Bruno Filipe Rodrigues Serafim
Bachelor's degree in Biology

Production of antibiotics and biopolymer by the bacterium *Pseudomonas chlororaphis* using glycerol

Dissertation for Master's degree in Biotechnology

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

Co-supervisor: Doctor Bárbara Fonseca de Almeida, UCIBIO-REQUIMTE, FCT-UNL

Jury
President: Professor Carlos Alberto Gomes Salgueiro
Examiner: Doctor Joana Oliveira Pais
Vogal: Doctor Cristiana Andreia Vieira Torres

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

September 2019

LOMBADA

Production of antibiotics and biopolymer by the bacterium *Pseudomonas chlororaphis* using glycerol
Bruno Serafim

2019

Bruno Filipe Rodrigues Serafim
Bachelor's degree in Biology

Production of antibiotics and biopolymer by the bacterium *Pseudomonas chlororaphis* using glycerol

Dissertation for Master's degree in Biotechnology

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

Co-supervisor: Doctor Bárbara Fonseca de Almeida, UCIBIO-REQUIMTE, FCT-UNL

Jury
President: Professor Carlos Alberto Gomes Salgueiro
Examiner: Doctor Joana Oliveira Pais
Vogal: Doctor Cristiana Andreia Vieira Torres

September 2019

Production of antibiotics and biopolymer by the bacterium *Pseudomonas chlororaphis* using glycerol

Agradecimentos

O meu primeiro agradecimento não poderia deixar de ir para Cristiana Torres, Bárbara Almeida e Filomena Freitas. Estas três pessoas não só contribuíram para este trabalho com os seus conselhos e esclarecimentos, como também me ajudaram a ter uma das mais desafiantes e interessantes experiências deste ano. No entanto, tenho ainda de acrescentar um especial obrigado à Cristiana pela sua paciência, e acima de tudo, pelas nossas incríveis conversas.

Um grande obrigado também a todas as outras pessoas do grupo BioEng, com quem partilhei horas no laboratório. Gostaria de agradecer em particular à Rita, ao João, à Patrícia e à avó Rita. As conversas que tivemos e os conselhos que me foram dando, tornaram este trabalho muito mais fácil.

Queria também agradecer a três pessoas do Laboratório de Análises - Nuno, Luz e Rodrigo. A paciência que tiveram para me ajudar a esclarecer algumas dúvidas foi muito importante.

O próximo agradecimento é para algumas pessoas que transformam as horas fora do laboratório em grandes momentos. Mauro, Asy, Tati, Ju, Mafa e Serrão, muito obrigado pelas histórias que tenho a contar sobre Lisboa.

A um Miguel que salta pocinhas, a um Valério que vai para casa cedinho e a mais treze incríveis pessoas da velhinha cidade, um grande obrigado. Tudo fica melhor num jantar de Panados.

Um obrigado muito especial à pessoa com quem mais gosto de perder tempo. Se existem pessoas que nunca deixaram de me apoiar, acreditar em mim e ajudar-me, tu és sem dúvida uma delas. Muito obrigado por tudo o que és para mim, Marta.

Por último, o meu enorme obrigado à minha família, em especial aos meus pais. Vocês são dos maiores responsáveis por aquilo que sou, e a minha maior motivação para fazer sempre mais e melhor. Muito obrigado por tudo o que fizeram por mim.

Abstract

Members of the *Pseudomonas* genus are known for their ability to produce multiple secondary metabolites, including bioactive metabolites, such as antibiotics. *Pseudomonas chlororaphis* subsp*. aurantiaca* DSM 19603 is a non-pathogenic bacterium widely used as plant growth-promoting rhizobacteria that has been reported to be able to produce three products of interest, namely, antibiotics (phenazines) and two biopolymers, medium-chain-length polyhydroxyalkanoates (mcl-PHA) and exopolysaccharides (EPS).

Phenazines are heterocyclic nitrogenous compounds that can act as antibiotic, antiparasitic or even anticancer agents. PHAs are a class of biobased and biodegradable polymers that depending on their composition and specific properties shows potential application on areas from thermoplastics to elastomers. EPS are a group of natural polymers mainly composed by sugars that due to their variety of physical and structural properties have many applications in industries such as food, pharmaceutical and cosmetics. This biopolymer could also be a good ally in bioremediation.

The main objective of this study was to develop and optimize the bioprocess for phenazines and biopolymers (mcl-PHA and EPS) co-production by *Pseudomonas chlororaphis* using glycerol as carbon source. Different nutrient (nitrogen and carbon) concentrations, pH control with NH_4OH and other conditions were tested in order to obtain higher productivities of all mentioned products.

The study showed that the same fermentation conditions (standard nutrients concentration and pH control with NH_4OH) enabled to achieve the best product concentrations, namely, a significant amount of phenazines (6.05 OD_{365nm}), an EPS concentration of 3.8 g/L and a mcl-PHA concentration of 8.43 g/L. According to literature, this mcl-PHA concentration is the highest achieved in *P. chlororaphis* fermentations. Further, carbon and nitrogen concentrations in culture medium seems to impact the mcl-PHA composition, which could guarantee different properties to this polymer.

Keywords

Pseudomonas chlororaphis; co-production; Phenazines; Medium-chain-length polyhydroxyalkanoates; Exopolysaccharides; Bioreactors.

Resumo

Membros do género *Pseudomonas* são conhecidos pela sua capacidade de produzir múltiplos metabolitos secundários, incluindo metabolitos bioativos, como antibióticos. *Pseudomonas chlororaphis* é uma bactéria não patogénica amplamente utilizada como rizobactéria promotora de crescimento de plantas, que pode produzir três produtos de interesse: antibióticos (fenazinas) e dois biopolímeros, polihidroxialcanoatos de cadeia média (mcl-PHA) e exopolissacarídos (EPS).

As fenazinas são compostos azotados heterocíclicos que podem atuar como antibióticos, antiparasitantes ou mesmo anticancerígenos. Os PHAs são uma classe de polímeros biodegradáveis de base biológica que, dependendo da sua composição e propriedades específicas, mostram potencial de aplicação em áreas dos termoplásticos aos elastómeros. Os EPS são um grupo de polímeros naturais compostos principalmente por açúcares que, devido à sua variedade de propriedades físicas e estruturais, têm muitas aplicações em indústrias como a alimentar, farmacêutica e cosmética. Este biopolímero também pode ser um bom aliado na área da biorremediação.

Este trabalho teve como principal objetivo desenvolver e otimizar o bioprocesso para coprodução de fenazinas e biopolímeros (mcl-PHA e EPS) por *Pseudomonas chlororaphis* subsp*. aurantiaca* DSM 19603 usando glicerol como fonte de carbono. Diferentes concentrações de nutrientes (azoto e carbono), controlo do pH com NH_4OH e outras condições foram testadas para se obter maiores produtividades de todos os produtos mencionados.

O estudo mostrou que, com as mesmas condições de fermentação (concentrações padrão de nutrientes e controle de pH com NH_4OH), foram alcançadas as melhores concentrações dos diferentes produtos, ou seja, uma quantidade significativa de fenazinas (6.05 OD_{365nm}), uma concentração de EPS de 3.8 g/L e uma concentração de mcl-PHA de 8.43 g/L. De acordo com a literatura existente, esta concentração de mcl-PHA é mais alta do que qualquer outra obtida em fermentações que também usaram estirpes de *P. chlororaphis*. As concentrações de carbono e azoto no meio de cultura influenciam a composição de mcl-PHA, o que poderia garantir propriedades diferentes a este polímero.

Palavras chave

Pseudomonas chlororaphis; coprodução; Fenazinas; Polihidroxialcanoatos de cadeia média; Exopolissacarídos; Biorreatores.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

CDW - Cell Dry Weight

DO - Dissolved Oxygen

EPS - Exopolysaccharide

GC - Gas Chromatography

HD - Hydroxydecanoate

HDd - Hydroxydodecanoate

HHx - Hydroxyhexanoate

HO - Hydroxyoctanoate

HPLC - High Performance Liquid Chromatography

HTd - Hydroxytetradecanoate

Mcl-PHA - Medium-chain-length Polyhydroxyalkanoates

MMC - Mixed Microbial Culture

OD - Optical Density

PHA - Polyhydroxyalkanoates

PKO - Palm Kernel Oil

QS - Quorum Sensing

RPM - Rotation Per Minute

Scl-PHA - Short-chain-length Polyhydroxyalkanoates

SFAE - Substrates derived From Animal Waste

SLPM - Standard Liters Per Minute

Chapter 1
Introduction and Motivation

1.1. *Pseudomonas chlororaphis*

Because of its abundance in the environment, *Pseudomonas* genus was first characterized long ago, and over the past hundred years, it has gone through many taxonomic revisions. Bacteria belonging to this genus are aerobic gram-negative rods that are found in biofilms or in planktonic forms. *Pseudomonad* bacteria could be further divided into subgroups due to its considerable heterogeneity based on pathogenicity or pigment production. Microorganisms of this genus inhabitants a range of environments including soil and water environments, in addition to plant and animal associations. Since they exhibit a vast metabolic versatility, these bacteria are known for their medical and biotechnological importance (Özen and Ussery, 2011).

One of the bacteria belonging to this genus is *Pseudomonas chlororaphis* (Anzai et al., 2000). This bacterium exhibits the capacity to produce several metabolites and products that are recognised as value-added products. Hydrogen cyanide, siderophores and phenazines are some of these metabolites associated to this bacterium (Nandi et al., 2015; Hohlneicher et al., 1995; Mavrodi et al. 2006). In 2007, Kasana et al. identify a strain of *P. chlororaphis* capable of transforming isoeugenol to vanillin. Besides these compounds, some strains of these bacteria also show ability to produce two kind of biopolymers, medium-chain-length polyhydroxyalkanoate (mcl-PHA) and exopolysaccharide (EPS) (Yun et al., 2003; Fett et al., 1996).

A work performed by Meneses, 2017 with the bacterium *Pseudomonas chlororaphis* subsp. *aurantiaca* DSM 19603 shows the potential of this strain to achieve a co-production of three products: phenazines, mcl-PHA and EPS.

1.1.1. Phenazines

Phenazines are heterocyclic nitrogen-containing compounds (Figure 1.1) produced by many bacterial species. These compounds show extraordinary potential due their physicochemical properties as redox potential and their bright pigmentation, which changes with pH and redox stat. Biochemical and structural studies demonstrate that several phenazine enzymes convert chorismic acid to phenazine-1,6-dicarboxylic acid (PDC) and phenazine-1-carboxylic acid (PCA). PDC and PCA then act as ‘‘core’’ phenazine that strain-specific enzymes convert to over 150 phenazine derivatives that have been isolated from natural sources. These compounds are coloured aromatic secondary metabolites and are excreted into the environment by bacteria Sometimes, bacteria produce them in such large quantities that they are visible to the naked eye (Figure 1.1) (Chincholkar and Thomashow, 2013; Yu et al., 2018).

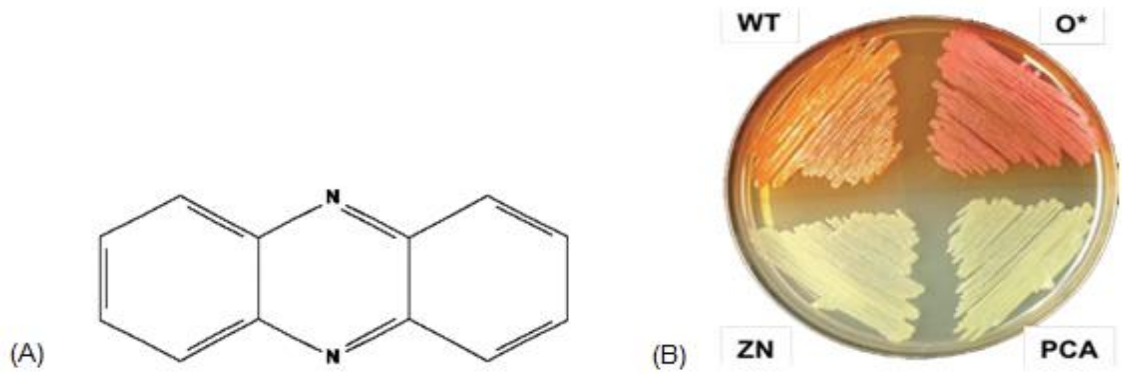


Figure 1.1 – (A) 2D phenazine structure (Pubchem.ncbi.nlm.nih.gov, 2019). (B) Agar plate containing *P. chlororaphis*: (WT) 30-84 wildtype; (O*) 30-84 2-OH-PCA overproducer; (ZN) 30-84 phenazine non-producer; (PCA) 30-84 PCA only producer (retrieved from Yu et al., 2018).

Phenazines present antifungal and antibacterial activities, being recognized as broad-spectrum antibiotics. Pierson and Pierson reported the ability of phenazines to eliminate the disease of wheat root caused by the fungus *Gaeumannomyces graminis var. graminis* (Pierson and Pierson, 1996). In literature is also demonstrated the capacity of phenazines to inhibit the growth of *Escherichia coli* and other bacteria (Saosoong et al., 2009). Furthermore, it was demonstrated, by different authors, that phenazines also exhibit antimalarial and anticancer activity (de Andrade-Neto et al., 2004; Imamura et al., 1997).

These antibiotic activities influenced researchers to increase the productivity of phenazine, although, chemical synthesis of phenazines shows relatively low productivity, requests harsh reaction process and produce toxic by-products as aniline (Wohl–Aue reaction) and azobenzoate (Chincholkar and Thomashow, 2013). However, phenazines can be produced by bacterial fermentation with extra benefit. Not only natural phenazines have proven to be more effective biocontrol agent than synthetic ones (Nansathit et al., 2009), as they show non-cytotoxicity, so they can be used as effective therapeutic agent for eukaryotic organisms (Laursen and Nielsen, 2004). Therefore, natural phenazines can be used for a wide range of biotechnological applications such as biocontrol agent, microbial fuel cell, antitumor agent, mineral reduction, oil degradation, anticandidal and food colorant (Chincholkar and Thomashow, 2013).

In order to produce phenazines in large scale, some parameters of phenazines fermentative processes have been studied. One of these parameters is the percentage of dissolved oxygen (DO) in medium. In 2010, Li et al., reported higher yields of phenazines production with DO at 20%. Another studied parameter is culture medium composition. Media components (e.g. sodium and potassium) influence the productivity of this metabolite during fermentation and suggested that phenazines production depending upon the nutritional conditions. Although phenazine production is an aged process, the impact of the nutritional composition is still not well documented (Pierson and Pierson, 2010; Chincholkar and Thomashow, 2013).

Phenazine producing bacteria are found all over nature, in association with plant and animal hosts that live in terrestrial, freshwater, and marine habitats. These metabolites play an important role in the ecosystems in which their producers and hosts live (Chincholkar and Thomashow, 2013). *Pseudomonas* genus, which have the ability to produce multiple secondary metabolites, is the principal phenazine producing bacteria with almost one-third of all known phenazines.

Fluorescent pseudomonads are the best studied phenazine producer which includes *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Pseudomonas chlororaphis*. *P. chlororaphis*, formerly known as *Pseudomonas aureofaciens* (Anzai et al., 2000), in addition to the core phenazine PCA, produces 2-Hydroxyphenazine-1-carboxylic acid (2-OH-PCA) and 2-Hydroxyphenazine (2-OH-PHZ) (Figure 1.2). As mentioned before, phenazines are coloured compounds, being that PCA presents a yellow colour, 2-OH-PCA presents an orange colour and 2-OH-PHZ presents a brick-red colour (Mavrodi et al. 2006).

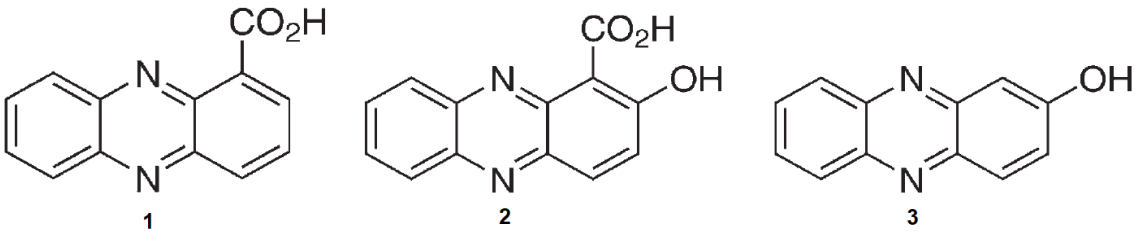


Figure 1.2 – (1) Phenazine-1-carboxylic acid. (2) 2-Hydroxyphenazine-1-carboxylic acid. (3) 2-Hydroxyphenazine (Beifuss et al., 2005).

In *Pseudomonas* sp., phenazines genes (phz) expression and the consequently phenazines production is under control of a two-component system, namely the quorum sensing (QS) mechanism and a small RNAs (sRNAs) system (Pierson III et al., 1998; De Maeyer et al., 2010; Haas and Défago, 2005; Bourret and Silversmith, 2010).

The sRNAs signalling is controlled by GacS sensor kinase and is highly important, because comprehend bacteria potential to directly sense environmental cues and then modulate the activities of downstream regulatory mechanisms or directly control phz expression. This occur when environmental conditions changes, and the phosphorylation status of the sensor protein is altered through binding of a small molecule or other environmental signal that activates conformational changes and affects activity. The phosphate group is then transported and activates the response regulator protein GacA. However, the cues that activates GacS have not been identified (Haas and Défago, 2005; Bourret and Silversmith, 2010; Chincholkar and Thomashow, 2013).

In QS, bacteria release small molecule or peptide signals into the environment, which can then interact with bacteria of the same strain and activate the expression of genes under the control of this mechanism. Molecules with diverse structures have been associated to this behaviour, but acyl homoserine lactones (AHLs) are the most relevant for phz expression. Monitoring the

quantity of compounds that are QS regulated, become clear that they arise when is reached a minimum cell concentration. Therefore, these signals enable bacteria to sense their own population size and to delay the expression of some genes until a specific cell density is achieved (Pierson III et al., 1998; De Maeyer et al., 2010; Bauer et al., 2016).

1.1.2. Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are polyesters of various hydroxyalkanoates (Figure 1.3) that are synthesized by many bacteria genera (Reddy et al., 2003), being *Pseudomonas* strains especially studied due to their potential to produce value-added compounds, such as PHAs (Mozejko-Ciesielska et al., 2019). Bacteria synthesize PHAs inside their cells as insoluble granules that form a reservoir of carbon (Figure 1.3) under nutrient limitation such as oxygen, nitrogen or phosphorus, and they do not accumulate PHAs in their growth phase (Mozejko-Ciesielska et al., 2019; Ward et al., 2005). Some bacteria are able to accumulate as much as 90% of their cell dry weigh in PHA (Mozejko-Ciesielska et al., 2019).

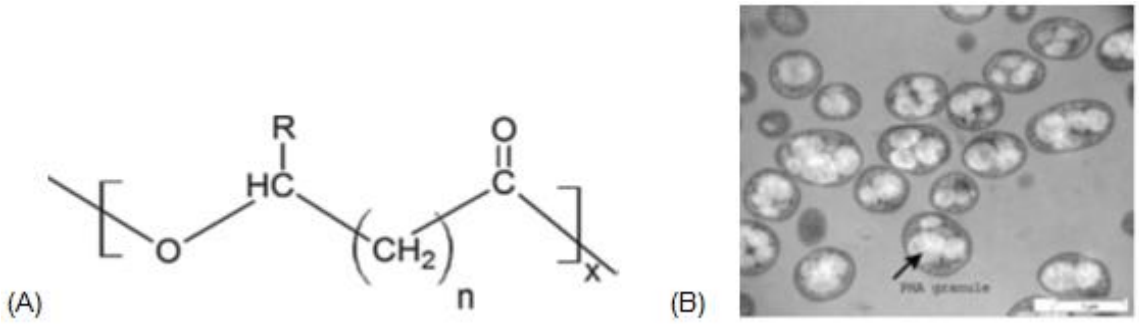


Figure 1.3 – (A) Structure of polyhydroxyalkanoates: R represents the side chain of the monomer, n-varies from 1 to 4, x varies from 100 to 300 000 (Lee, 1996; Mozejko-Ciesielska et al., 2016). (B) Transmission electron micrograph of PHA granules (retrieved from Ward et al., 2005).

These polyesters are biocompatibility, bio-based and biodegradable, and can present a wide range of physical and mechanical properties depending on their chemical composition. Further, they are stable in air, inert, resistant to moisture, water insoluble and can be completely degraded by the microorganisms present in soil, sea, lake water and sewage (Anjum et al., 2016; Jacquel et al., 2007).

According to “Report European Bioplastics, 2018”, PHAs are considered innovative biopolymers, with a growth in the field of bio-based and biodegradable plastics market. In 2018, PHAs represented 1.4 % of the global 2.11 million tonnes of bioplastics produced. It is estimated that the production capacity can quadruple in the next five years.

This biopolymer can be produced by mixed or single microbial cultures. Use mixed microbial culture (MMC) make the process economically more attractive, since they do not require the need to work under sterile conditions and improve the use of carbon sources like industrial wastes (Agustín Martinez et al., 2015; Serafim et al., 2008). PHA content accumulated by MMC is usually less than 20% and to increase the bacterial PHA content there is a cellular growth phase followed by a PHA accumulation phase. PHA production by MMC is usually a two or three-stage process depending on the type of substrate used as feedstock (Serafim et al., 2008), which represents a disadvantage when compared to single cultures on which the whole process is performed in one reactor. Another disadvantage of using MMC is that this strategy only allows to produce scl-PHA, for example poly(3-hydroxybutyrate) (P(3HB)) and poly(3-hydroxyvalerate) (P(3HV)) (Serafim et al., 2008; Moita and Lemos, 2010).

Production with single cultures usually have better productivity and higher PHA content - as it has been referred, about 90% of cellular dry weight (Reddy et al., 2003; Mozejko-Ciesielska et al., 2019). The produced polymer is a simple, well-defined polymer, since there is only one type of bacteria producing it and single cultures can produce an enormous variety of PHA ranging from scl-PHA to mcl-PHA (Cruz et al., 2016). The need to use pure carbon sources has been overcome since carbon-rich wastes have successfully used for PHA production by single cultures (Cruz et al., 2015, 2016). The major disadvantage of using single cultures is that this strategy demand sterile conditions, in order to guarantee the absence of contaminations and the existence of only one bacteria strain in culture (Agustín Martinez et al., 2015).

Depending on the number of carbon atoms in the monomer units, these polyesters can be divided into two groups: short-chain-length polyhydroxyalkanoates (scl-PHAs) and medium-chain-length polyhydroxyalkanoates (mcl-PHAs). Scl-PHAs contain from 3 to 5 carbon atoms in each monomer units and mcl-PHAs contain from 6 to 14 carbon atoms in each unit (Mozejko-Ciesielska et al., 2019). These polymers can also be classified as homopolymers, when composed by one monomer, or heteropolymers when composed by two or more monomers. Poly(3-hydroxyhexanoate) (P(3HHx)) and poly(3-hydroxyoctanoate) (P(3HO)) are examples of homopolymers, and the copolymer P(3HHx-co-3HO) is an example of a heteropolymer. The produced monomers depend on the bacterial strain, the carbon source and PHA synthases substrate specificity (Anjum et al., 2016). The nomenclature and carbon number for PHA compounds is determined by the R group of the monomer (Table 1.1) (Lee, 1996; Tan et al., 2014).

The composition and quantity of PHA polymer can be determined by gas chromatography after methanolysis (Lee, 1996). These different types of polymers with differences in monomers content can be produced depending on selected bacteria species, fermentation process, growth and nutrient conditions (Keshavarz et al., 2010).

Table 1.1 – PHA monomers produced by bacteria (Tan et al., 2014).

R group	Carbon number	PHA polymer	Abbreviation
methyl	C_4	Poly(3-hydroxybutyrate)	P3HB
ethyl	C_5	Poly(3-hydroxyvalerate)	P3HV
propyl	C_6	Poly(3-hydroxyhexanoate)	P3HHx
butyl	C_7	Poly(3-hydroxyheptanoate)	P3HH
pentyl	C_8	Poly(3-hydroxyoctanoate)	P3HO
hexyl	C_9	Poly(3-hydroxynonanoate)	P3HN
heptyl	C_{10}	Poly(3-hydroxydecanoate)	P3HD
octyl	C_{11}	Poly(3-hydroxyundecanoate)	P3HUD
nonyl	C_{12}	Poly(3-hydroxydodecanoate)	P3HDd
decyl	C_{13}	Poly(3-hydroxytridecanoate)	P3HTriD
undecyl	C_{14}	Poly(3-hydroxytetradecanoate)	P3HTd
dodecyl	C_{15}	Poly(3-hydroxypentadecanoate)	P3HPD
tridecyl	C_{16}	Poly(3-hydroxyhexadecanoate)	P3HHxD

As mentioned before, these biopolymers can present different properties depending on their chemical composition. Therefore, scl-PHAs are rigid, brittle and possess a high degree of crystallinity in the range of 60–80%, while mcl-PHAs are flexible and elastic materials with low crystallinity (25%), low tensile strength, high elongation to break and low melting temperatures. These characteristics make them much more elastic and flexible materials and provide them a larger variety of applications as glues, rubbers or adhesives (Anjum et al., 2016; Mozejko-Ciesielska et al., 2019).

1.1.2.1. Medium-chain-length PHAs

Pseudomonas putida, formerly known as *Pseudomonas oleovorans*, was the first mcl-PHA producer strain to be discovered and has been widely investigated. Other *Pseudomonas* strains such as *P. aeruginosa, P. marginalis* or *P. chlororaphis* are also known to be mcl-PHA producers (Sun et al., 2007; Tan et al., 2014; Pereira et al., 2019).

Several substrates had already been tested for mcl-PHA fermentation processes. Some of them are methanol, octane, nonanoic acid, glucose, fructose and glycerol. Most of them are efficient carbon sources for mcl-PHA synthesis, being possible to achieve 75% of cell dry weigh in PHA (Lee, 1996; Sun et al., 2007; Tan et al., 2014). However, some of those substrates presents low solubility in water and some are toxic to bacteria at quite low concentrations (Sun et al., 2007). In 1991, Ramsay et al., reported that the growth of *P. putida* GPo1 was inhibited with an octane concentration of 4.65 g/L. Sugars, like glucose and fructose, are effective carbon sources for use in mcl-PHA fermentation processes but their high price represents most of the costs for the PHA production. Consequently, the use of wastes or by-products as carbon sources (e.g. crude glycerol from biodiesel production), could reduce PHA production costs (Cruz et al., 2016).

1.1.3. (Exo)Polysaccharide

Polysaccharides are the main group of natural polymers in the world. They are high molecular weight polymeric biomaterials with a large structural diversity, are obtained from plants, algae, animals and could also be produced by bacteria (Elnashar, 2011).

The polysaccharide production by microorganisms ensure higher growth rates and high yields of one specific polymer. Moreover, by changing the cultivation conditions it is possible to achieve higher growth rates and productivities (Sutherland, 2009). Additionally, microbial production is not dependent on climate changes or seasonality (Cruz et al., 2011).

Microbial polysaccharides can be divided into intracellular (e.g. glycogen), structural cell wall components (e.g. chitin) and extracellular polysaccharides. Extracellular polysaccharides or exopolysaccharides (EPS) are polymers secreted by the cells, either as a capsule firmly attached to the bacterial cell surface, or may be observed as a free slime secreted by the microorganisms that is loosely bound to the cell (e.g. xanthan, gellan, hyaluronic acid) (Kumar et al., 2007; Rehm, 2010). On solid surfaces and exposed to aqueous environments, bacterial growth is seen as biofilms, in which the microbial cells are associated with large amounts of EPS. These aqueous environments can be fresh water and salty oceans, or even the simple moisture of a human or animal body (Sutherland, 2009). This capacity of creating stable biofilms controlled by EPS (Figure 1.4), give them some self-protection in case of predation, effects of antibiotics, antimicrobial substances and antibodies, when under stress. Moreover, helps the adherence to other bacteria, animal cells and plant tissue (Vijayendra and Shamala, 2013; Ates, 2015).

The biosynthesis of most exopolysaccharides closely resembles the process by which the bacterial wall polymers peptidoglycan and lipopolysaccharide are formed. Indeed, the three types of macromolecule share the characteristic of being formed by carbohydrates and associated monomers, being synthesised at the cell membrane and exported to final sites external to the cytoplasmic membrane. Formation of the precursors for polysaccharide synthesis occurs inside the cytoplasm. This is probably a need to ensure that are easily available, as in many cases they are utilised for several different polymer-synthesising systems. As they are freely soluble in the cytoplasm, they can quickly guide to the appropriate biosynthetic process occurring inside of the cytoplasmic membrane (Sutherland, 2009).

EPS is produced by microorganisms under many different stress conditions, being affected by physical and chemical factors. The most important physical parameters are temperature, aeration, agitation and fermentation period, while the vital chemical factors are media composition, carbon and nitrogen source and concentration, trace elements, pH and dissolved oxygen (Ates, 2015).

These polymers are composed of monomers such as neutral sugars and/or acidic or amino sugars (e.g. N-acetyl-D-glucosamine and N-acetyl-D-galactosamine), commonly containing non-sugar components, such as acyl groups (e.g. acetyl, pyruvil, succinyl). The most common sugar residues in EPS structures are glucose and galactose. However, certain EPS have an increased value due to their content in some rare sugars (e.g. fucose), which occur rarely in Nature (Torres et al., 2011; Sutherland, 2009).

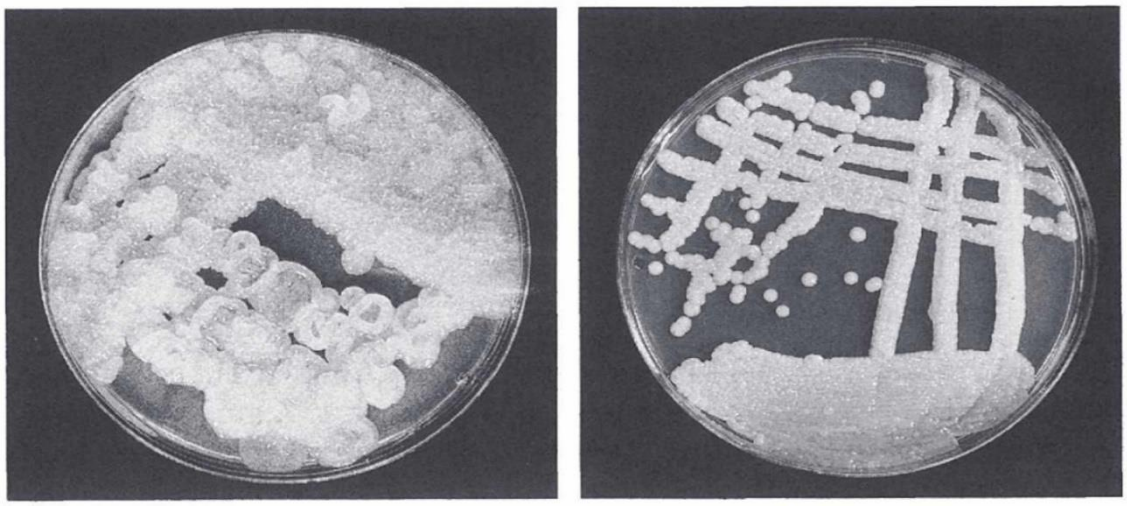


Figure 1.4 – Appearance of two polysaccharide-producing pseudomonads (retrieved from Sutherland, 2009).

If the monomers of sugar that constitute the polymer are only of one kind, this EPS will be classified as a homopolysaccharide. In other hand, if the polymer presents two to four different monosaccharides, it is classified as a heteropolysaccharide. In these last-mentioned biopolymers, the different monosaccharides are usually arranged into a repeating unit constituted by ten or less monomers (Finore et al., 2014). There some exceptions, such as alginate that has no defined repeating unit, with the number of monomers and their sequential distribution varies along the polymer chain and depends on the source of the alginate (Sutherland, 2009).

In batch culture, the molecules are probably polydisperse and the range of molecular mass obtained, may depend to a large extent on the microbial species under study. Many bacterial have been reported as being capable of producing EPS, which are usually characterized by having high diversity in terms of chemical structure, and composition (e.g. alginate, dextran and xanthan) (Sutherland, 2009). Due to variety of physical and structural properties, EPSs have many applications in food, pharmaceutical, cosmetics, paints, explosives, paper and oil industries, and can be used as structuring, gelling, stabilizing, binding, emulsifying or flocculating agents (Cruz et al., 2011).

1.2. Motivation

“Natural products” is a designation that comprise a lot of chemical compounds derived and isolated from biological sources. These compounds produced by living beings are used in medicine, agriculture, cosmetics, food, etc, from earliest to modern societies and cultures around the world. Therefore, the capability to access/produce natural products and understand their values and applications has been a major priority in the field of natural product research.

Since mankind and their societies are greatly increasing, Biotechnology and other scientific fields are challenged with the development of sustainable systems able to produce natural products. Therefore, systems capable of substitute outdated processes and products are continuously growing. *Pseudomonas chlororaphis* subsp*. aurantiaca* DSM 19603 fermentation could be an example of an alternative to produce three interesting compounds, since it seems capable of co-produce three products with high value: phenazines, mcl-PHA and EPS. Consequently, the purpose of this work study was to achieve the highest yields and productivities for of each product in a single fermentation.

After performing a bioreactor with standard conditions, it was confirmed the potential of *Pseudomonas chlororaphis* subsp*. aurantiaca* DSM 19603 to produce the antibiotics and biopolymers mentioned before. Therefore, the next step was test and evaluate the best concentrations of nitrogen and glycerol (carbon source) to achieve higher cellular growth, and consequently higher quantity of phenazines. With the best concentrations of these nutrients defined, the last and longer step was to perform several bioreactors with different operational conditions, selecting the one that exhibit the highest concentrations, yields and productivities for all products. The changed parameters were cultivation mode, feeding strategy, dissolved oxygen concentration and base for pH control.

Chapter 2
Material and Methods

2.1 Bacterial strain and culture media

The bacterial strain used in this work was *Pseudomonas chlororaphis* subsp*. aurantiaca* DSM 19603, cryopreserved in 20% (v/v) glycerol at -80 °C.

Lysogeny broth (LB) medium: tryptone, 10.0 g/L; yeast extract, 5.0 g/L; NaCl, 10.0 g/L.

Medium E*: K_2HPO_4, 5.8 g/L; KH_2PO_4, 3.7 g/L; $(NH_4)_2HPO_4$, 3.3 g/L; 10 mL/L of $MgSO_4$, 10 mL/L of mineral solution diluted (1:10) containing $FeSO_4.7H_2O$, 2.78 g/L; $MnCl_2.4H_2O$, 1.98 g/L; $CoSO_4.7H_2O$, 2.81 g/L; $CaCl_2.2H_2O$, 1.67 g/L; $CuCl_2.2H_2O$ 0,17 g/L; $ZnSO_4.7H_2O$, 0.29 g/L.

Glycerol (99% w/w, Scharlau) was used as substrate, at initial concentrations of 40 g/L.

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

2.2 Inoculum preparation

Inoculum were prepared by adding 2 mL of the cryopreserved sample (stored at -80 °C) to 200 mL of LB medium, in a 500 mL baffled shake flask. The baffled shake flask was incubated in an orbital shaker for 24 hours, at 30 °C and 200 rpm.

2.3 Effect of nitrogen and carbon concentration

Baffled shake flasks cultivations were performed with a working volume of 500 mL.

Nitrogen-concentration test was performed with 200 mL of medium E*, with different concentrations of $(NH_4)_2HPO_4$, supplemented with glycerol at concentration of 40 g/L. The $(NH_4)_2HPO_4$ concentrations tested were: 3.3 g/L; 6.6 g/L; 9.9 g/L; 13.2 g/L; 16.5 g/L; 19.8 g/L; 23.1 g/L; 26.4 g/L, corresponding to the following nitrogen concentrations: 0.7 g/L; 1.4 g/L; 2.1 g/L; 2.8 g/L; 3.5 g/L; 4.2 g/L; 4.9 g/L; 5.6 g/L, respectively.

Glycerol-concentration test was performed with 200 mL of medium E*, with 16.5 g/L concentration of $(NH_4)_2HPO_4$, supplemented with different concentrations of glycerol. The concentration values of glycerol tested were: 40 g/L; 60 g/L; 80 g/L; 100 g/L.

For all assays, the temperature and agitation were kept at 30 °C and 200 rpm, respectively, in an incubator shaker. The assays run for 66-67h and culture broth samples were taken three times a day for quantification of cell dry weight, phenazines, PHA, ammonia and glycerol. The samples (2 mL) taken periodically were centrifuged (10000 rpm, 20 min, 4 °C; Sigma 4-16KS), the cell-

free supernatant was stored at -20 ºC for phenazines, EPS, ammonia and glycerol quantification, and the pellet was lyophilized for biomass and mcl-PHA quantification.

2.4 Bioreactor Fermentations

P. chlororaphis cultivations were performed in a bioreactor (BioStat®B-Plus, Sartorius) with a working volume of 2 L. Runs were initiated with 1.8 L of Medium E* supplemented with glycerol at a concentration of 40 g/L. The temperature and the pH were controlled at 30 ± 0.1 ˚C and 7.0 ± 0.1, respectively. The pH was controlled by the automatic addition of NaOH 5M or NH_4OH (25%, v/v), and 2 M HCl solutions. A constant aeration rate of 2 SLPM (standard liters per minute) was kept during all experiments. The dissolved oxygen concentration (DO) was controlled at 30% of air saturation by automatically adjusting the stirring speed between 300 and 900 rpm. Foam formation was automatically suppressed by addition of Antifoam A (Sigma/VWR). Samples were periodically taken from the bioreactor for quantification of cell dry weight, phenazines, PHA, ammonia and glycerol. Culture broth samples (15 mL) taken periodically during the cultivation runs were centrifuged (10000 rpm, 20 min, 4 °C; Sigma 4-16KS), the cell-free supernatant was stored at -20 ºC for phenazines, EPS, ammonia and glycerol quantification, and the pellet was lyophilized for biomass and mcl-PHA quantification.

2.4.1 Fed-batch Fermentations

Several runs with different strategies were performed in fed-batch fermentation. All conditions of these strategies are described in table 2.1. The remaining conditions of the cited assays were performed as described in section 2.4.

Table 2.1 – Different conditions of all fed-batch assays performed in this study.

Strategy	$(NH_4)_2HPO_4$ (g/L)	Initial glycerol (g/L)	Glycerol pulse (g/L)	pH control base	DO control
Standard	3.3	40	40	NaOH 5M	Stirring
5-fold N	16.5	40	30	NH4OH (25%, v/v)	Stirring
DO-glycerol	16.5	40	-	NH4OH (25%, v/v)	Stirring (0-14h) Glycerol addition (14-47h)
DO-20	16.5	40	30	NH4OH (25%, v/v)	20% (Stirring 250-700 1.7 SLPM)
NH_4OH-base	3.3	40	40	NH4OH (25%, v/v)	Stirring

2.4.2 Batch Fermentation

In this run the initial glycerol and $(NH_4)_2HPO_4$ concentrations were 40 g/L and 8.25 g/L, respectively. The pH was controlled with NH_4OH (25%, v/v) and 2 M HCl solutions. The remaining conditions of the assay were performed as described in section 2.4.

2.5 Analytical techniques

2.5.1 Cellular growth

Cellular growth was monitored during all trials by measuring the optical density of the cultivation broth at 600 nm (OD_{600nm}), according to Helou et al., 2013. Samples were diluted with deionised water whenever necessary, in order to have the OD_{600nm} value below 0.250. Deionised water was used as zero reference and all measurements were done in duplicate.

2.5.2 Biomass Quantification

The cell dry weight (CDW), dry weight of the cells per litre of fermentation broth, was determined by gravimetry, according to Wang et al., 2012, with slight modifications. The cell pellets, obtained as described above, were washed once by resuspension in deionised water (5 mL) and centrifuged again (10000 rpm, 20 min, 4 °C; Sigma 4-16KS). The washed cell pellets were frozen in liquid nitrogen and lyophilized for 48 hours at -108 °C. The CDW was obtained by weighing the lyophilized cell pellets. The analysis was done in duplicate.

2.5.3 Phenazines quantification

Phenazines produced over the cultivation time were measured by spectrophotometry, according to Bauer et al., 2016, with slight modifications. 2 mL of cell-free supernatant diluted in deionised water, in a 1:20 ratio, were acidified with 0.2 mL of HCl (1 N) and phenazines were extracted with 2 mL of chloroform. Scans (Appendix 1) were performed in several samples at wavelengths between 190 and 400 nm (quartz cuvette), and absorbance were measured at 365 nm (glass cuvette) in a UV/vis spectrophotometer (INSIGHT™2 Software Scan, Thermo Scientific Evolution 201 UV-Visible Spectrophotometers).

2.5.4 Ammonium Quantification

Ammonium concentration was determined by colorimetry, using a flow segmented analyser (Skalar 5100, Skalar Analytical, The Netherlands), according to Carvalho et al., 2007. Ammonium chloride (Sigma) was used as standard at concentrations between of 5 and 20 mg/L. The cell-free supernatant was diluted in deionized water to achieve an ammonium concentration lower than 20 ppm.

2.5.5 Glycerol Quantification

Determination of glycerol concentration was made by high performance liquid chromatography (HPLC) with a VARIAN Metacarb 87H column coupled to an infrared (IR) detector, according to Torres et al., 2011. The analysis was performed at 50 °C using H_2SO_4, 0.01 N, as eluent with a flow rate of 0.6 mL/min. Samples were prepared by diluting the cell-free supernatant, in the eluent (H_2SO_4, 0.01 N), in a 1:50 proportion. All samples were filtered with using VWR centrifuge filters (0.2 µm). A standard cultivation curve was constructed using glycerol (Scharlau, 99% w/w) solutions as standards, in a concentration ranging from 0.0625 to 1 g/L.

2.5.6 PHA Quantification and Composition

To determine the polymer's content in the biomass and its monomer composition, gas chromatography (GC) analysis was performed, according to Cruz et al., 2016. Dried cell samples (1 to 10 mg) 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) standards were prepared at 1 g/L and then diluted to give concentrations in the range 0.05 and 1 g/L.

2.5.7 EPS quantification

For EPS quantification, the cell-free supernatant was dialysed against deionised water, according to Freitas et al., 2011, with slight modifications. For this process, dialysis membranes (ZelluTrans Carl Roth, MWCO 12000 - 14000) with 4 mL of supernatant, were placed in a 5 L beaker with deionised water, under constant stirring. Sodium azide (10 ppm) was added to the water to prevent biological contamination and sample degradation. The water was changed every two to three hours during the 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 deionised water conductivity (after approximately 72 hours when conductivity was lower than 5 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.

2.5.8 EPS characterisation

The EPS was characterized in terms of its sugar monomers composition, according to Freitas et al., 2011. 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%), for 2 hours at 120°C. After cooling down to room temperature, the samples were filtered using VWR centrifuge filters (0.2 µm).

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-(+)-trehalose dehydrate (99%, Alfa Aesan), D-glucuronic acid (98%, Alfa Aesan) and D-(+)-galacturonic acid monohydrate (97%, Fluka), were used as standards (5 to 50 ppm).

2.6 Calculus

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 mcl-PHA at that time t (h).

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

$$\mu = LN\left(\frac{X_t}{X_0}\right) \quad \text{Eq. 2}$$

where LN is natural logarithm, X_t is active biomass concentration (g/L) at a certain time t (h) and X_0 is active biomass concentration (g/L) at the beginning of fermentation.

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

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

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

where PHA is the mcl-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), mcl-PHA on substrate (Ypha/s, g/g) and EPS on substrate (Yeps/s, g/g) were determined by the following equations:

$$Y_{x/s} = \frac{\text{x f} - \text{x i}}{\text{sf} - \text{si}} \quad \text{Eq. 5}$$

$$Y_{\text{PHA}/s} = \frac{\text{PHA f} - \text{PHA i}}{\text{sf} - \text{si}} \quad \text{Eq. 6}$$

$$Y_{\text{EPS}/s} = \frac{\text{EPS f} - \text{EPS i}}{\text{sf} - \text{si}} \quad \text{Eq. 7}$$

where xf 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 mcl-PHA produced, EPSf and EPSi are the final and initial EPS produced, respectively.

Chapter 3
Results and Discussion

3.1. *Pseudomonas chlororaphis* cultivation: Bioreactor assays and Nutrients concentration tests

3.1.1 Standard conditions assay

In order to study the phenazines production by *Pseudomonas chlororaphis*, it was cultivated on a fed-batch mode, for 44 hours in a 2 L bioreactor with medium E* and glycerol (40 g/L) as carbon source.

Phenazines production is growth associated and is regulated by the quorum sensing (QS) mechanism, achieving the maximum production in the end of the exponential phase (Chincholkar and Thomashow, 2013; Bauer et al., 2016). Therefore, the bioreactor inoculation was made taking this in consideration and the lag and early exponential phases took place overnight.

Figure 3.1 shows the glycerol (A) and nitrogen (B) consumption, both compared with cell dry weight (CDW) concentration over cultivation time. Figure 3.1.C shows the production profile of the three products associated to *P. chlororaphis*: phenazines, mcl-polyhydroxyalkanoates (mcl-PHA) and exopolysaccharide (EPS).

The growth of *P. chlororaphis* ended after 14 hours (Figure 3.1), when nitrogen concentration become 0 g/L, limiting the growth of the bacteria. At this time, a CDW of 4.33 g/L was achieved with a μ_{max} of 0.229 h^{-1}. A phenazines amount of 3.19 OD_{365nm} were reached and nearly 10 g/L of glycerol were consumed, at a consumption rate of 0.71 g/L·h. The glycerol was mostly directed to cellular growth and phenazines and EPS production, since at that point the accumulated mcl-PHA was only 0.3% of the biomass content. This content corresponding to a mcl-PHA concentration of 0.01 g/L, a yield of 0.001 g/g and a volumetric productivity of $7.1x10^{-4}$ g/L·h. The EPS concentration was slightly higher presenting a value of 1.9 g/L, which represent a yield of 0.19 g/g and a volumetric productivity of 0.14 g/L·h.

In order to increase the production of the two biopolymers, after 24 hours of cultivation, a glycerol pulse (40 g/L) was fed to the culture and the run was prolonged for more 20 hours. At the end, 34.03 g/L of glycerol were consumed at a rate of 0.77 g/L·h. PHA was accumulated inside the cell between 14 h and 44 h (Figure 3.1.C), to a content of 14.36%, corresponding to a concentration of 1.15 g/L, explaining the CDW still increase to 7.98 g/L, which was partly due to mcl-PHA accumulation. This concentration of polymer presents a yield of 0.034 g/g and a volumetric productivity of 0.026 g/L·h.

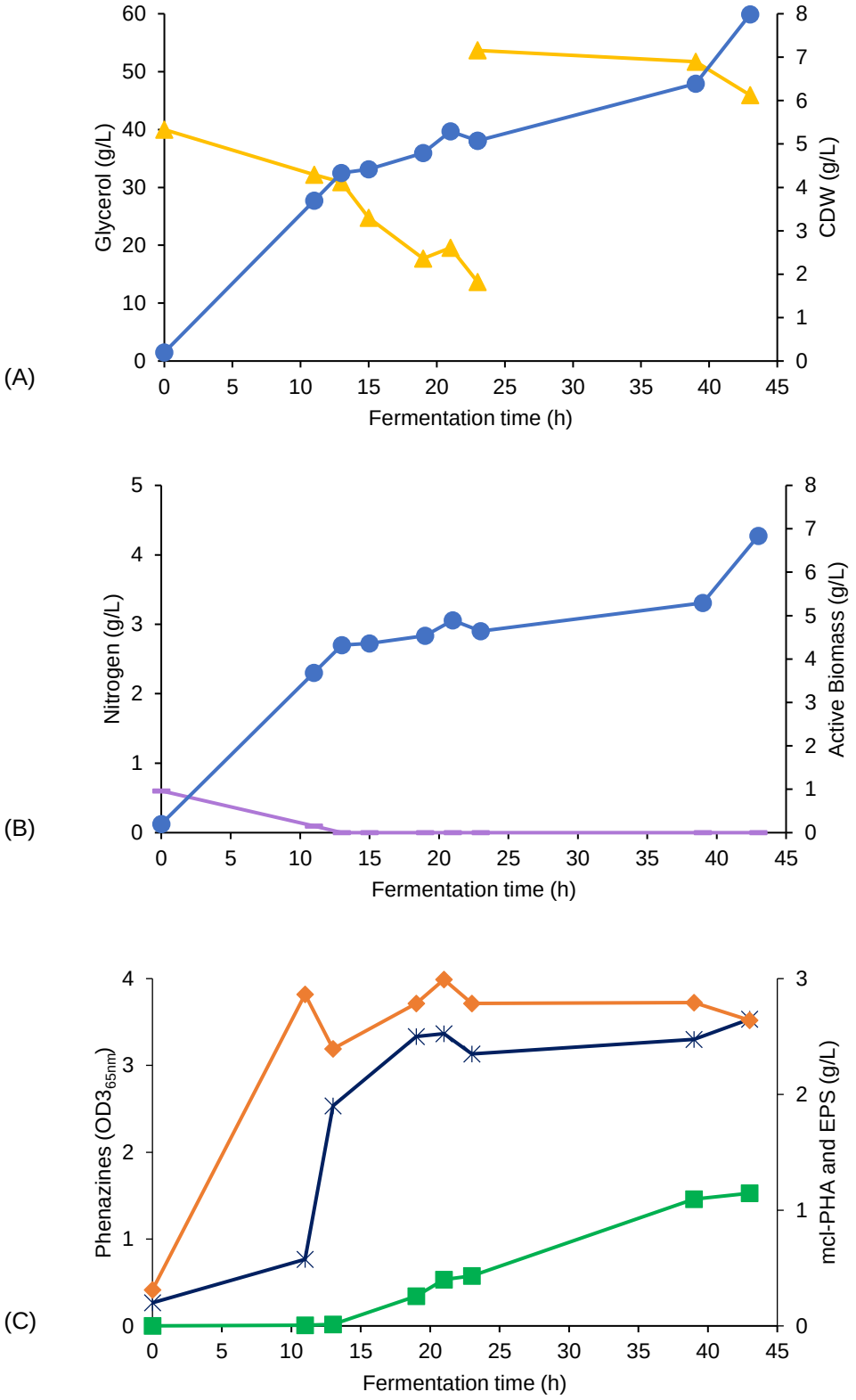


Figure 3.1 – Cultivation profile of *Pseudomonas chlororaphis* subsp. aurantiaca DSM 19603 during standard assay. (A) CDW (●) and glycerol concentration (▲) in (g/L); (B) Active biomass (●) and nitrogen concentration (-) in (g/L); (C) production profiles of phenazines (♦) in OD_{365nm}, mcl-PHA (■) and EPS (X) in (g/L).

This concentration of accumulated polymer indicate that, at the end of the assay, active biomass concentration was 6.84 g/L (Figure 3.1.B). These results suggest that bacteria keep growing, even after the concentration of nitrogen reached 0 g/L. However, several bacteria belonging to phyla *Actinobacteria* and *Proteobacteria*, where *Pseudomonas* genus is integrated, have genes that give them the ability to degrading phenazines (Costa et al., 2015). Since phenazines are nitrogen-containing compounds, some bacteria able to degrade this antibiotic, also exhibit the capacity to use them as nitrogen and carbon source (Yang et al., 2007; Costa et al., 2015). Therefore, seems probable that during this assay, in lack of nitrogen, bacteria started degrading their own phenazines to ensure a nitrogen source.

Figure 3.1.C shows the production curves of both polymers, in which is observed that EPS had a higher production from 12 to 20 hours run, being its concentration already 2.5 g/L by that time. This polymer reached a concentration of 2.65 g/L, which correspond to a yield of 0.078 g/g and a volumetric productivity of 0.06 g/L·h. During this assay, it is possible to see that phenazines amount, CDW, mcl-PHA and EPS concentrations increased, while glycerol concentration decreased. Therefore, is reasonable to consider that glycerol was consumed for bacterial growth, mcl-PHA accumulation, and phenazines and EPS production.

Figure 3.2 – Picture of the bioreactor with *Pseudomonas chlororaphis* subsp. *aurantiaca* DSM 19603 culture, during standard assay at 12 h fermentation.

After 12 hours of fermentation and until the end of the assay, the culture inside the bioreactor presented an orange bright colour (Figure 3.2), that become slightly darker until the end of the run. As cited in the introduction section, phenazines produced by *Pseudomonas* genus presents yellow, orange and brick-red colours. Since phenazine-1-carboxylic acid (PCA) originate the 2-Hydroxyphenazine-1-carboxylic acid (2-OH-PCA), which originates the 2-Hydroxyphenazine (2-

OH-PHZ) (Delaney et al., 2001), it is expected that the orange pigment results of a combination of this different molecules, instead of being produced by only one phenazine derivate.

The results obtained by absorbance reading (Figure 3.1.C), show that a near maximum value of phenazines (3.82 OD_{365nm}) was achieved in the end of exponential phase, at 12 hours run. In the following hours of fermentation, as the culture started to enter in stationary phase, the amount of phenazines in fermenter stays practically the same until the end of the run. This information suggest that bacteria start the production of phenazines during exponential phase. Bauer et al. (2016), described a similar pattern of phenazine production that goes along with cellular growth. This pattern seems be explained by other results of that study, since that the quorum sensing (QS) signal molecules in study, namely acyl homoserine lactones (AHLs), reached a maximum at the end of the exponential phase and decrease until the end of the assay. These results are not quantitative, but represent the amount of this compound and show that exist a dependence between cellular concentration and phenazine production.

3.1.2 Impact of nitrogen and carbon concentration on *P. chlororaphis* cultivation

As mentioned in introduction, phenazines are compounds that are controlled by QS. Therefore, its production should be higher with an increase of QS signal molecules induced by a higher cellular growth. Since nitrogen is an important element to bacterial growth, but can also negatively affect this growth depending on the quantity that is supplemented (Treseder, 2008), the next step was test the best nitrogen concentration to improve *P. chlororaphis* growth.

Along with nitrogen, carbon is also a very important nutrient that can limit bacterial growth (Treseder, 2008). Furthermore, as cited in introduction, this element is a vital nutrient for PHA accumulation. Therefore, the best carbon concentration for *P. chlororaphis* growth was also tested.

3.1.2.1 Effect of Nitrogen-concentration – shake flask tests

Eight different nitrogen concentrations (0.7 g/L; 1.4 g/L; 2.1 g/L; 2.8 g/L; 3.5 g/L; 4.2 g/L; 4.9 g/L; 5.6 g/L) were tested. These tests were performed in 500 mL baffled shake flasks, for 66 hours, in duplicate. Figure 3.3.A and 3.3.B shows the OD and phenazines amount, respectively, achieved in all fermentations.

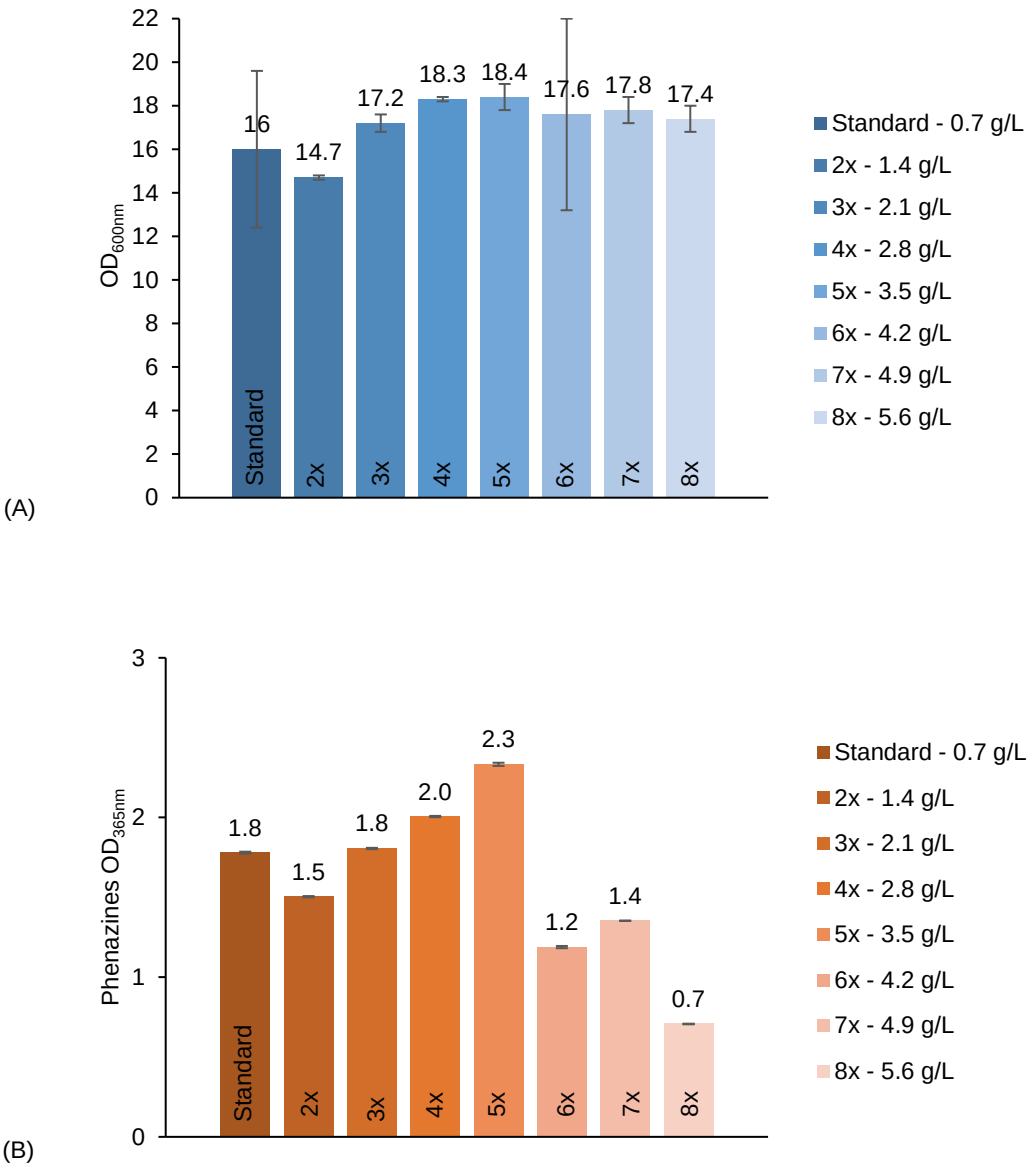


Figure 3.3 – (A) OD at 66 hours run of nitrogen-concentration test; (B) phenazines amount at 66 hours run of nitrogen-concentration test.

By looking at OD values obtained in the experiments (Figure 3.3.A), it is possible to observe that cultures with standard and 6-fold nitrogen concentrations, (0.7 and 4.2 g/L, respectively), presented quite different values among duplicates. Despite that, the runs that presented lower OD values were the standard and 2-fold concentrations (0.7 and 1.4 g/L, respectively).

In the remaining tests, OD_{600nm} values ranged between 17.2 and 18.4, which indicate quite similar cellular growth. In these runs, the cultures that presented the higher growth, i.e. higher values of OD, were the ones with 4 and 5-fold concentration (2.8 and 3.5 g/L, respectively) tests. In table 3.1, is also visible that the changes in N concentrations did not affect the specific growth rates, since μ_{max} values are almost equal in all runs.

Table 3.1 – Specific growth rates (μ_{max} (h^{-1})) for the different nitrogen concentration tested.

Parameter	Nitrogen concentration (g/L)							
	0.7	1.4	2.1	2.8	3.5	4.2	4.9	5.6
μ_{max} (h^{-1})	0.116	0.125	0.124	0.132	0.133	0.122	0.116	0.113

Beyond cellular growth, phenazines production was also evaluated, since it is a growth associated metabolite. Consequently, phenazines protocol was performed over all samples taken at 66 hours run, and in figure 3.3.B, is possible to compare all the values acquired from the absorbance reading ($OD_{365\ nm}$). It is possible to observe that despite the similar growth and μ_{max} (h^{-1}), the higher amount of phenazines was achieved with a 5-fold nitrogen concentration (3.5 g/L). On the other hand, phenazines amount was lower in all runs with N concentrations higher than 5-fold.

3.1.2.2 Effect of Glycerol concentration – shake flask tests

After increasing the N concentration for 3.5 g/L, the effect of different glycerol concentrations was evaluated.

In this assay, runs were performed for 67 hours, in 500 mL baffled shake flasks with 200 mL of medium E*, containing 16.5 g/L concentration of $(NH_4)_2HPO_4$. Four different concentrations of glycerol (40 g/L, 60 g/L, 80 g/L and 100 g/L) were tested and compared with standard conditions, all in duplicates. Figure 3.4.A and 3.4.B presents the OD and phenazines amount, respectively, achieved in all the concentrations tested.

In this assay, the highest OD, corresponding to the highest cellular growth, was achieved in the run with 40 g/L of glycerol and 3.5 g/L of nitrogen (Figure 3.4.A). Further, it is possible to observe that the increase in the glycerol concentration affects negatively the bacteria metabolism (Figure 3.4.A), by decreasing the cellular growth, achieving lower OD, as well as lowering specific growth rates (μ_{max}) (Table 3.2).

By comparing standard with the remaining tests, it is perceived that once more the nitrogen concentration of 3.5 g/L lead to a higher cellular growth than standard nitrogen concentration.

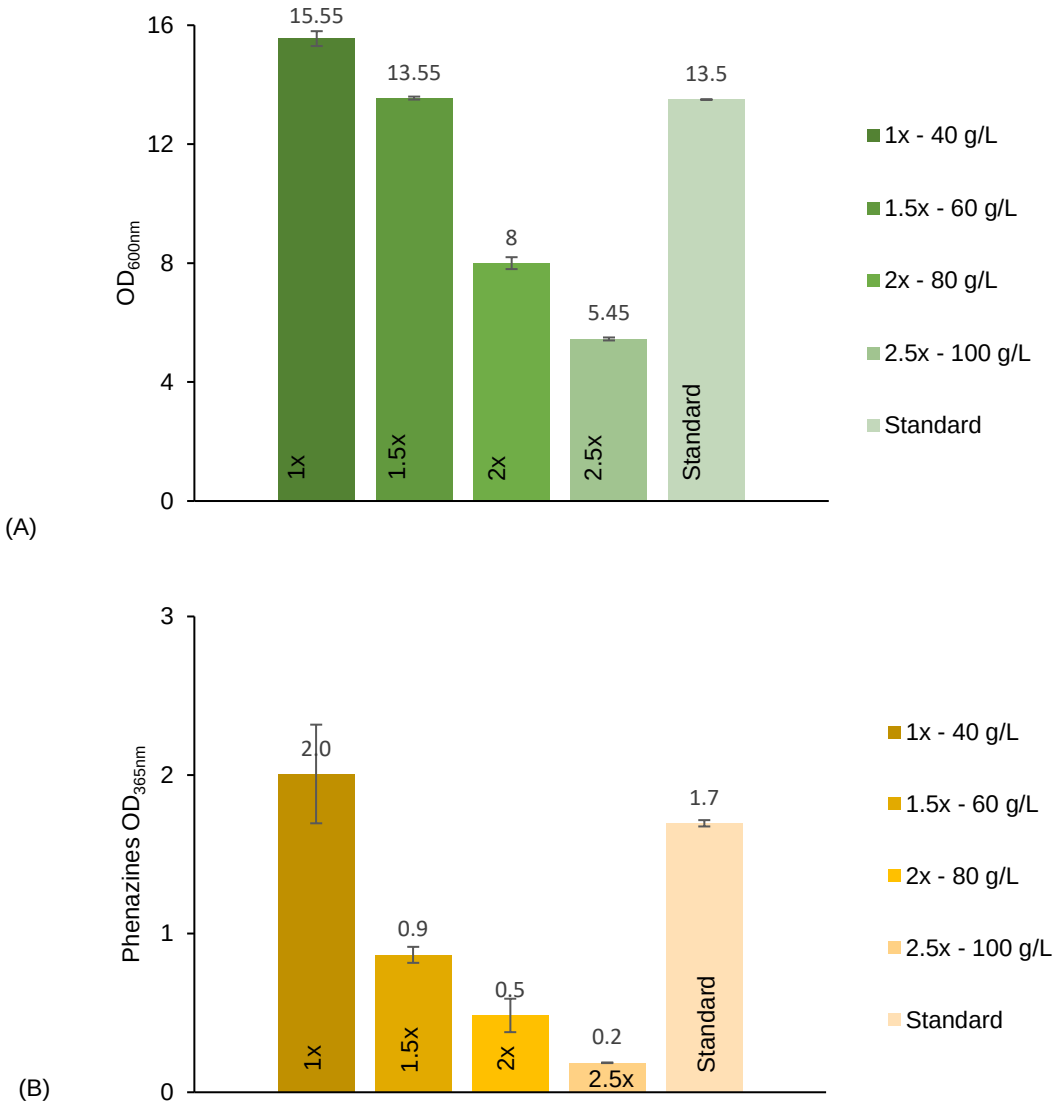


Figure 3.4 – (A) OD at 67 hours run of glycerol-concentration test; (B) phenazines amount at 67 hours run of glycerol-concentration test.

Phenazines production was evaluated once again (Figure 3.4.B). This time, the OD_{365nm} values show that the amount of phenazines produced goes along with cellular growth (Figure 3.4.A), being the run with glycerol concentration of 40 g/L, the one that presented better results.

Table 3.2 – Specific growth rates (μ_{max} (h^{-1})) for the different glycerol concentration tested (*Standard - 40 g/L of glycerol with 0.7 g/L of N).

Parameter	**Glycerol concentration (g/L)**				
	40	60	80	100	40*
μ_{max} (h^{-1})	0.128	0.109	0.095	0.089	0.115

3.1.3 Bioreactor assay with 3.5 g/L of Nitrogen

The concentrations of nitrogen and glycerol that showed the best results, in the shake flask tests, were selected to perform the next bioreactor fermentation. These concentrations were 3.5 g/L of nitrogen and 40 g/L of glycerol. In order to guarantee nitrogen availability across the experiment, the base used for pH control was changed from NaOH to NH_4OH.

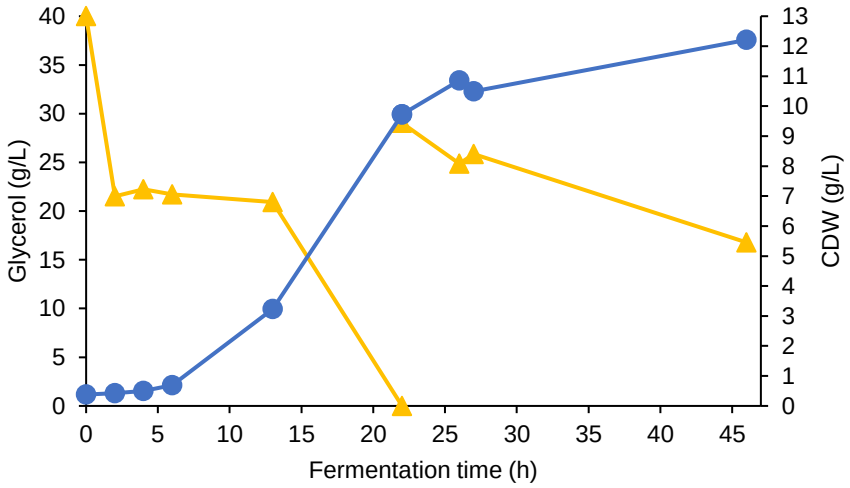
Therefore, a fed-batch fermentation was performed during 46 hours in a 2 L bioreactor with medium E*., with 16.5 g/L of $(NH_4)_2HPO_4$, 40 g/L of glycerol as carbon source and NH_4OH as base for pH control. After a lag phase of 6 hours, *P. chlororaphis* entered in the exponential growth phase that lasted around 16 hours (Figure 3.5). At 13 hours run, the culture inside fermenter start to acquire the orange colour already mentioned in standard assay. Once again, this colour become slightly darker until the end of the run.

Around the 22 hours of run, culture entered in stationary phase. This run was performed with 5-fold initial concentration of nitrogen and the pH controlled with NH_4OH, enabling nitrogen availability across all the runs (Figure 3.5.B) which enabled to achieve a CDW at the end of exponential phase of 9.73 g/L, which is a higher cellular growth than the one achieved at the end of the standard assay (7.98 g/L). During the exponential phase, the culture presented a μmax of 0.131 h^{-1}, which is lower than the one presented by standard assay. Further, biomass kept increasing until 12.21 g/L (Figures 3.5.A and 3.5.B).

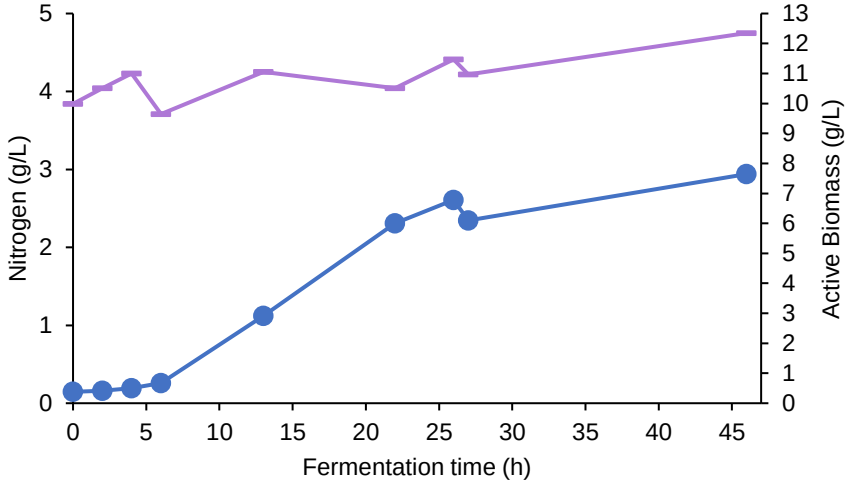
Since this assay start with 3.5 g/L of nitrogen and the pH was controlled with NH_4OH, is complicated to take for sure the quantity of nitrogen that was consumed during this run just with the data displayed in Figure 3.5.B. However, values taken from the bioreactor controller, point to a enter of 3.63 g/L of nitrogen during the run, which suggest a consumption of 2.72 g/L of nitrogen. At 22 hours run, *P. chlororaphis* seemed to have consumed all of glycerol. Due to that reason, a 30 g/L glycerol pulse was added to the bioreactor.

Phenazines production increased relatively to production obtained in the standard conditions run. The maximum value, achieved at 22h run, was 5.78 $OD_{365nm.}$ Although, this value start to decrease when the culture entered in stationary phase, and at the end of the assay the phenazines amount measured was 2.41 OD_{365nm} (Figure 3.5.C). In 2015, Chen et al. indicate that reduced phenazines presents lower absorption values in UV-vis analysis than before reduction. Phenazines could be reduced by obtaining two electrons (Sullivan et al., 2011), whom could be released during ammonia oxidation. Therefore, this decrease in phenazines amount could be explained by the presence of a higher concentration of ammonia in the fermenter.

(A)



(B)



(C)

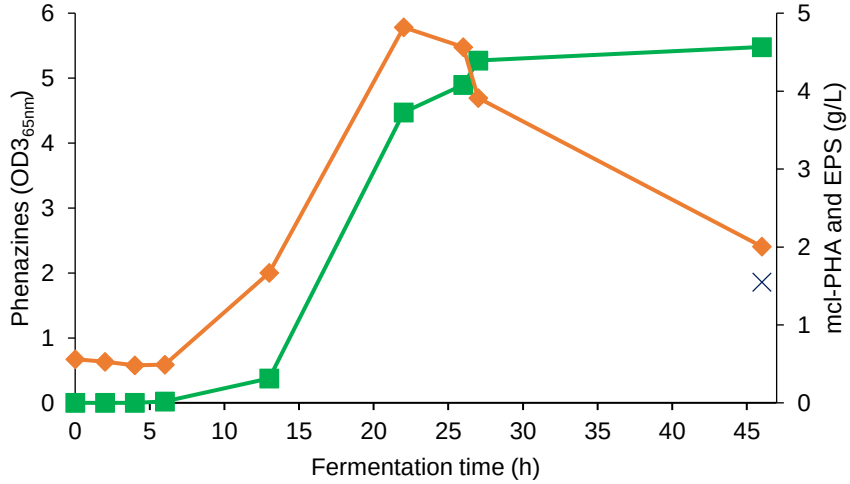


Figure 3.5 – Cultivation profile of *Pseudomonas chlororaphis* subsp. aurantiaca DSM 19603 during 5-fold N assay. (A) CDW (●) and glycerol concentration (▲) in (g/L); (B) Active biomass (●) and nitrogen concentration (-) in (g/L); (C) production profile of phenazines (♦) in OD_{365nm} and mcl-PHA (■); final concentration of EPS (X) in (g/L).

Related to the PHA production, at 27 hours of fermentation, the culture had accumulated 41.89% of its weight in mcl-PHA, corresponding to a concentration of 4.39 g/L. This value is 4 times higher than the mcl-PHA accumulated in the run performed with standard conditions (1.11 g/L), resulting in a volumetric productivity of 0.162 g/L·h.

EPS concentration at the end of the run was 1.55 g/L. This concentration means a volumetric productivity of 0.034 g/L·h, being these values lower than the ones achieved in standard assay.

Table 3.3 – Kinetic and stoichiometric parameters for all the different bioreactor experiments performed in this study.

Parameter	Standard assay	5-fold N assay	Batch assay	DO-glycerol assay	DO-20 assay	NH_4OH-base assay
Bacterial strain	*P. chlororaphis* DSM 19603	*P. chlororaphis* DSM 19603	*P. chlororaphis* DSM 19603	*P. chlororaphis* DSM 19603	*P. chlororaphis DSM 19603*	*P. chlororaphis DSM 19603*
Carbon source	Glycerol	Glycerol	Glycerol	Glycerol	Glycerol	Glycerol
Fermentation mode	Fed-batch	Fed-batch	Batch	Fed-batch	Fed-batch	Fed-batch
CDW (g/L)	7.98	12.21	11.60	9.01	10.41	15.29
Phenazines (OD_{365nm})	3.99	5.78	3.98	4.80	5.48	6.05
Active Biomass (g/L)	6.84	7.64	7.84	8.05	7.34	6.86
$Y_{x/s}$ ($g_{Biomass}/g_{subs}$)	0.195	0.115	0.264	0.214	0.158	0.100
PHA (%)	17.24	41.89	32.41	10.62	29.50	55.13
PHA (g/L)	1.11	4.39	3.76	0.96	3.07	8.43
$Y_{PHA/s}$ (g_{PHA}/g_{subs})	0.034	0.081	0.128	0.026	0.066	0.148
$r_{p\,PHA}$ (g/L·h)	0.026	0.162	0.078	0.020	0.068	0.211
EPS (g/L)	2.67	1.55	2.40	1.18	1.33	3.80
$Y_{EPS/s}$ (g_{EPS}/g_{subs})	0.072	0.025	0.082	0.032	0.029	0.071
$r_{p\,EPS}$ (g/L·h)	0.056	0.034	0.049	0.025	0.029	0.101

3.1.4 Batch fermentation assay

Taking into consideration that maximum phenazines amount was achieved at the end of exponential phase, and the biopolymers production was not so much increased after the pulse addition, it was performed a *Pseudomonas chlororaphis* cultivation on a batch mode, in a 2 L bioreactor. Nitrogen initial concentration was halved to 1.75 g/L and glycerol initial concentration was 40 g/L to allow mcl-PHA accumulation. Once more, NH_4OH was used as base for pH control.

In this assay, the culture did not present a lag phase and entered in stationary phase after 14 hours of exponential phase (Figure 3.6). During this phase the μ_{max} value was 0.200 h^{-1}, which was higher than in previous assay (0.131 h^{-1}). Therefore, the stationary phase and the orange colour inside the fermenter were reached faster than it was in the assay with 3.5 g/L of Nitrogen. At the end of exponential phase, CDW concentration was 9.7 g/L and almost 8 g/L of glycerol had been consumed at a rate of 0.57 g/L·h. At that time, the amount of phenazines was 3.98 OD_{365nm}. This value was the highest of the assay, but lower than the amount reached in the previous run, with 5 times more nitrogen concentration (5.78 OD_{365nm}) and similar to the one achieved in the standard assay (3.99 OD_{365nm}). Once again, phenazines amount decrease until the end of the run. On the other hand, the concentrations of mcl-PHA and EPS were 1.04 g/L and 0.55 g/L, respectively (Figure 3.6.C).

After that, the assay continued for 30 hours more, consuming 21.46 g/L of glycerol at a rate of 0.71 g/L·h. CDW achieved a concentration of 11.6 g/L, from which 32.41% was mcl-PHA. Therefore, the active biomass concentration, at the end of the run, was 7.84 g/L (Figure3.6.B) with a yield of biomass on glycerol ($Y_{X/S}$ = 0.264).

The mcl-PHA concentration was 3.76 g/L, which was lower than the one reached in the assay 5-fold nitrogen (4.57 g/L), however higher than the concentration achieved in the standard (1.11 g/L). This concentration of polymer presents a yield of 0.128 g/g and a volumetric productivity of 0.078 g/L·h. In contrast to mcl-PHA, EPS reached a concentration of 2.4 g/L, that is a higher concentration than the one achieved in previous assays, but similar to the concentration achieved by Meneses (2017) (Table 3.4). These values correspond to a $Y_{EPS/S}$ of 0.082 g/g and a volumetric productivity of 0.049 g/L·h. These values of r_p are lower than the achieved in the standard assay and higher than the ones achieved in the run with 3.5 g/L of nitrogen.

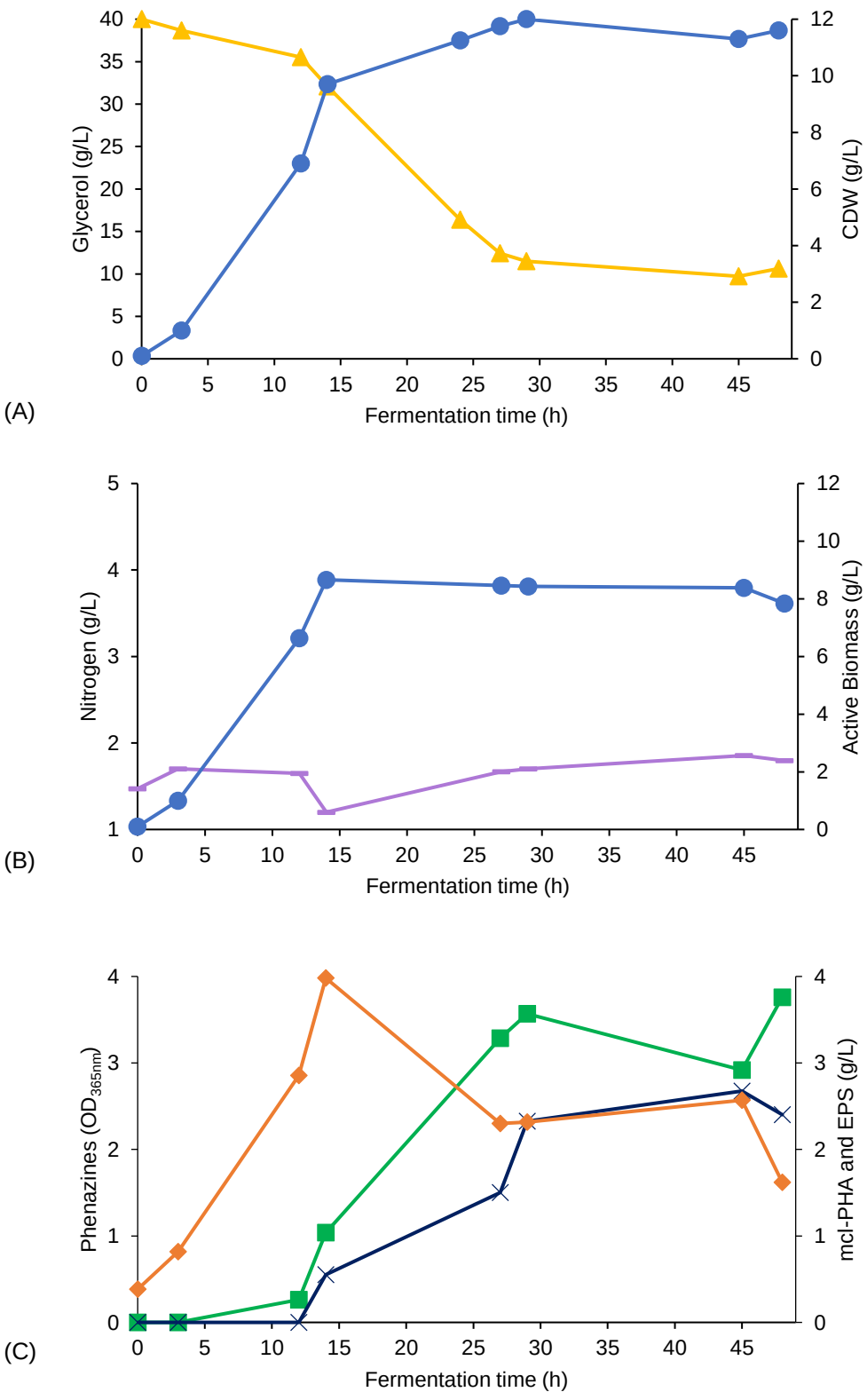


Figure 3.6 – Cultivation profile of *Pseudomonas chlororaphis* subsp. aurantiaca DSM 19603 during batch assay. (A) CDW (●) and glycerol concentration (▲) in (g/L); (B) Active biomass (●) and nitrogen concentration (-) in (g/L); (C) production profile of phenazines (♦) in OD_{365nm}, mcl-PHA (■) and EPS (X) in (g/L).

The results obtained with this approach presented higher EPS production, but lower phenazines production and mcl-PHA accumulation than the previous run. Moreover, despite the availability of nitrogen and glycerol across the experiment results were not improved, hence other feeding strategy was evaluated.

3.1.5 Dissolved Oxygen controlled with glycerol

As mentioned in introduction, glycerol is a carbon source that stimulate phenazines production and mcl-PHA accumulation (Tan et al., 2014). In the attempt to provide more carbon to the culture without slowdown its metabolism, a new feeding strategy was planned. During fermentation, stirring speed controlled dissolved oxygen (DO) at 30 %, however when the initial glycerol finished the DO start to increase for values above 30%. Therefore, strategy used was, when the exponential phase ending, DO was controlled by the automatic addition of glycerol. It was also decided to use 3.5 g/L of initial nitrogen concentration and 40 g/L of glycerol, since phenazines production was much higher using these conditions. Therefore, a fed-batch fermentation in a 2 L bioreactor, with medium E*, containing 16.5 g/L of $(NH_4)_2HPO_4$, 40 g/L of glycerol as carbon source and NH_4OH as base for pH control, was performed.

After 2 hours fermentation, the culture entered in exponential phase (Figure 3.7.A). The μ value was 0.247 h^{-1}, which was higher than all of others assays. At the end of the exponential phase the DO start increasing and for 2 hours, until 14 hours run, the DO control system was changed a few times between stirring speed and glycerol adding. After that, the system seemed to be adding low quantities of glycerol in order to control the DO at 30%. However, all the 250 g of glycerol available for DO control enter in the bioreactor in 12 h.

At the end of the exponential growth phase (14h) the CDW was 7.39 g/L. At the same time, the maximum amount 4.80 OD_{365nm} of phenazines were achieved. This value is similar to the ones achieved in the previous assays (Table 3.3). However, as in the other assay with high nitrogen concentration available, during stationary phase the amount of phenazines measured decrease.

During the rest of the assay the CDW concentration increased for 9.01 g/L, from that 8.05 g/L were active biomass (Figure 3.7.B) corresponding to a yield of 0.214 $g_{CDW}/g_{glyecrol}$. On the other hand, mcl-PHA concentration was very low (0.96 g/L), as well as the EPS production, which reached a concentration of 1.18 g/L. These lowest biopolymers concentration achieved could be related with the high glycerol concentration (134 g/L) presented in the medium during the stationary phase, which may have slow down the culture metabolism.

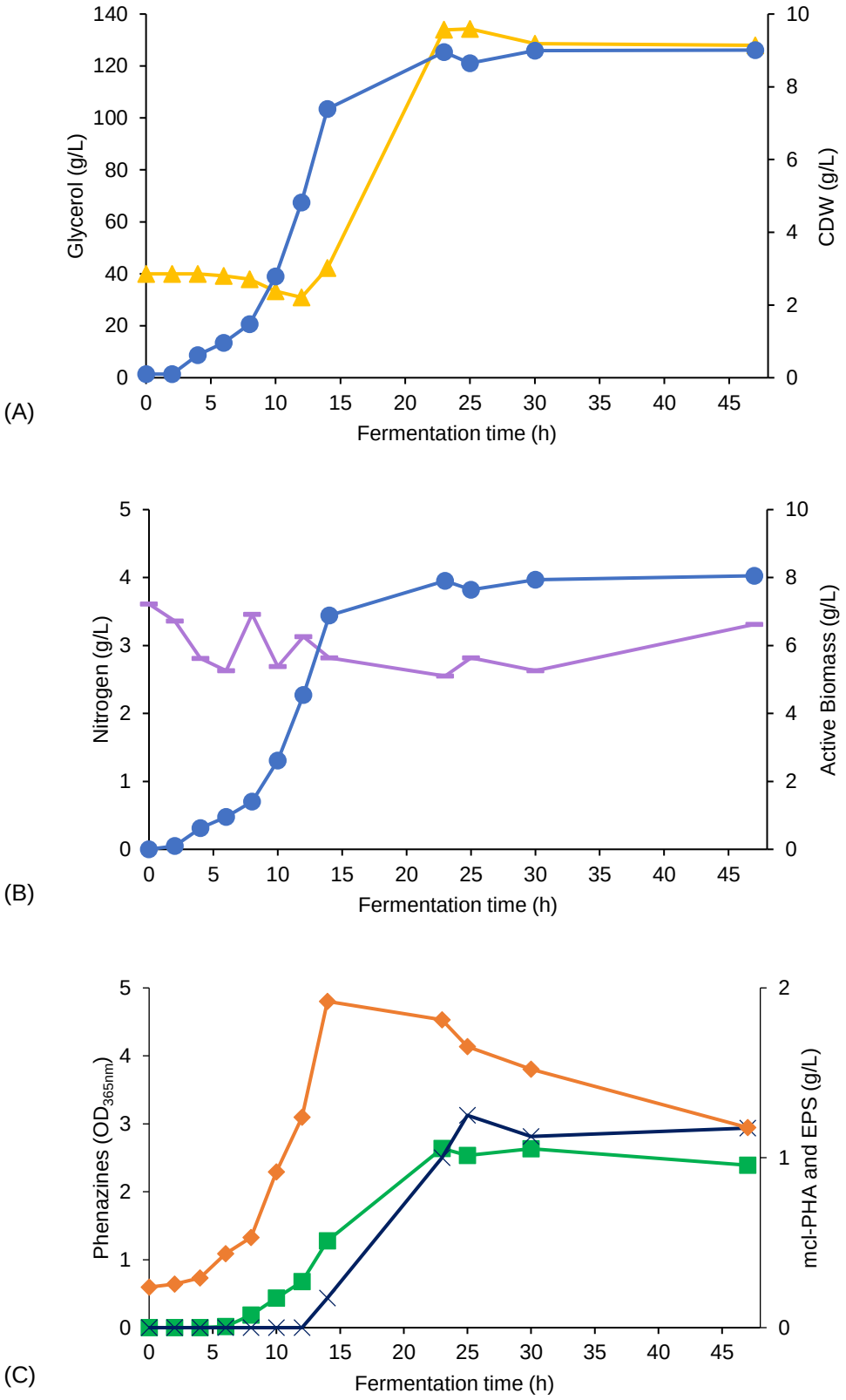


Figure 3.7 – Cultivation profile of *Pseudomonas chlororaphis* subsp. aurantiaca DSM 19603 during DO-glycerol assay. (A) CDW (●) and glycerol concentration (▲) in (g/L); (B) Active biomass (●) and nitrogen concentration (-) in (g/L); (C) production profile of phenazines (♦) in OD_{365nm}, mcl-PHA (■) and EPS (X) in (g/L).

3.1.6 Dissolved Oxygen controlled at 20%

As was already mentioned, bacteria increase the production of some defence products, such as EPS, under stress conditions (Vijayendra and Shamala, 2013; Ates, 2015). Hence, in order to test the impact of suboptimal conditions in *P. chlororaphis* metabolites production, the fermentation was designed in order to promote the culture growth in a medium with less 10% of available air. Therefore, the DO was controlled at 20% of air saturation by adjusting aeration to 1.7 SLPM and automatically adjusting the stirring speed between 250 and 700 rpm. The fed-batch fermentation was performed during 45 hours in a 2 L bioreactor with medium E*, with 16.5 g/L of $(NH_4)_2HPO_4$, 40 g/L of glycerol as carbon source and NH_4OH as base for pH control.

In this assay, the lag phase lasted for 6 hours. After that, the culture entered in exponential phase, which ended at 25 hours of fermentation. At this time, the CDW achieved by the culture was 9.15 g/L and nearly 31 g/L of glycerol had been consumed at an average consumption rate of 1.24 g/L·h. Providing a yield of 0.295 $g_{CDW}/g_{glycerol}$. By that time, the concentrations of mcl-PHA and EPS were 1.82 g/L and 1 g/L, respectively (Figure 3.8.C). The specific growth rate was 0.117 h^{-1} (Figure 3.8.A), which is a lower value than all others presented in previous assays. Therefore, it seems that the decrease of available O_2 lead to a lower specific growth rate of *P. chlororaphis*. In 1971, Maclennan et al., also verified that oxygen limitation led to a decline in growth rate of a bacterium from *Pseudomonas* genus.

After that, a 30 g/L glycerol pulse was fed to the bioreactor and the run continued for more 22 hours. During that time, more 15.26 g/L of glycerol were consumed at an average consumption rate of 0.69 g/L·h. In the end, CDW concentration was 10.41 g/L, being 29.5% of that weight in mcl-PHA accumulation. Therefore, active biomass concentration at the end of the run was 7.34 g/L (Figure 3.8.B) and presented a yield of 0.158 $g_{biomass}/g_{glycerol}$.

At 11 hours run, the bioreactor started to exhibit the orange colour characteristic of this culture. Although, the maximum value of phenazines amount was only achieved at 23 hours run 5.48 OD_{365nm} (Figure 3.8.C). This antibiotic amount was quite lower than the maximum amount reached on the assay with 3.5 g/L of nitrogen. However, the major difference this time, is that the amount of this compound did not decrease as much as in that assay. At the end of fermentation, phenazines amount in DO-20% assay was more than twice the amount of the assay with 5-fold nitrogen concentration at the same time.

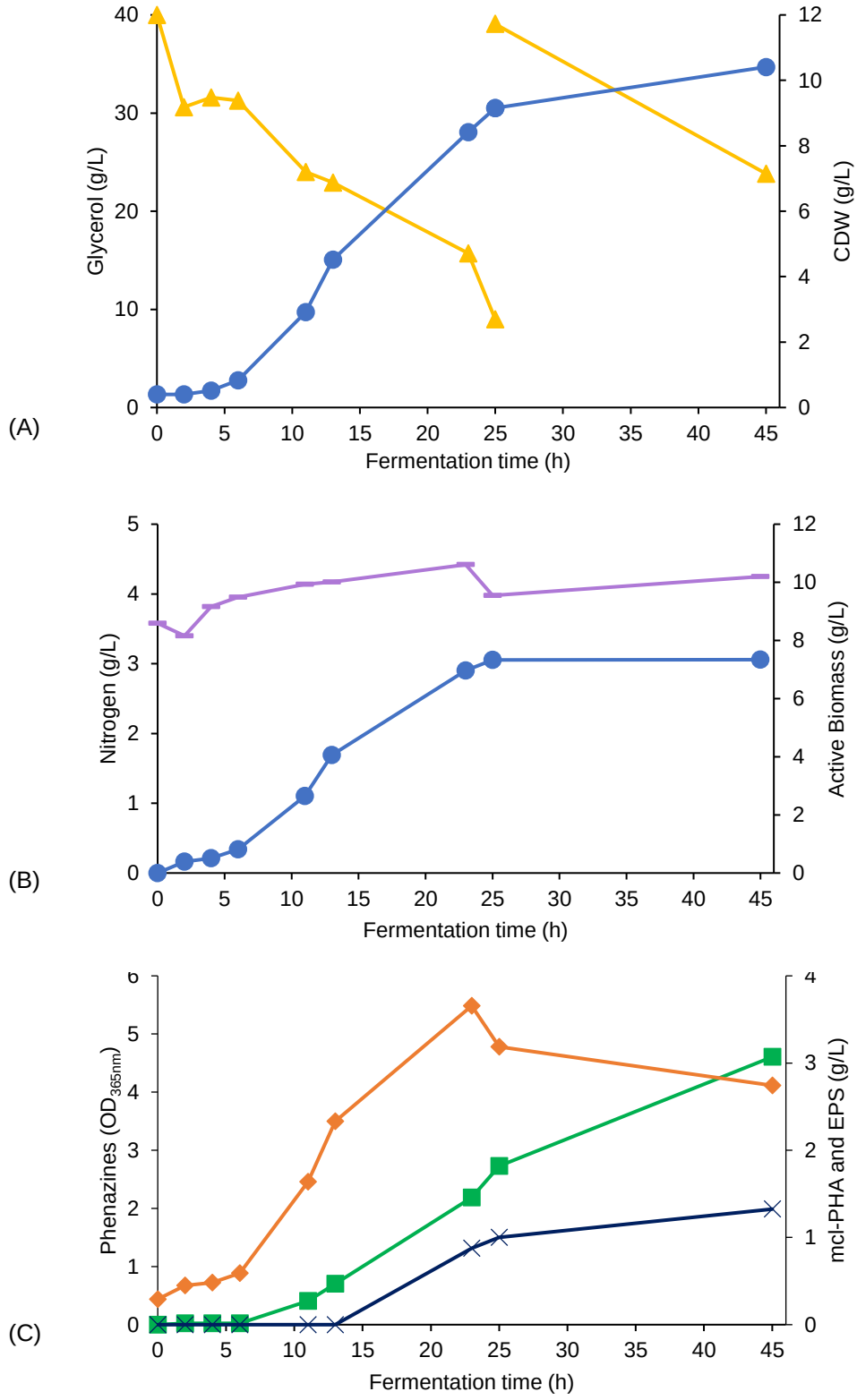


Figure 3.8 – Cultivation profile of *Pseudomonas chlororaphis* subsp. aurantiaca DSM 19603 during DO-20 assay. (A) CDW (●) and glycerol concentration (▲) in (g/L); (B) Active biomass (●) and nitrogen concentration (-) in (g/L); (C) production profile of phenazines (♦) in OD_{365nm}, mcl-PHA (■) and EPS (X) in (g/L).

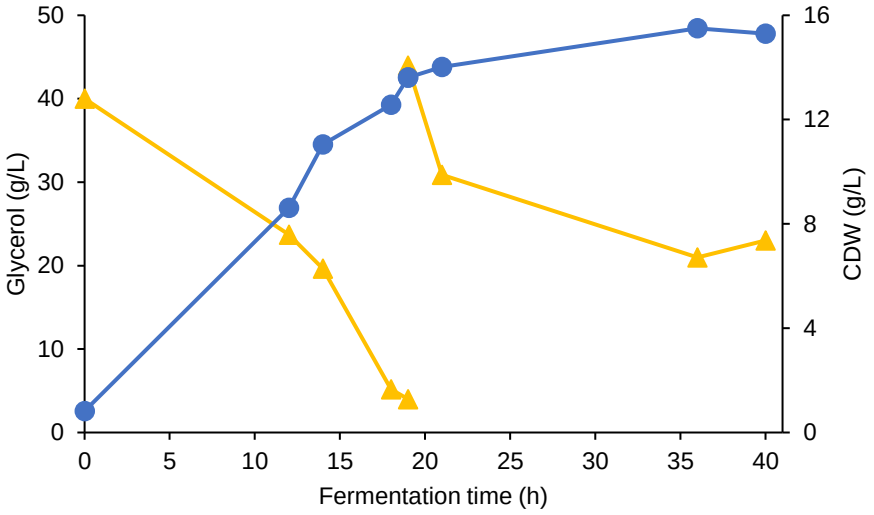
In the end of cultivation, mcl-PHA concentration was 3.07 g/L, which was lower than the one reached in nitrogen assay (4.39 g/L) (Table 3.3). This concentration of polymer presents a yield of 0.066 g/g and a volumetric productivity of 0.068 g/L·h. Regarding EPS production, it was achieved 1.33 g/L, which was also lower than the concentration achieved in the 5-fold nitrogen concentration and batch assays (1.55 and 2.40 g/L, respectively). This concentration of EPS corresponds to a yield of 0.029 g/g and a volumetric productivity of 0.029 g/L·h of this polymer, which are under the range of those obtained in the assay with 3.5 g/L of nitrogen. Therefore, the lower availability of oxygen in the cultivation medium did not promote metabolites production by *P. chlororaphis*.

3.1.7 Ammonium Hydroxide-base assay

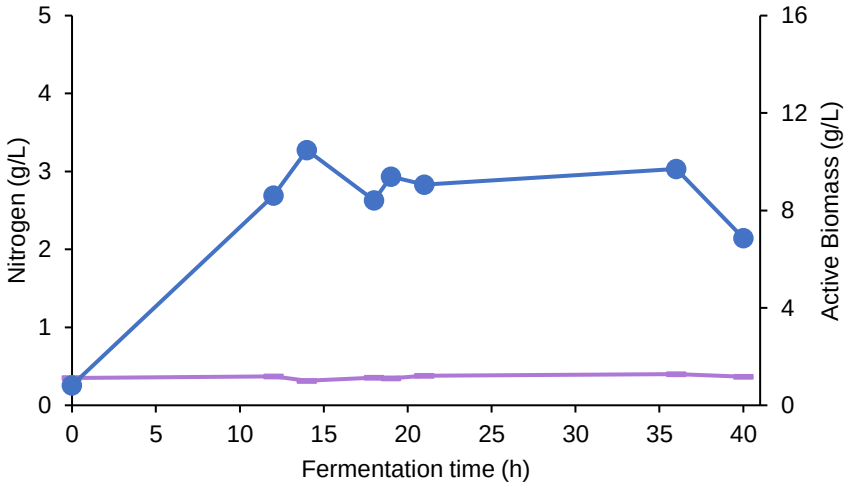
In the attempt to increase the production of mcl-PHA, without affect as much as possible the phenazines production, and taking into consideration the results of the previous experiments, a new fermentation strategy was planned. In order to provide non-limiting conditions of nitrogen to promote a higher growth of the culture, ammonium hydroxide (NH_4OH) was kept for pH control. However, initial $(NH_4)_2HPO_4$ concentration was changed to 3.3 g/L, which correspond to the initial standard nitrogen concentration (0.7 g/L). Since bacteria synthesize PHAs under nutrient limitation such as oxygen, nitrogen or phosphorus, and they do not accumulate PHAs in their growth phase (Mozejko-Ciesielska et al., 2019; Ward et al., 2005), this change was made in the attempt to direct the culture to mcl-PHA accumulation after cellular growth, due to limitation of phosphorus.

A fed-batch fermentation was performed during 40 hours in a 2 L bioreactor with medium E*, 40 g/L of glycerol as carbon source and NH_4OH as base for pH control. At 18 hours run, a 40 g/L glycerol pulse was added.

(A)



(B)



(C)

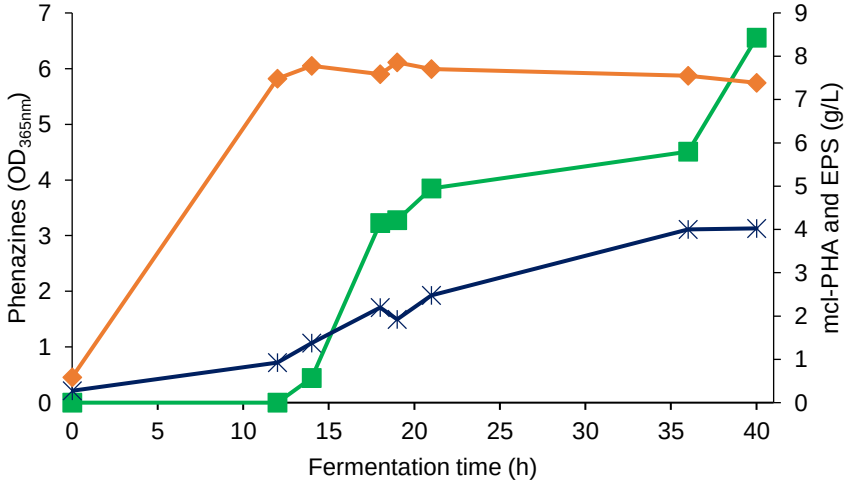


Figure 3.9 – Cultivation profile of *Pseudomonas chlororaphis* subsp. *aurantiaca* DSM 19603 during NH_4OH-base assay. (A) CDW (●) and glycerol concentration (▲) in (g/L); (B) Active biomass (●) and nitrogen concentration (-) in (g/L); (C) production profile of phenazines (♦) in OD_{365nm}, mcl-PHA (■) and EPS (X) in (g/L).

In this assay, *P. chlororaphis* entered in stationary phase after 14 hours run (Figure 3.9). A CDW of 11.05 g/L and nearly 20 g/L of glycerol were consumed at an average consumption rate of 1.43 g/L·h. The glycerol was mostly directed to cellular growth and phenazines production, since at that point the concentration of accumulated mcl-PHA was 0.57 g/L. The EPS concentration was slightly higher presenting a value of 1.38 g/L (Figure 3.9.C).

After the absorbance reading, was possible to see that phenazines amount reached a maximum value of 6.05 OD_{365nm}, at 12 hours run (Figure 3.9.C). By this time, the culture already exhibited an orange colour. This time the orange colour was more intense than in any other bioreactor (Figure 3.10). This colour became a little darker and intense until the end of the run, as it is possible to see in cell-free samples (Figure 3.11).

Figure 3.10 – Bioreactor with *Pseudomonas chlororaphis* subsp. aurantiaca DSM 19603 culture, during NH_4OH -base assay, at 14 h fermentation.

After 19 hours of cultivation, a 40 g/L glycerol pulse was added to the culture and the assay was prolonged for more 21 hours. At the end, 57 g/L of glycerol were consumed at an average glycerol consumption rate of 1.43 g/L·h. Subsequently, CDW still increased to 15.3 g/L, which was partly due to mcl-PHA accumulation that reached 55.13%. This implicate an active biomass concentration of 6.86 g/L (Figure 3.9.B), which provided a cellular growth yield of 0.100 g/g. At the end of the assay, mcl-PHA concentration was 8.43 g/L. This concentration of polymer presents a yield of 0.148 g/g and a volumetric productivity of 0.211 g/L·h., The other produced polymer, EPS, had a concentration of 4.02 g/L, which correspond to a yield of 0.071 g/g of and a volumetric productivity of 0.101 g/L·h.

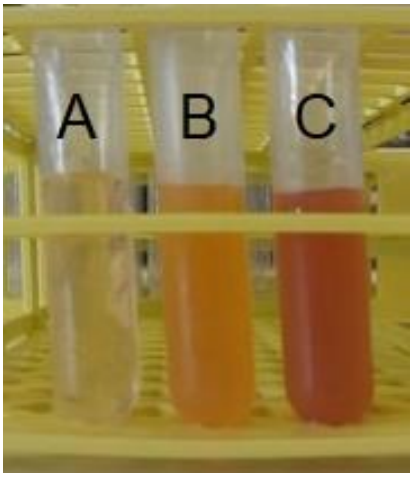


Figure 3.11 – Cell-free samples taken from the bioreactor with *Pseudomonas chlororaphis* subsp. aurantiaca DSM 19603 culture, during NH_4OH-base assay. (A) at 0 hours run; (B) at 14 hours run; (C) at 40 hours run.

The NH_4OH-based assay strategy enabled to reach the best results about cellular growth, phenazines, mcl-PHA and EPS production (Table 3.3). The phenazines amount in this assay, not only was the highest, as it was reached faster (14 hours run) than the amount achieved in the 5-fold N assay (22 hours run). In relation to mcl-PHA, this study exhibited the highest concentration of polymer and the highest volumetric productivity. The EPS concentration achieved was also the highest (4.02 g/L), as well as volumetric productivity.

The results achieved in this run are better than the ones achieved by other authors reported in the literature, for *P. chlororaphis* strains (Table 3.4). Muhr et al. (2013), used saturated biodiesel fractions (SFAE) as sole carbon source and achieved a cellular concentration much higher (42.2 g/L) than the one achieved in this study, but the mcl-PHA content of the cells was much lower (15.2%). A mcl-PHA content of 45% was achieved by Yun et al., 2003, using palm kernel oil (PKO) as sole carbon source, however, cellular concentration was very low (3.3 g/L).

By comparing these results with a previous work performed by Meneses, 2017 with the same bacteria, is possible to conclude that in this study, were achieved higher concentrations of the two polymers produced by *P. chlororaphis*. The phenazines amount was slightly higher as well.

Table 3.4 – Kinetic and stoichiometric parameters for NH_4OH-base assay performed in this study compared to other studies that use *P. chlororaphis* strains to mcl-PHA production; (values with * were estimated based on the values given in the literature; value with ** was recalculated with the same phenazines protocol performed during this study; SFAE – saturated biodiesel fractions; PKO – palm kernel oil).

Parameter	NH_4OH-base assay	Pereira, 2016	Meneses, 2017	Muhr et al., 2013	Yun et al., 2003
Bacterial strain	*P. chlororaphis* DSM 19603	*P. chlororaphis* DSM 19603	*P. chlororaphis* DSM 19603	*P. chlororaphis* DSM-50083	*P. chlororaphis HS21*
Carbon source	Glycerol	Glycerol waste	Glycerol	SFAE	PKO
Fermentation mode	Fed-batch	Fed-batch	Fed-batch	Fed-batch	Batch
CDW (g/L)	15.29	9.35	13.58	42.20	3.30
Phenazines (OD_{365nm})	6.05	-	**5.38	-	-
Active Biomass (g/L)	6.86	*8.42	10.95	*35.79	*1.81
$Y_{x/s}$ **($g_{Biomass}/g_{subs}$)**	0.100	0.30	0.20	0.66	0.62
PHA (%)	55.13	10.00	19.00	15.20	45.00
PHA (g/L)	8.43	0.93	2.64	*6.41	1.49
$Y_{PHA/s}$ **(g_{PHA}/g_{subs})**	0.148	0.03	0.05	0.10	-
$r_{p\,PHA}$ **(g/L·h)**	0.211	0.02	*0.05	0.14	0.07
EPS (g/L)	3.80	-	2.40	-	-
$Y_{EPS/s}$ **(g_{EPS}/g_{subs})**	0.071	-	0.04	-	-
$r_{p\,EPS}$ **(g/L·h)**	0.101	-	*0.05	-	-

3.2 Composition of mcl-PHA accumulated by *P. chlororaphis*

The mcl-PHA monomer composition was evaluated by gas chromatography. Although all samples have been analysed, in table 3.5 are represented the monomer composition of the last sample of each assay.

All samples of mcl-PHA presented in their composition the same 5 monomers: 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDd) and 3-hydroxytetradecanoate (3HTd). By comparing the monomers percentage of each experiment, the major difference between them is the composition of mcl-PHA obtained in standard assay, which presents a considerable percentage of 3HHx, an insignificant quantity of 3HDd and more than half of this polymer is composed by 3HD. On the other hand, the remain four polymers exhibit an insignificant percentage of 3HHx, and the main part of their composition is the monomer 3HDd. The 5-fold N and NH_4OH-base assays presented an mcl-PHA monomer composition very similar (Table 3.5).

Table 3.5 – Mcl-PHA monomer composition (%) of the last sample of each assay of this study, compared to other studies that use *P. chlororaphis* subsp. aurantiaca DSM 19603 to mcl-PHA production.

Assay	Monomers (%)				
	3HHx	3HO	3HD	3HDd	3HTd
Standard	5.0	24.6	51.0	0.8	18.6
5-fold N	0.7	5.4	25.2	65.0	3.7
Batch	1.0	8.1	26.3	60.1	4.5
DO-glycerol	0.2	5.3	14.0	78.1	2.4
DO-20	0.8	7.1	21.3	67.7	3.1
NH_4OH-base	0.7	5.7	27.6	61.5	4.5
Meneses, 2017	3	17	50	13	17
Pereira, 2016	6.1	26.9	54.1	13	-

The main difference between the cultivation of the standard mcl-PHA to the other ones was that in standard fermentation the concentration of the nitrogen source hit the 0 g/L. Therefore, results suggest that the mcl-PHA composition, and consequently its properties, can be modified by varying the nutrient composition/ concentration of cultivation medium. These results could be supported by the study performed by Hong et al., 2000, where the monomers composition of mcl-

PHA produced by *Pseudomonas mendocina* 0806 and *Pseudomonas pseudoalkaligenus* YSl, was successfully affected by C:N molar ratio in the cultivation medium. These changes in monomers composition occur especially in heteropolymers, which is the category where mcl-PHA produced by *Pseudomonas chlororaphis* subsp. aurantiaca DSM 19603 is integrated.

By comparing the results of this work with Meneses, 2017 and Pereira, 2016, is perceived that the composition of mcl-PHA of these two studies was similar to the composition of mcl-PHA from standard assay. Once again, this could be explained by C:N molar ratio in the cultivation medium. In both of these studies, during stationary phase, the base used to control pH was NaOH. Therefore, it seems that nitrogen concentrations inside the fermenters in these two studies were lower than in all assays of this work, with exception to standard assay.

Since mcl-PHA is a polymer that shows different properties depending on its monomer's composition, the ability of changing its composition is a real added value. 3HHx polymers, when in significant proportions (>10%) can maximize toughness and elongation at break without severe loss in tensile strength (Lim et al., 2013). Even better is a polymer with higher 3HDd percentage (>30%), since presents remarkable change in mechanical properties, becoming normal elastic materials that are not sticky (Liu and Chen, 2007).

3.3 Characterisation of EPS produced by *P. chlororaphis*

To understand the sugar composition of the EPS produced by *P. chlororaphis*, the samples were hydrolysed and analysed by HPLC. The chromatogram obtained from that analysis is shown in figure 3.12.

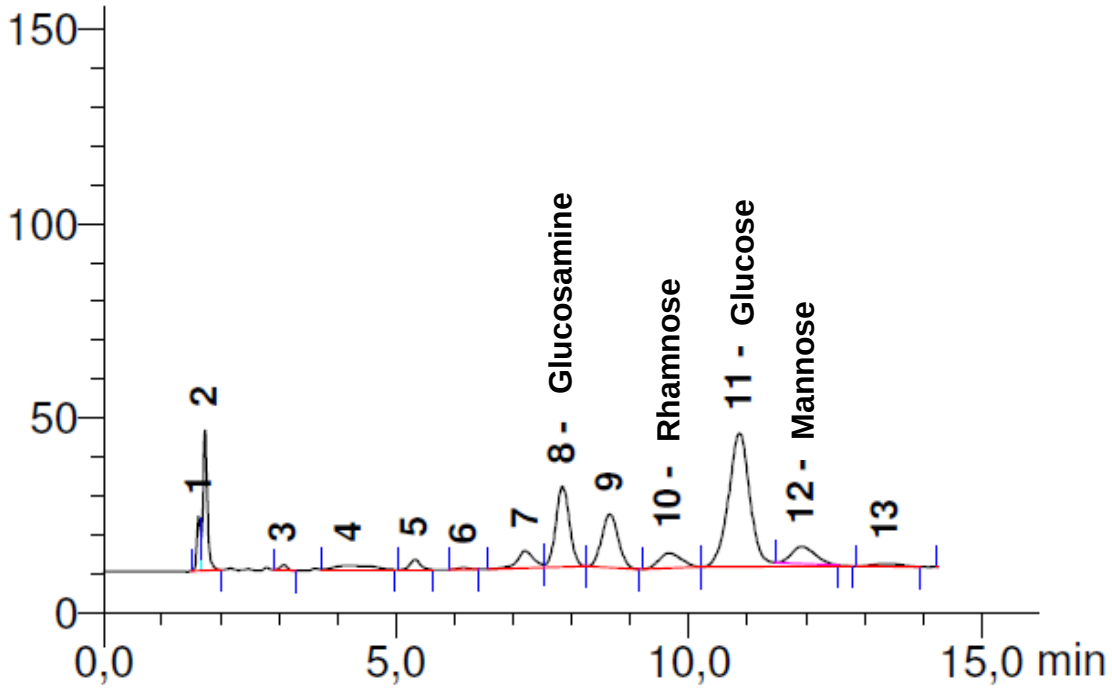


Figure 3.12 – Chromatogram of an EPS sample produced by *Pseudomonas chlororaphis* subsp. *aurantiaca* DSM 19603 from glycerol.

From the chromatogram, only four major peaks were identified. Glucose, that was the main sugar monomer of the EPS in all sample, appearing at retention time RT=10.86 min. Glucosamine, appearing at retention time RT=7.84 min. Rhamnose, appearing at retention time RT=9.66 min. And the last one, mannose, appearing at retention time RT=11.93 min. It was not possible to identify the other peaks. The composition of each EPS sample with the identified sugar is in table 3.6.

By look at table 3.6, is possible to perceived that the sugar composition of each sample of this work does not present significant differences and in contrast to mcl-PHA, seems not be changed by nitrogen and carbon concentrations. In all samples, the identified sugar in higher percentage is glucose, being that most of the polymer remain undefined. The sugars identified on the EPS samples of this work are the same that were identified in Meneses, 2017 study.

The available information about EPS secreted by *P. chlororaphis* strains is very limited. An extracellular polymer produced by *P. chlororaphis* NRRL B-2075 containing alginate was identified by Fett et al., 1996. Despite major differences, the mentioned EPS exhibit two similarities with the one produced during this work: the presence of neutral sugars such as

mannose in a range between 1 and 14%, and the presence of hexosamines (glucosamine sugar kind).

In 2018, Mi Cho et al. also mentioned an EPS produced by a *P. chlororaphis* strain. However, by the date that the work was published, the extracellular polymer composition was not known. Nevertheless, it was anticipated the possibility of this EPS also contain alginate, like the EPS produced by *Pseudomonas aeruginosa*.

Table 3.6 – EPS monomer sugar composition (%) of the last sample of each assay of this study.

Assay	**Sugar (%)**				**Undefined (%)**
	Glucosamine	Rhamnose	Glucose	Mannose	
Standard	2.34	0.6	14.95	0.86	81.25
5-fold N	10.46	3.17	28.49	3.79	54.09
Batch	3.93	1.16	25.46	1.84	67.61
DO-glycerol	1.20	0.37	9.60	0.38	88.45
DO-20	1.68	0.59	33.44	1.78	62.51
NH_4OH-base	18.52	5.05	26.14	4.72	45.57

3.4 Conclusions and Future Work

P. chlororaphis is able to co-produce phenazines, mcl-PHA and EPS, using glycerol as carbon source. Phenazines production are growth associated, ending or slowing down when the culture entered in stationary phase. On the other hand, biopolymers (mcl-PHA and EPS) production occur during the stationary phase, under nutrient limiting conditions. The production of the 3 products was affected by the fermentation conditions, namely nitrogen and glycerol concentration, and dissolved oxygen.

P. chlororaphis achieved the highest phenazines production and the highest mcl-PHA and EPS concentrations (8.43 and 3.8 g/L, respectively), as well as volumetric productivities, in fed-batch cultivation using 40 g/L glycerol, 0.7 g/L nitrogen and controlling the pH with ammonium hydroxide.

The mcl-PHA was composed of five monomers: 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDd) and 3-hydroxytetradecanoate (3HTd). 3HDd was the monomer present in higher amount in almost every mcl-PHA sample, but the amount of each monomer varied with the cultivation conditions. The monomer composition achieved seems to guarantee desirable properties to the mcl-PHA. Therefore, their detailed characterization and properties evaluation should be performed.

Regarding EPS, the other polymer produced by *P. chlororaphis*, it was composed of glucosamine, rhamnose, glucose and mannose. The EPS has to be characterized about its physicochemical properties, such as molecular weight.

The mechanisms that make the culture direct its metabolism to produce either one or another polymer need to be better understood. Nevertheless, it seems that the carbon source concentration and limitation of phosphate, are some of the reasons that may influence. Hence, tests are required to confirm these hypotheses.

Phenazines production was measured by a spectrophotometric method. Therefore, quantitative tests should be performed in order to ensure the real quantity of phenazines in the fermenter. The phenazines produced by *P. chlororaphis* are described to have some very interesting properties, such as antibiotic, antiviral or anticancer agent. It would also be interesting to try to purify and, perhaps, find new areas where they can be applied.

Chapter 4
References

Agustín Martinez, G., Bertin, L., Scoma, A., Rebecchi, S., Braunegg, G. and Fava, F. (2015). Production of polyhydroxyalkanoates from dephenolised and fermented olive mill wastewaters by employing a pure culture of *Cupriavidus necator*. *Biochemical Engineering Journal*, 97, pp.92-100.

Anjum, A., Zuber, M., Zia, K., Noreen, A., Anjum, M. and Tabasum, S. (2016). Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: A review of recent advancements. *International Journal of Biological Macromolecules*, 89, pp.161-174.

Anzai, Y., Kim, H., Park, J., Wakabayashi, H. and Oyaizu, H. (2000). Phylogenetic affiliation of the *pseudomonads* based on 16S rRNA sequence. *International Journal of Systematic and Evolutionary Microbiology*, 50(4), pp.1563-1589.

Ates, O. (2015). Systems Biology of Microbial Exopolysaccharides Production. *Frontiers in Bioengineering and Biotechnology*, 3.

Bauer, J., Hauck, N., Christof, L., Mehnaz, S., Gust, B. and Gross, H. (2016). The Systematic Investigation of the Quorum Sensing System of the Biocontrol Strain *Pseudomonas chlororaphis* subsp. *aurantiaca* PB-St2 Unveils aurI to Be a Biosynthetic Origin for 3-Oxo-Homoserine Lactones. *Plos One*, 11(11), p.e0167002.

Beifuss, U. and Tietze, M. (2005). Methanophenazine and Other Natural Biologically Active Phenazines. *ChemInform*, 36(37).

Bourret, R. and Silversmith, R. (2010). Two-component signal transduction. Current Opinion in *Microbiology*, 13(2), pp.113-115.

Carvalho, G., Lemos, P., Oehmen, A. and Reis, M. (2007). Denitrifying phosphorus removal: Linking the process performance with the microbial community structure. *Water Research*, 41(19), pp.4383-4396.

Castellane, T., Persona, M., Campanharo, J. and de Macedo Lemos, E. (2015). Production of exopolysaccharide from rhizobia with potential biotechnological and bioremediation applications. *International Journal of Biological Macromolecules*, 74, pp.515-522.

Chen, W., Liu, X., Qian, C., Song, X., Li, W. and Yu, H. (2015). An UV–vis spectroelectrochemical approach for rapid detection of phenazines and exploration of their redox characteristics. *Biosensors and Bioelectronics*, 64, pp.25-29.

Chincholkar, S. and Thomashow, L. (2013). *Microbial Phenazines*. 1st ed. Heidelberg New York Dordrecht London: Springer.

Cruz, M., Araújo, D., Alves, V., Freitas, F. and Reis, M. (2015). Characterization of medium chain length polyhydroxyalkanoate produced from olive oil deodorizer distillate. *International Journal of Biological Macromolecules*, 82, pp.243-248.

Cruz, M., Freitas, F., Paiva, A., Mano, F., Dionísio, M., Ramos, A. and Reis, M. (2016). Valorization of fatty acids-containing wastes and byproducts into short- and medium-chain length polyhydroxyalkanoates. *New Biotechnology*, 33(1), pp.206-215.

Cruz, M., Freitas, F., Torres, C., Reis, M. and Alves, V. (2011). Influence of temperature on the rheological behavior of a new fucose-containing bacterial exopolysaccharide. *International Journal of Biological Macromolecules*, 48(4), pp.695-699.

de Andrade-Neto, V., Goulart, M., da Silva Filho, J., da Silva, M., Pinto, M., Pinto, A., Zalis, M., Carvalho, L. and Krettli, A. (2004). Antimalarial Activity of Phenazines from Lapachol, β-Lapachone and Its Derivatives Against *Plasmodium falciparum* in vitro and *Plasmodium berghei* in vivo. *ChemInform*, 35(26).

De Maeyer, K., D'aes, J., Hua, G., Perneel, M., Vanhaecke, L., Noppe, H. and Hofte, M. (2010). N-Acylhomoserine lactone quorum-sensing signalling in antagonistic phenazine-producing *Pseudomonas* isolates from the red cocoyam rhizosphere. *Microbiology*, 157(2), pp.459-472.

Delaney, S., Mavrodi, D., Bonsall, R. and Thomashow, L. (2001). phzO, a Gene for Biosynthesis of 2-Hydroxylated Phenazine Compounds in *Pseudomonas aureofaciens* 30-84. *Journal of Bacteriology*, 183(1), pp.318-327.

Elnashar, M. (2011). *Biotechnology of biopolymers*. 1st ed. Rijeka: InTech.

Fett, W., Cescutti, P. and Wijey, C. (1996). Exopolysaccharides of the plant pathogens *Pseudomonas corrugata* and *Ps. flavescens* and the saprophyte *Ps. chlororaphis*. *Journal of Applied Bacteriology*, 81(2), pp.181-187.

Finore, I., Di Donato, P., Mastascusa, V., Nicolaus, B. and Poli, A. (2014). Fermentation Technologies for the Optimization of Marine Microbial Exopolysaccharide Production. *Marine Drugs*, 12(5), pp.3005-3024.

Freitas, F., Alves, V., Torres, C., Cruz, M., Sousa, I., Melo, M., Ramos, A. and Reis, M. (2011). Fucose-containing exopolysaccharide produced by the newly isolated *Enterobacter* strain A47 DSM 23139. *Carbohydrate Polymers*, 83(1), pp.159-165.

Haas, D. and Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature Reviews Microbiology*, 3(4), pp.307-319.

Helou, C., Marier, D., Jacolot, P., Abdennebi-Najar, L., Niquet-Léridon, C., Tessier, F. and Gadonna-Widehem, P. (2013). Microorganisms and Maillard reaction products: a review of the literature and recent findings. *Amino Acids*, 46(2), pp.267-277.

Hohlneicher, U., Hartmann, R., Taraz, K. and Budzikiewicz, H. (1995). Pyoverdin, Ferribactin, Azotobactin -a New Triade of Siderophores from *Pseudomonas chlororaphis* ATCC 9446 and Its Relation to *Pseudomonas fluorescens* ATCC 13525. *Zeitschrift für Naturforschung C*, 50(5-6), pp.337-344.

Hong, K., Chen, G., Yu, P., Zhang, G., Liu, Y. and Chua, H. (2000). Effect of C:N Molar Ratio on Monomer Composition of Polyhydroxyalkanoates Produced by *Pseudomonas mendocina* 0806 and *Pseudomonas pseudoalkaligenus* YS1. *Applied Biochemistry and Biotechnology*, 84-86(1-9), pp.971-980.

Imamura, N., Nishijima, M., Takadera, T., Adachi, K., Sakai, M. And Sano, H. (1997). New Anticancer Antibiotics Pelagiomicins, Produced by a New Marine Bacterium *Pelagiobacter variabilis*. *The Journal of Antibiotics*, 50(1), pp.8-12.

Jacquel, N., Lo, C., Wu, H., Wei, Y. and Wang, S. (2007). Solubility of polyhydroxyalkanoates by experiment and thermodynamic correlations. *AIChE Journal*, 53(10), pp.2704-2714.

Kasana, R., Sharma, U., Sharma, N. and Sinha, A. (2007). Isolation and Identification of a Novel Strain of *Pseudomonas chlororaphis* Capable of Transforming Isoeugenol to Vanillin†. *Current Microbiology*, 54(6), pp.457-461.

Keshavarz, T. and Roy, I. (2010). Polyhydroxyalkanoates: bioplastics with a green agenda. *Current Opinion in Microbiology*, 13(3), pp.321-326.

Laursen, J. and Nielsen, J. (2004). Phenazine Natural Products: Biosynthesis, Synthetic Analogues, and Biological Activity. *ChemInform*, 35(21).

Lee, S. (1996). Bacterial polyhydroxyalkanoates. *Biotechnology and Bioengineering*, 49(1), pp.1-14.

Li, Y., Jiang, H., Du, X., Huang, X., Zhang, X., Xu, Y. and Xu, Y. (2010). Enhancement of phenazine-1-carboxylic acid production using batch and fed-batch culture of gacA inactivated *Pseudomonas* sp. M18G. *Bioresource Technology*, 101(10), pp.3649-3656.

Lim, J., Park, K., Chung, G. and Kim, J. (2013). Effect of composition ratio on the thermal and physical properties of semicrystalline PLA/PHB-HHx composites. *Materials Science and Engineering: C*, 33(4), pp.2131-2137.

Liu, W. and Chen, G. (2007). Production and characterization of medium-chain-length polyhydroxyalkanoate with high 3-hydroxytetradecanoate monomer content by fadB and fadA knockout mutant of *Pseudomonas putida* KT2442. *Applied Microbiology and Biotechnology*, 76(5), pp.1153-1159.

Maclennan, D., Ousby, J., Vasey, R. And Cotton, N. (1971). The Influence of Dissolved Oxygen on *Pseudomonas* AM1 Grown on Methanol in Continuous Culture. *Journal of General Microbiology*, 69(3), pp.395-404.

Mavrodi, D., Blankenfeldt, W. and Thomashow, L. (2006). Phenazine Compounds in Fluorescent *Pseudomonas* Spp. Biosynthesis and Regulation. *Annual Review of Phytopathology*, 44(1), pp.417-445.

Meneses, L. (2017). *Evaluation of sub-, supercritical fluids as solvents for extraction and impregnation of polyhydroxyalkanoates*. Master thesis. Universidade Nova de Lisboa.

Mi Cho, S., J. Anderson, A. and Cheol Kim, Y. (2018). Extracellular Polymeric Substances of *Pseudomonas chlororaphis* O6 Induce Systemic Drought Tolerance in Plants. *Res. Plant Dis.*, 24(3), pp.242-247.

Moita, R. and Lemos, P. (2010). Biopolymers production from mixed cultures and pyrolysis by-products. *Journal of Biotechnology*, 150, pp.36-36.

Możejko-Ciesielska, J. and Kiewisz, R. (2016). Bacterial polyhydroxyalkanoates: Still fabulous?. *Microbiological Research*, 192, pp.271-282.

Mozejko-Ciesielska, J., Szacherska, K. and Marciniak, P. (2019). *Pseudomonas* Species as Producers of Eco-friendly Polyhydroxyalkanoates. *Journal of Polymers and the Environment*, 27(6), pp.1151-1166.

Muhr, A., Rechberger, E., Salerno, A., Reiterer, A., Malli, K., Strohmeier, K., Schober, S., Mittelbach, M. and Koller, M. (2013). Novel Description of mcl-PHA Biosynthesis by *Pseudomonas chlororaphis* from Animal-Derived Waste. *Journal of Biotechnology*, 165(1), pp.45-51.

Nandi, M., Selin, C., Brassinga, A., Belmonte, M., Fernando, W., Loewen, P. and de Kievit, T. (2015). Pyrrolnitrin and Hydrogen Cyanide Production by *Pseudomonas chlororaphis* Strain PA23

Exhibits Nematicidal and Repellent Activity against Caenorhabditis elegans. *Plos One*, 10(4), p.e0123184.

Nansathit, A., Apipattarakul, S., Phaosiri, C., Pongdontri, P., Chanthai, S. And Ruangviriyachai, C. (2009). Synthesis, Isolation of Phenazine Derivatives and Their Antimicrobial Activities. *Walailak J Sci & Tech*, 6(1), pp.79-91.

Özen, A. and Ussery, D. (2011). Defining the *Pseudomonas* Genus: Where Do We Draw the Line with *Azotobacter*?. *Microbial Ecology*, 63(2), pp.239-248.

Pereira, J. (2016). *Investigation of the Adhesive Properties of Bacterial Medium-Chain-Length Polyhydroxyalkanoates (mcl-PHA) for Medical Applications*. Master thesis. Universidade Nova de Lisboa.

Pereira, J., Araújo, D., Marques, A., Neves, L., Grandfils, C., Sevrin, C., Alves, V., Fortunato, E., Reis, M. and Freitas, F. (2019). Demonstration of the adhesive properties of the medium-chain-length polyhydroxyalkanoate produced by *Pseudomonas chlororaphis* subsp. *aurantiaca* from glycerol. *International Journal of Biological Macromolecules*, 122, pp.1144-1151.

Pierson III, L., Wood, D. and Pierson, E. (1998). Homoserine Lactone-Mediated Gene Regulation In Plant-Associated Bacteria. *Annual Review of Phytopathology*, 36(1), pp.207-225.

Pierson, L. and Pierson, E. (1996). Phenazine antibiotic production in *Pseudomonas aureofaciens*: role in rhizosphere ecology and pathogen suppression. *FEMS Microbiology Letters*, 136(2), pp.101-108.

Pierson, L. and Pierson, E. (2010). Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes. *Applied Microbiology and Biotechnology*, 86(6), pp.1659-1670.

Pubchem.ncbi.nlm.nih.gov. (2019). Phenazine. [online] Available at: https://pubchem.ncbi.nlm.nih.gov/compound/4757 [Accessed 02 Aug. 2019].

Ramsay, B., Saracovan, I., Ramsay, J. and Marchessault, R. (1991). Continuous Production of Long-Side-Chain Poly-β-Hydroxyalkanoates by *Pseudomonas oleovorans*. *Appl Environ Microbiol*, 57(3), pp.625–629.

Raza, Z., Tariq, M., Majeed, M. and Banat, I. (2019). Recent developments in bioreactor scale production of bacterial polyhydroxyalkanoates. *Bioprocess and Biosystems Engineering*, 42(6), pp.901-919.

Reddy, C., Ghai, R., Rashmi and Kalia, V. (2003). Polyhydroxyalkanoates: an overview. *Bioresource Technology*, 87(2), pp.137-146.

Rees, G., Vasiliadis, G., May, J. and Bayly, R. (1992). Differentiation of polyphosphate and poly-Î²-hydroxybutyrate granules in an *Acinetobacter* sp. isolated from activated sludge. *FEMS Microbiology Letters*, 94(1-2), pp.171-173.

Report European Bioplastics, 2018. *Bioplastics market data,* 2018.

Saosoong, K., Wongphathanakul, W., Poasiri, C. and Ruangviriyachai, C. (2009). Isolation and Analysis of Antibacterial Substance Produced from *P. aeruginosa* TISTR 781. *KKU Sci J,* 37:163–172

Serafim, L., Lemos, P., Albuquerque, M. and Reis, M. (2008). Strategies for PHA production by mixed cultures and renewable waste materials. *Applied Microbiology and Biotechnology*, 81(4), pp.615-628.

Sullivan, N., Tzeranis, D., Wang, Y., So, P. and Newman, D. (2011). Quantifying the Dynamics of Bacterial Secondary Metabolites by Spectral Multiphoton Microscopy. *ACS Chemical Biology*, 6(9), pp.893-899.

Sun, Z., Ramsay, J., Guay, M. and Ramsay, B. (2007). Fermentation process development for the production of medium-chain-length poly-3-hyroxyalkanoates. *Applied Microbiology and Biotechnology*, 75(3), pp.475-485.

Sutherland, I. (2009). *Biotechnology of Microbial Exopolysaccharides*. 1st ed. Cambridge, GBR: Cambridge University Press.

Tan, G., Chen, C., Li, L., Ge, L., Wang, L., Razaad, I., Li, Y., Zhao, L., Mo, Y. and Wang, J. (2014). Start a Research on Biopolymer Polyhydroxyalkanoate (PHA): A Review. *Polymers*, 6(3), pp.706-754.

Torres, C., Marques, R., Antunes, S., Alves, V., Sousa, I., Ramos, A., Oliveira, R., Freitas, F. and Reis, M. (2011). Kinetics of production and characterization of the fucose-containing exopolysaccharide from *Enterobacter* A47. *Journal of Biotechnology*, 156(4), pp.261-267.

Treseder, K. (2008). Nitrogen additions and microbial biomass: a meta-analysis of ecosystem studies. *Ecology Letters*, 11(10), pp.1111-1120.

Vijayendra, S. and Shamala, T. (2013). Film forming microbial biopolymers for commercial applications—A review. *Critical Reviews in Biotechnology*, 34(4), pp.338-357.

Wang, Y., Zhou, X., Hu, J. and Zhang, Q. (2012). Study on Extraction and Identification of PHB from H_2 Production Fermentation Residue by Photosynthetic Bacteria. *Advanced Materials Research*, 518-523, pp.108-115.

Ward, P., de Roo, G. and O'Connor, K. (2005). Accumulation of Polyhydroxyalkanoate from Styrene and Phenylacetic Acid by *Pseudomonas putida* CA-3. *Applied and Environmental Microbiology*, 71(4), pp.2046-2052.

Yu, J., Wang, D., Pierson, L. and Pierson, E. (2018). Effect of Producing Different Phenazines on Bacterial Fitness and Biological Control in *Pseudomonas chlororaphis* 30-84. *The Plant Pathology Journal*, 34(1), pp.44-58.

Yun, H., Kim, D., Chung, C., Kim, H., Yang, Y. and Rhee, Y. (2003). Characterization of a Tacky Poly(3-Hydroxyalkanoate) Produced by *Pseudomonas chlororaphis* HS21 from Palm Kernel Oi. *J. Microbiol. Biotechnol*, 13(1), pp.64-69.

Zhang, J., Shishatskaya, E., Volova, T., da Silva, L. and Chen, G. (2018). Polyhydroxyalkanoates (PHA) for therapeutic applications. *Materials Science and Engineering: C*, 86, pp.144-150.

Chapter 5
Appendices

Appendix 1 – Phenazines scan.

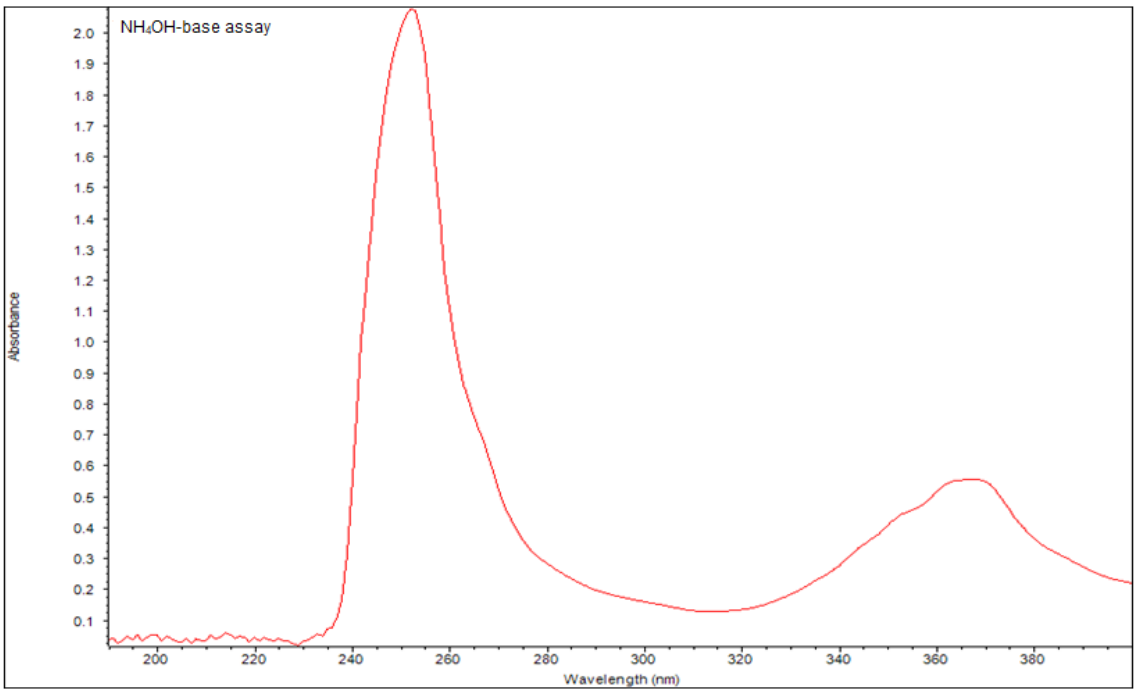


Figure 5.1 – Phenazines scan performed over the sample taken during NH_4OH-base assay, at 14 hours run.