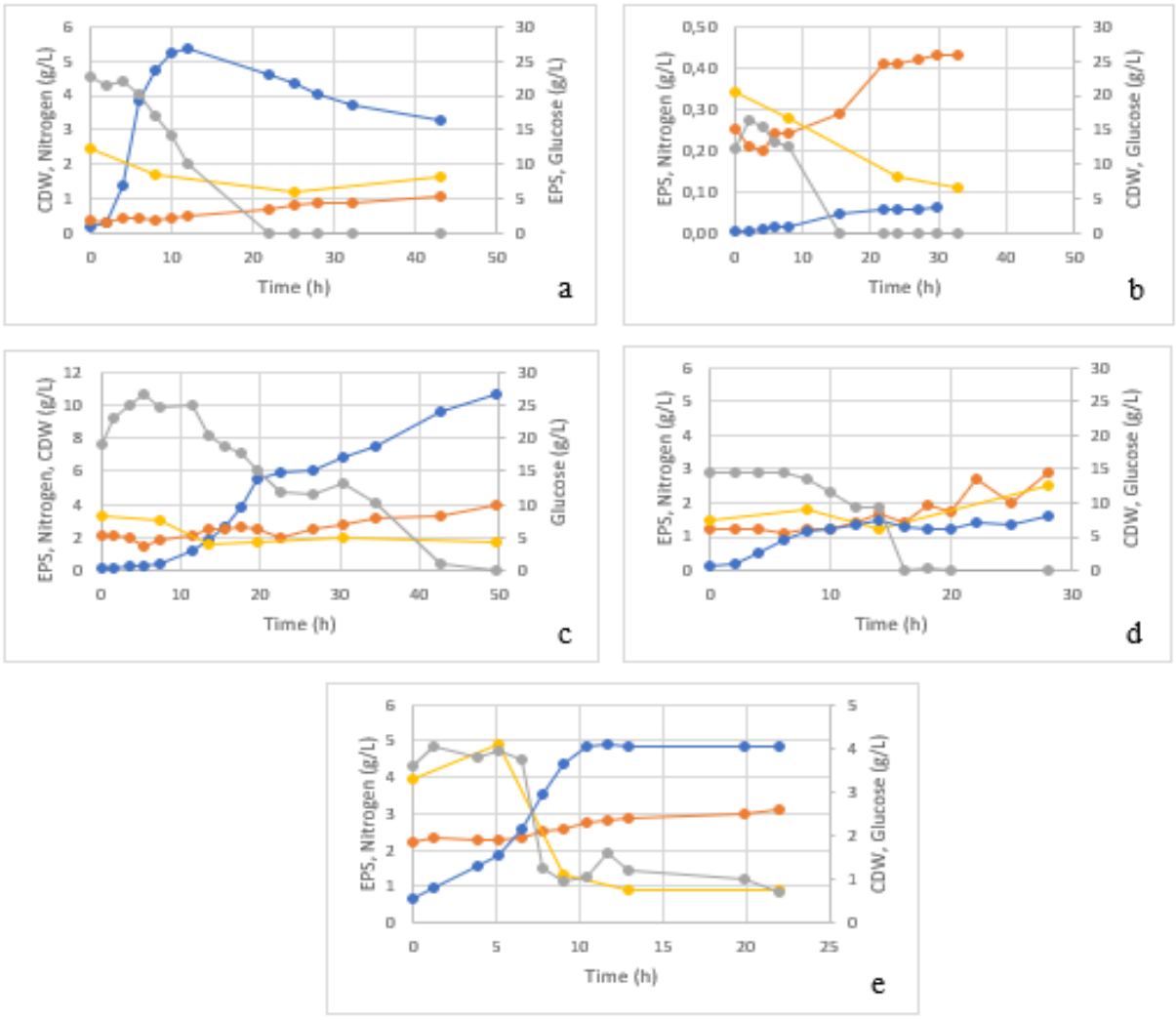

Figure 3.6 - Cultivation profile of *Pseudomonas arsenicoxydans* ACM1 with TSB medium (a), and with E medium (b), *Pseudomonas mandellii* ACM7 with TSB medium at 16ºC (c), and at 30ºC (d), *Pseudomonas* sp. REST10 (e), wherein experimental results of CDW (●), EPS (●), glucose (●) and nitrogen (●) are represented throughout the cultivation run.

Pseudomonas arsenicoxydans ACM1 presented a higher cellular growth in TSB medium than in medium E (Figure 3.6 a and b), achieving 5.27 g/L and 3.89 g/L of CDW, respectively. This result is different from that obtained in the shake flask assays (Table 3.8), which could be related with the nitrogen concentration available in each medium, for the cultivation in TSB the initial nitrogen concentration was 2,4g/L, while for medium E the initial nitrogen concentration was 0.4 g/L. In the assay with the TSB medium, there was practically no lag phase, having grown very quickly until reaching the maximum CDW that was achieved after 12h, using glucose to grow at a specific growth rate (μ) of 0.61 h^{-1} (Table 3.9). In medium E the culture grew at a μ of 0.17 h^{-1} ending the

exponential growth phase at 16 h of assay when glucose was depleted. In medium E the glucose was all consumed during cellular growth, the same was true for TSB medium, but after that CDW increases probably due to some PHA accumulation. Regarding EPS production, in TSB medium, EPS starts after the ending of cellular growth until to be produced, but it is at 19h when its production is most pronounced, reaching the maximum concentration of 5.33 g/L. At this same time, the cell concentration starts to drop, and the glucose concentration is greatly reduced, being depleted at 22h. The decrease in CDW is probably due to PHA being consumed as a carbon source for cell maintenance. Despite that, from 22h until the end of the assay the EPS continue being synthesized probably because bacteria used other carbon source available in the TSB medium. The culture when cultivated in medium E synthesized only 0.43 g/L of EPS, which is probably related with the depletion of glucose at the end of exponential growth phase. Hence, the bacterium had no carbon source available for EPS production which can be surpassed by increasing the initial glucose concentration or bay using a fed-batch strategy feeding a rich carbon solution continuously or by pulses.

Table 3.9 - Kinetic and stoichiometric parameters for all the different bioreactor experiments performed in this study.

Parameter	*Pseudomonas arsenicoxydans* ACM1		*Pseudomonas mandellii* ACM7		*Pseudomonas sp.* REST10
Medium	E	TSB	TSB	TSB	TSB
Temperature (ºC)	30	30	30	16	22
glucose(g/L)	20	20	20	20	2.5
CDW (g/L)	3.89	3.27	8.11	10.7	4.06
μ (h^{-1})	0.17	0.61	0.37	0.13	0.98
$Y_{X/s}(g_{biomass}/g_{subs})$	0.29	0.40	0.52	0.56	---
Glucose consumption rate (g/L.h)	0.08	0.7	0.05	0.38	0.13
EPS (g/L)	0.43	5.33	3.01	3.95	3.22

$Y_{EPS/S}$ (g_{EPS}/g_{subs})	0.01	0.15	0.14	0.10	0.32
rP (g/L.h)	0.01	0.12	0.11	0.08	0.15

Pseudomonas mandellii ACM7 were cultivated in bioreactor using TSB medium with 20 g/L of glucose at 16ºC (Figure 3.6 c), and at 30ºC (Figure 3.6. d) The culture at 30ºC start growing exponentially, at a μ of 0.37 h^{-1} 2 hours after inoculation, attaining the maximum cellular growth (8.11 g/L) after 14 hours of run, at this time almost all the glucose was consumed. EPS production seems to be partially associated with growth, since it increases after the end of the exponential phase, reaching a maximum value of 3.01 g/L at the end of cultivation time, corresponding to a volumetric productivity of 0.11 g/L.h, which is similar to those obtained for *Pseudomonas arsenicoxydans* ACM1 in TSB medium at 30ºC (0.12 g/L.h; Table 3.9).Since glucose finishes after 16h of run and the EPS was being produced across the stationary phase, bacteria was consuming other carbon sources provided by peptone, and probably due to that uptake the N present in the proteins of the peptones is being released to the medium increasing the N concentration (Figure 3.6 d) or due to protein production.
Pseudomonas mandellii ACM7 when cultivated at 16ºC (Figure 3.6 c) showed a longer lag phase (about 10h) since it needs to adapt to the low temperature.

After 12h initiates the exponential growth phase at a μ of 0.13 h^{-1}, which is a lower rate than in the assay at 30ºC, which is related with a low metabolism rate due to the low temperature. During exponential phase glucose start being consumed at a rate of 0.38 g/L, and during this phase EPS synthesis also. The bioreactor was finished when the glucose was just depleted. The end of exponential phase occurred near the 20 h of assay with a CDW of 6 g/L, however until the end of the cultivation run the CDW was always increasing reaching a concentration of 10.7 g/L, the highest value obtained from all the bioreactors performed in this work (figure 3.6.d). This increase in CDW could also be related with a possible PHA accumulation, since the culture presented fluorescence when observed at microscope (Figure3.7). Further, the EPS was also increasing slowly attaining a concentration of 3.95 g/L. These values were slightly higher than those obtained for the same bacterium at 30ºC (8.11 g/L CDW and 3.01 g/L EPS), however, the reactor's lifetime was longer (48h instead of 28h), resulting in a lower volumetric productivity (0.08 g/L.h), this difference not being significant enough to make up for the time spent and the economic costs.

In the cultivation of *Pseudomonas* sp. REST10 with TSB medium the exponential growth phase begins at 5 h of fermentation attaining 4.06 g/L of CDW, as well as the production of EPS, its synthesis being growth associated. Glucose consumption also starts at 5 h of cultivation, being consumed at a rate of 0.13 g/L.h. The bacterium reaches the stationary phase after 11 h, where a great reduction in the concentration of glucose can be observed. After that, glucose was no longer being consumed, nevertheless the EPS production continued probably by consuming some carbon from the medium, which seems that glucose is not the more suitable carbon source for polymer synthesis. The maxiumum EPS concentration 3.22 g/L was attained at the end of the run, corresponding to a rP of 0.15 g/L.h.

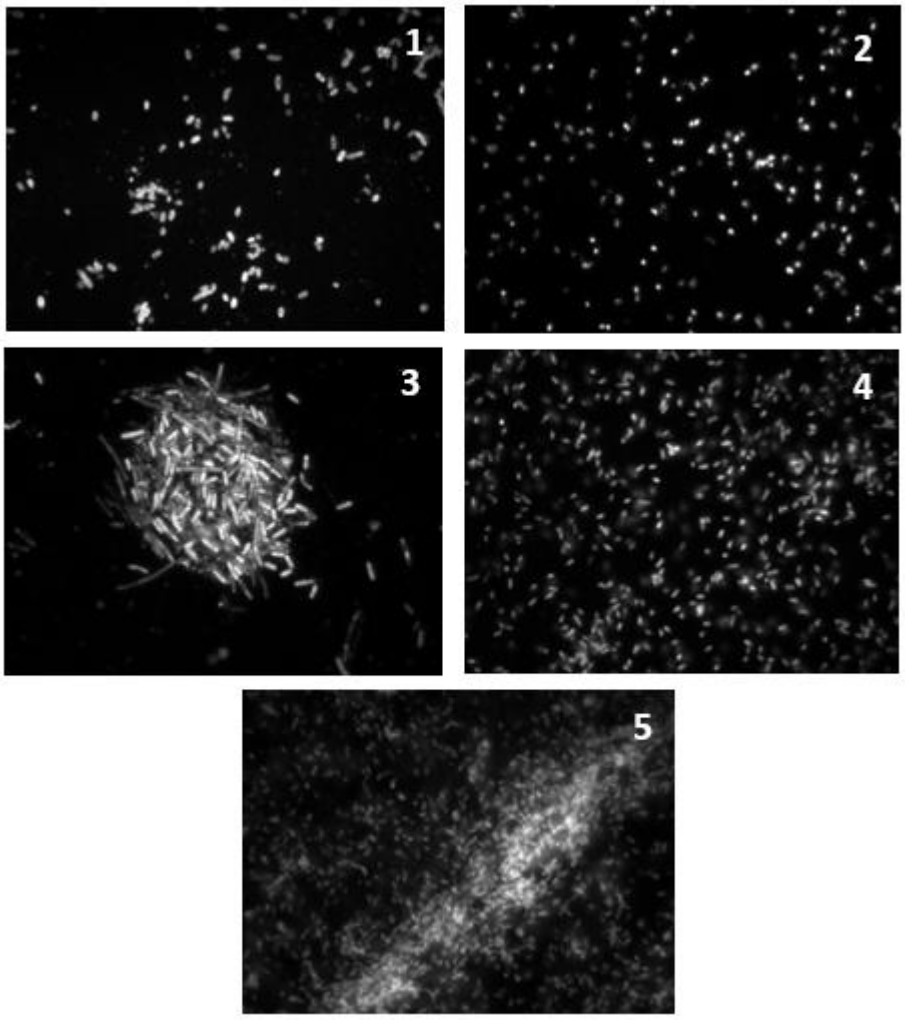

Figure 3.7 - Fresh samples of *Pseudomonas arsenicoxydans* ACM1 (1) in TSB medium and (2) in medium E, *Pseudomonas mandellii* ACM7 (3) in TSB medium at 30ºC and at 16ºC (4) and *Pseudomonas sp*. REST10 (5) in TSB medium at 22ºC stained with Nile Blue under the microscope (1000x).

In conclusion *Pseudomonas arsenicoxydans* ACM1 cultivated in TSB medium demonstrated to have a great potential for EPS production since it achieved a very good EPS production (5.33 g/L) in a batch mode (Table 3.9). Moreover, *Pseudomonas mandelli* ACM7 and *Pseudomonas* REST 10 also demonstrated potential for bioprocess optimization, reaching similar EPS concentration (3.01 - 3.22 g/L) with good volumetric productivities at 30ºC (0.11 - 0.15 g/L.h).

When looking at the EPS values (Figure 3.6) it is possible to verify that when bacteria were cultivated in TSB medium, the initial EPS concentration value immediately starts very high, and that the same does not occur in the assay carried out in medium E. One hypothesis would be that some proteins of the medium were not being eliminated during the purification step (dialysis) due to the high molecular weight. For that purpose, the protein content in some supernatant samples over the bioreactors assays with the TSB medium was evaluated, where it was found that its value was always higher in first sample (0.89 g/L - *Pseudomonas* sp. REST10, 1.17 g/L – *Pseudomonas arsenicoxydans* ACM1; 1.31g/L – *Pseudomonas mandellii* ACM7 at 30ºC , 1.36 g/L *Pseudomonas arsenicoxydans* ACM7 at 16ºC) and that throughout the assay it decreased (0.71 g/L - *Pseudomonas* sp. REST10, 0.65 g/L – *Pseudomonas arsenicoxydans* ACM1; 0.65 g/L – Pseudomonas mandellii ACM7 at 30ºC, 0.68 g/L Pseudomonas arsenicoxydans ACM7 at 16ºC), because the bacteria was consuming it. Then the amount of protein was also determined for the first and last EPS samples from the bioreactors. In these it was found that the amount of protein was always higher in the last sample compared to the first, meaning that some protein could be being produced. The values achieved were also in agreement with the concentration of EPS produced in each bioreactor, so the largest amount of protein in the last sample was that of the *Pseudomonas arsenicoxydans* ACM1 with the TSB medium (1.78 g / L), which obtained the highest value of EPS, followed by *Pseudomonas mandellii* ACM7 at 16ºC (1.69g / L), *Pseudomonas sp.* REST10 (1.57 g / L) and lastly *Pseudomonas mandellii* at 30ºC (0.76 g / L).

Considering the cell dry weight and EPS values obtained by the species *Pseudomonas* in the literature, some authors reported that *Pseudomonas oleovorans* reached a CDW value of 9.55 g / L at 30ºC and 3.47g/L at 20ºC, with an EPS production of 11.82 g /L and 1.90 g/L, respectively (Freitas et al., 2010). In another article it was reported that *Pseudomonas citronelloli* obtained a CDW value of 4.0 g/L (Rebocho et al., 2019). Looking at marine bacteria it was reported that *Pseudoalteromonas haloplanktis* was able to obtain 11g/L of CDW and 4.4 g/L of EPS with a volumetric productivity of 0.25 g/L.h in a fed-batch process (Wilmes et al., 2010). When comparing the values obtained in this work in a batch mode with those found in the literature, it can be concluded that some values were similar, showing the potential of these bacteria to produce

EPS. Nevertheless, the bioprocess for each bacterium needs to be optimized, after which they will surely obtain better results.

3.2.2. EPS and PHA characterization

The sugar monomer composition of the EPS produced with the *Pseudomonas* species in the bioreactors assays is represented in figure 3.8, where, in general, the composition seems very similar among all bioreactors performed. The same sugars are present for all results, except for *Pseudomonas arsenicoxydans* ACM1 grown in medium E (1) that does not contain arabinose and *Pseudomonas mandellii* ACM7 (3, 4) that does not include galacturonic acid in its constitution.

The sugar with the highest percentage is galactose (13 to 37 % mol), followed by glucose (13 to 30 % mol). Fucose, a rare sugar of great interest, is found in all EPS in the range of 4 to 8 % mol, it concentration increased when compared with the shake flask assays. Arabinose (12 to 26 % mol) and galacturonic acid (6 to 22 % mol), also considered a rare sugar, have a significant share in the composition of EPS, making them very attractive. Glucuronic acid that exists in the shake flask assay was not detected in the EPS synthesized in the bioreactor cultivations.

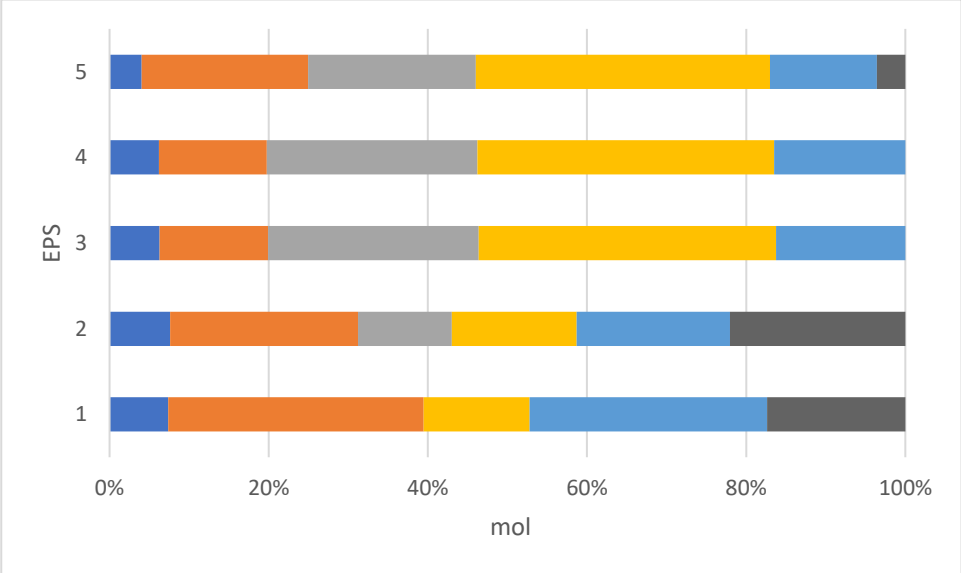

Figure 3.8 - Sugar monomers composition (% mol) profile for the EPS synthesized by the bacteria *Pseudomonas arsenicoxydans* ACM1 when cultivated in medium E (1) and TSB medium (2), *Pseudomonas mandellii* ACM7 at 30ºC (3) and 16ºC (4) and *Pseudomonas sp.* REST10 (5) cultivated in bioreactor assays. Dark blue – fucose; Orange - rhamnose; Light grey – arabinose; Yellow – galactose: Light blue – glucose; Dark grey – galacturonic acid.

Pseudomonas arsenicoxydans ACM1 was the one with the highest percentage of rare sugars in the EPS composition. This bacterium when grown in E medium was not able to produce arabinose. From the composition obtained from *Pseudomonas mandellii* ACM7, it is possible to verify that temperature does not influence the composition of EPS. The EPS of *Pseudomonas mandellii* ACM7 and *Pseudomonas* sp. REST10 ended up being very similar, the only most notable differences being the small presence of galacturonic acid and a higher percentage of rhamnose in *Pseudomonas* sp. REST10.

All the EPS synthesized have acyl groups in its composition, namely acetate, pyruvate and succinate. There are other EPS in the literature that contains acyl groups such as fucose-containing exopolysaccharide (EPS) produced by the bacterium *Enterobacter A47* and sphingan secreted by marine *Sphingomonas sp*. WG (Li et al., 2016; Torres et al., 2011).

In the literature, *Pseudomonas oleovorans* in batch reactor had a sugar composition consisting of galactose (68%), mannose (17%), glucose (13%) and rhamnose (2%). Compared with the EPS produced by the bacteria cultivated in bioreactors of this work, they exhibit a similar composition, also presenting in their constitution galactose (in a smaller percentage), glucose in a similar proportion and rhamnose with a higher percentage, which is very interesting because it is a rare sugar, however, they were not able to produce mannose. To compensate they are composed of other sugars, namely arabinose (except *Pseudomonas arsenicoxydans* ACM1 when cultivated in medium E), fucose and galacturonic acid (except *Pseudomonas mandellii* ACM7), which are rare sugars with great potential (Alves et al., 2011)

Fucopol, a commercial exopolysaccharide with several applications, namely used as thickening agent, gelling agent or emulsion stabilizer for food, cosmetic or pharmaceutical products, is composed of fucose (32-36 mol%), glucose (28-37 mol%), galactose (25-26 mol%), glucuronic acid (9-10 mol%) and acyl groups, namely succinyl (2-3 wt.%), pyruvyl (13-14 wt.%) and acetyl (3-5 wt.%). When analyzing and comparing the EPSs obtained in this work with fucopol, it can be noticed that they have the same sugar monomers as fucopol with the exception of glucuronic acid, which in turn presents galacturonic acid. The proportion of monomers is not the same, but with future optimizations

it is possible to make it more similar, showing the potential interest of these EPS (Freitas et al., 2011; Torres et al., 2015).

Gum arabic (GA), an edible, dried, gummy, with a wide industrial use in food, textile, pottery, lithography, cosmetics and pharmaceutical industries as stabilizer, thickening and emulsifier agent, is comprised of 39–42% galactose, 24–27% arabinose, 12–16% rhamnose, 15–16% glucuronic acid,1.5–2.6% protein, 0.22–0.39% nitrogen, and 12.5–16.0% moisture (Ali et al., 2009). When comparing the percentages of sugars, these were very similar for galactose, arabinose and rhamnose in the bacteria *Pseudomonas* sp. REST10 and *Pseudomonas mandellii* ACM7 at 30°C and 16°C (figure 3.8), showing again that these bacteria may have potential as marketable products.

In terms of PHA, it is known that the bacteria were able to produce it by observing them at the microscope with Nile Blue dye, where fluorescence was detected in all samples. PHA monomer composition was evaluated by gas chromatography, however due to some technical problems it was not possible to quantify them.

The bacteria presented all the monomers tested for both PHA (3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDd) and 3-hydroxytetradecanoate (3HTd)), as for PHB (3HB (3-hydroxybutyrate) and 3HV (3-hydroxyvalerate)). However, it is important to repeat the analyses and determine the percentages of each monomer in order to understand the potential of the polymer produced and its possible applications.

Chapter 4 :
Conclusions and Future Work

4.1. Conclusions and Future Work

Pseudomonas mandellii ACM7, *Pseudomonas arsenicoxydans* ACM1, *Pseudomonas* sp. REST10, *Marinomonas* sp. A3A and *Pantoea cypripedi* NE1 are able to produce PHA and EPS under all conditions tested. The TSB medium was the one that allowed to obtain the best results, being the most suitable to be used in these bacteria.

Pseudomonas arsenicoxydans ACM1 cultivated in TSB medium achieved the highest EPS concentration (5.33 g/L). The CDW value in this work was maximum with *Pseudomonas mandellii* ACM7 grown in TSB medium at 16ºC (10.7 g/L) in batch cultivation.

Regarding EPS, the sugar composition obtained by the bacteria was very similar in the reactor assays, with the monomers of galactose, glucose, rhamnose, arabinose, fucose and galacturonic acid being present. *Pseudomonas arsenicoxydans* ACM1 when cultivated in E medium obtained the most interesting composition as it was the richest in rare sugars, which present great value to the market. EPS, as well as PHA, has to be characterized about its molecular weight.

PHA was composed of all monomers tested (3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDd), 3-hydroxytetradecanoate (3HTd)), 3HB (3-hydroxybutyrate) and 3HV (3-hydroxyvalerate)). It is necessary to repeat the analysis to check these values and determine the percentage of each monomer in the polymer to understand if it exhibits good properties with potential market interest.

This study was a preliminary work as a proof of concept, thus much remains to be done, namely, reactor optimization, studying the source of carbon and nitrogen, and conducting further tests to clarify how cultivation conditions affect and influence the bacterial growth and polymer production.

Another important aspect to be accomplished in the future would be to study the other polymers that these bacteria are capable of producing, namely ectoin by *Marinomonas* sp. A3A and alginate by *Pseudomonas* sp. REST10 and to determine the taxonomy of these species.

It would also be important to study and discover more suitable growing conditions for *Marinomonas* sp. A3A, as it contaminates very easily, probably because the ideal conditions for its growth were not met.

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