Universidade do Minho
Escola de Engenharia

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Biotech strategies to improve the quality of crude oils

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Dissertação de Mestrado
Mestrado em Bioengenharia

Trabalho Efetuado sob a orientação do
Professor Doutor José António Couto Teixeira
e do
Doutor Eduardo José Gudiña Pérez

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DECLARAÇÃO

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ABSTRACT

The isolation and identification of microorganisms with potential for bioconversion in petroleum fields is of great relevance, since these organisms may be related to a decrease in petroleum quality in the reservoirs or cause damages in the production facilities; furthermore, microorganisms with beneficial effects and activities can also be found. In that sense, a petroleum sample was obtained from a Brazilian oil field (oil well CLB), with the aim of isolating microorganisms and evaluating their ability to degrade different hydrocarbons, in particular long chain *n*-alkanes. Twenty-three isolates were obtained from the oil sample: fourteen bacterial isolates and nine filamentous fungi. Five isolates were identified as *Pseudomonas* sp. according to their macroscopic characteristics. The *Pseudomonas* sp. strains were selected due to their high biosurfactant-producing ability. The biosurfactants produced by those isolates reduced the surface tension of water from 72 to 30 mN/m, and exhibited emulsifying activities of almost 60%. Furthermore, one of the filamentous fungi isolated, identified through morphological and molecular techniques as *Scedosporium* spp., produced a bioemulsifier growing in a medium containing 30% (v/v) of oil mill wastewater (OMW). This bioemulsifier exhibited high stability at extreme conditions of pH and salinity. Oil treatments using microorganisms to improve the API degree of heavy crude oils constitute an innovative approach. In that sense, the Mukhaizna crude oil (heavy oil) was subjected to treatments with filamentous fungi, *Pseudomonas* sp. and *Bacillus subtilis* strains, all of them isolated from oil samples. However, none of the assays performed improved the quality of this oil. Other promising biotechnological application is Microbial Enhanced Oil Recovery (MEOR), which is potentially useful to increment oil recovery from reservoirs beyond primary and secondary recovery operations using microorganisms and their metabolites. In this work, a sand-pack column model was used to simulate the oil recovery operations and evaluate the mobilization of residual oil by three filamentous fungi isolated from the oil sample, with additional oil recoveries ranging from 1.9 to 9.2%.

In conclusion, although the main objective of this work couldn't be achieved (improve the quality of Mukhaizna crude oil), the results obtained show that microorganisms isolated from oil reservoirs can be useful for several applications, not only in the oil industry, but also in other areas such as waste management and the production of surface-active compounds for several applications.

SUMÁRIO

O isolamento e identificação de microrganismos com potencial de bioconversão nos campos petrolíferos é de grande relevância, devido ao relacionamento dos organismos com a diminuição da qualidade do petróleo nos reservatórios ou dos danos causados nas instalações de produção. Além disso, microrganismos com actividades benéficas podem também ser encontrados. Nesse sentido, foi recolhida uma amostra de um campo de petróleo brasileiro (poço petrolífero CLB), com o objectivo de isolar microorganismos e avaliar a capacidade desses degradarem diferentes compostos. Vinte e três isolados foram isolados a partir da amostra de óleo: catorze bactérias e nove fungos filamentosos. Cinco isolados foram identificados como *Pseudomonas* sp. de acordo com as suas características macroscópicas e apresentaram capacidade de produzir biossurfatantes. Os biossurfatantes produzidos por estes isolados reduziram a tensão superficial da água de 72 para 30 mN/m e exibiram actividades emulsionantes de quase 60%. Além disso, um dos fungos filamentosos isolados, identificado através de técnicas moleculares e morfológicas como *Scedosporium* spp. produziu um bioemulsionante num meio contendo 30% (v/v) de águas ruças. Este bioemulsionante exibiu alta estabilidade em condições extremas de pH e salinidade. Os tratamentos de petróleo utilizando microrganismos como estratégia para melhorar o grau API de petróleo pesado constituem uma abordagem inovadora. Nesse sentido, o petróleo oriundo do poço do Mukhaizna foi submetido a tratamentos com fungos filamentosos, *Pseudomonas* sp. e estirpes de *Bacillus subtilis*, todos eles isolados a partir de amostras de óleo. No entanto, nenhum dos ensaios realizados melhorou a qualidade deste petróleo. Outra aplicação biotecnológica promissora é a recuperação de petróleo com utilização de microrganismos (MEOR), que é potencialmente útil para aumentar a recuperação de petróleo num reservatório. Neste trabalho, um modelo de coluna com areia empacotada foi concebido para simular as operações de recuperação de petróleo e avaliar a mobilidade de óleo residual inoculado pelos três fungos filamentosos isolados a partir da amostra de óleo, onde as recuperações adicionais de óleo variaram entre 1.9 e 9.2%.

Em conclusão, embora o principal objectivo deste trabalho não tenha sido alcançado (melhorar a qualidade do petróleo do Mukhaizna), os resultados obtidos mostram quem os microrganismos isolados de reservatórios de petróleo podem ser uteis para diversas aplicações, não só na industria petrolífera, mas também em outras áreas como a gestão de resíduos e a produção de compostos tensioactivos para diversas aplicações.

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LIST OF ABBREVIATIONS

ABTS	2,2'-azinobis-(3-ethylbenzothianoline-6-sulphonic acid)
ANOVA	Analysis of variance
AOR	Additional Oil Recovery
API	American Petroleum Institute
BLASTn	Nucleotide-nucleotide blast network service
BOD	Biochemical Oxygen Demand
BRM	Basidiomycetes Rich Medium
BRM +	BRM medium supplemented with glucose (10g/l)
BRM -	BRM medium without glucose
BSA	Bovine Serum Albumin
BTEX	Benzene, Toluene, Ethylbenzene and Xylene
CMC	Critical Micelle Concentration
COD	Chemical Oxygen Demand
CSL	Corn Steep Liquor
E_{24}	Emulsification Index
E_{24}^{-1}	Emulsification Index of a sample 10 times diluted
GC	Gas Chromatography
LB	Luria Bertani Medium
LiP	Lignin Peroxidase
MA+	MA medium supplemented with glucose (10g/l)
MA-	MA medium without glucose
MEOR	Microbial Enhanced Oil Recovery
MnP	Manganese Peroxidase
MSM	Mineral Salt Medium
MSM+	MSM medium supplemented with glucose (12g/l)
MSM-	MSM medium without glucose

$MSM_{1/2}$	MSM medium supplemented with glucose (6g/l)
MSS	Mineral Salt Solution Medium
MSS+	MSS medium supplemented with sucrose (10g/l)
MSS-	MSS medium without sucrose
MSS-CSL	MSS with CSL 10% (v/v) without sucrose
NCBI	National Centre for Biotechnology Information
OA	Oatmeal Agar medium
OMW	Oil Mill Wastewater
OOIP	Original Oil In Place
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar medium
PV	Pore Volume
rpm	Rotations per Minute
SD	Standard Derivation
S_{oi}	Initial Oil Saturation
S_{or}	Residual Oil Saturation
S_{orwf}	Oil Recovered after Water Flooding
ST	Surface Tension
ST^{1}	Surface Tension of a sample 10 times diluted
ST^{2}	Surface Tension of a sample 100 times diluted
S_{wi}	Initial Water Saturation
Wunder+	Wunder medium supplemented with glucose (10g/l)
Wunder-	Wunder medium without glucose
η	Dinamic Viscosity

CONTEXT AND MOTIVATION

Crude oil is the major source of energy worldwide. As the price of petroleum increases and crude oil reserves collapse, exploitation of oil resources in mature reservoirs is essential for meeting future energy demands. Conventional oil recovery methods currently used have become inefficient to provide the increasing demand of crude oil; for that reason, there is a continuous need of developing new technologies to increase oil production from mature oil fields. Microbial enhanced oil recovery (MEOR) is a cost-effective and eco-friendly important tertiary oil recovery method which can contribute to improve oil production from mature reservoirs.

On the other hand, the treatment of heavy oils (API gravity below 20.0°) is also a priority. The degradation of heavy oils using biological treatments as a strategy to improve their API (American Petroleum Institute) degree constitutes an innovative approach with enormous potential. Typically the heavy oils are characterized by exhibiting high viscosity and density. Their biological treatment is an environmentally friendly alternative and/or a complement to other existing processes. Additionally, these treatments require less severe operational conditions and are highly selective and specific as compared to the conventional chemical methods. The use of microorganisms as biocatalysts in these treatments presents several advantages, such as stability, specificity and selectivity; furthermore, microorganisms can use a wide range of substrates and are recyclable.

In summary, MEOR process and the biological treatment of Mukhaizna crude oil will help to overcome the economic barriers and will allow the exploration of oils that, at this moment, are not available.

REASERCH AND AIMS:

The main goal of this work was the application of biotech strategies to improve the quality of heavy crude oils using different microorganisms. At the same time, other studies were performed, as described below:

- ✓ Isolation of microorganisms from the crude oil sample;
- ✓ Evaluation of biosurfactants/bioemulsifiers production by the different microorganisms and study of their production profiles;
- ✓ Optimization of biosurfactants and bioemulsifiers production;
- ✓ Study of alternative substrates (oil mill wastewaters (OMW)) for bioemulsifier production;
- ✓ Characterization of the bioemulsifier produced (stability studies);
- ✓ Study of ligninolytic enzymes production by the filamentous fungi isolated from the oil sample;
- ✓ Treatments to improve the quality of Mukhaizna oil;
- ✓ Microbial Enhanced Oil Recovery assays.

1.INTRODUCTION

1.1. Crude oil composition: problems and opportunities for improvement

Crude oil formed from the thermal cracking of kerogen is a complex mixture of aliphatic, aromatic and naphthenic oils, as well as other compounds containing sulfur, oxygen, nitrogen and organometallic constituents complexed with nickel and vanadium. Among these compounds highlight the biomarkers and geochemical fossils originating from living organisms, which are classified as linear, and branched alkanes, isoprenoids, cycloalkanes, terpanes, steranes and aromatic compounds [1].

Biodegradation is a process of change in crude oil due to the activity of different microorganisms. Early in the process, the hydrocarbons are used by microorganisms as energy source (electron donor), while nutrients (molecular oxygen, nitrate, sulphate or ferric ion) are required as electron acceptors for microbial activity. At the end of the biodegradation process, the hydrocarbons are converted into metabolites such as organic acids and/or CO_2, resulting in a reduction in the amount of saturated compounds and the API degree, and a progressive increase of the content of asphaltenes, metals, sulphur, acidity (due to the formation of carboxylic acids and phenols), density and viscosity. These changes affect the characteristics of the petroleum production and the efficiency of refinery process, increasing the production costs [2].

The American Petroleum Institute gravity, or API gravity, measures the gravity of liquid petroleum products. The API gravity or degree is calculated as follows (Equation 1):

$$^{o}API = \frac{141.5}{\rho} - 131.5 \qquad \text{Equation 1}$$

Where ρ is the density of the oil. Although the API gravity has no units, the measuring scale is calibrated in terms of degrees API.

Crude oils are classified as light, medium, heavy or extra heavy, according to their API gravity [3]:

- Condensed oil is defined as having an API gravity higher than 45.0 °API;
- Light oil is defined as having an API gravity between 20.0 and 45.0 °API;
- Heavy crude oil is defined as having an API gravity between 20.0 and 10.0 °API;
- Extra heavy oil or natural bitumen has an with API gravity below 10.0 °API.

Figure 1 represents a schematic biodegradation mechanism within an oil reservoir and the changes in API degree during the process.

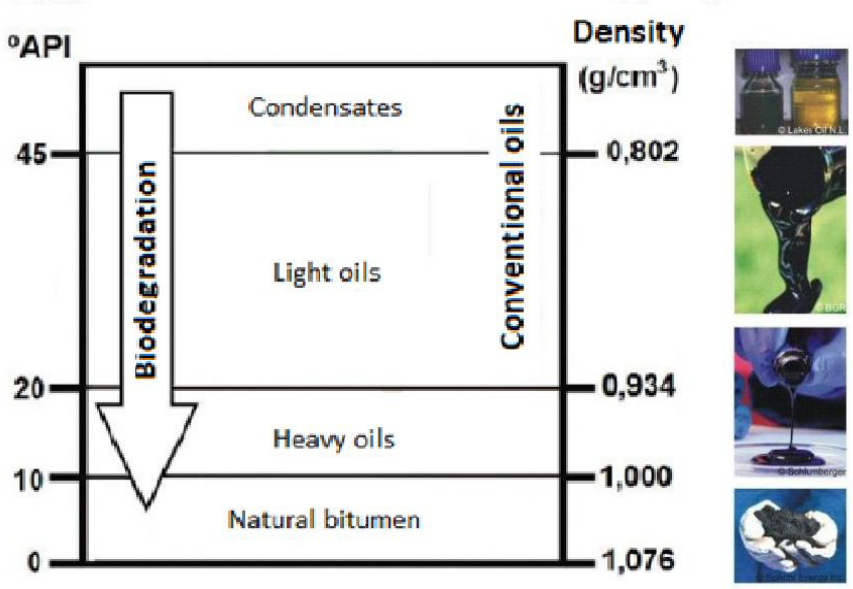


Figure 1: Schematic representation of the biodegradation process in an oil reservoir, leading to changes in their capacity to decrease the API degree. Adapted from da Cruz and Marsaioli [2].

Most of the World's oil is entirely or partially biodegraded in the reservoirs, and this process can take millions of years to occur [4]. To have a better understanding of the mechanisms of oil biodegradation it is necessary to use optimal conditions for the process to occur at short intervals compared to geological time [2]. In general, biodegradation requires: i) the presence of electron acceptors and inorganic nutrients required for the beginning of the process and the maintenance of the microorganisms, respectively; ii) the source rock must have sufficient porosity and permeability to allow the diffusion of nutrients and the mobility of microorganisms [5]; iii) the reservoir temperature should not exceed 80 ^{O}C to avoid the "paleopasteurisation" or "sterilization" of the oil, that would kill the biodegrading microorganisms. Considering the typical geothermal gradient (25-30 ^{O}C/Km), this temperature is reached at about 2-3 Km deep; iv) the salinity of formation water should be in the range of 100-150 ‰. Oils in reservoirs with higher salinity are typically non-biodegradable [4]; v) the presence of microorganisms capable of degrading the oil components [6]; vi) the absence of "natural poisons": microorganisms without the ability of degrading hydrocarbons which are tolerant to the

reservoir conditions and inhibit or limit the growth and enzymatic activity of degrading microorganisms. If all these conditions are present, the oil is modified by the microorganisms, decreasing its quality.

Biodegradation of petroleum hydrocarbons is a complex process that depends on the nature and the amount of the hydrocarbons present. Petroleum hydrocarbons can be divided into four classes: saturates, aromatics, asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and resins (pyridines, quinolines, carbazoles, sulfoxides, and amides). Hydrocarbons differ in their susceptibility to microbial degradation, that can be generally ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes [7]. Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all [8].

The microorganisms present in oil reservoirs exhibit preferences regarding their metabolic and enzymatic capacities to degrade different oil components. In appropriate conditions, in the presence of inorganic nutrients and electron acceptors, oil in reservoirs is often biologically degraded in geologic time scales by microorganisms that destroy hydrocarbons and other components, producing new compounds that modify the physical properties of the oil, turning it into “heavy oil” and reducing its economic value [2]. Rapid urbanisation and industrialisation led to a multifold increase in petroleum consumption, and the demand for petroleum-derived products continues growing [9].

Heavy oil and bitumen resources dominate the World’s oil inventory. These resources are estimated to comprise about five times the remaining conventional oil reserves: in other words, 5.60 trillion versus 1.02 trillion barrels, respectively [10]. As most of the World’s oil is biodegradable, alternative solutions to recover “heavy oils” are continually being evaluated [2].

1.2. Biological treatments to improve the quality of the oils

The bioconversion of crude oils leads to an enrichment in lighter hydrocarbons and an overall redistribution of these hydrocarbons. The interactions of microorganisms with crude oils are variable and depend on the microbial species and the chemical composition of crude oils. Different interactions may influence the efficiency of processes in which single or mixed microbial species are used for the oil treatment and may also suggest possible combinations of biological and chemical technologies. In bioconversion, heavy oil fractions are converted into lighter ones by microorganisms, reducing the viscosity of crude oil [6].

The bioconversion processes depend on many factors including pH, temperature, oxygen concentration, microbial population, degree of acclimation, accessibility of nutrients, chemical structure of the oil, cellular transport properties, and chemical partitioning in growth medium [11].

Microbial consortia have been widely used in laboratory studies. It is generally accepted that microbial consortia are more effective than pure cultures in bioconversion of hydrocarbons [12]. Mixed microbial communities exhibit a powerful biodegradative potential because the genetic information of more than one organism is necessary to convert the complex mixtures of organic compounds present in crude oil [13]. This is possible because a broader enzymatic capacity is achieved, and the formation of toxic intermediate metabolites is counteracted by the conversion of these dead-end products by co-metabolism processes [14]. Bouchez et al. [15] found that a microbial consortia could readily mineralize a mixture of five PAHs; however, mixed cultures of two or three pure strains possessing the capacity to mineralize each PAHs achieved a limited conversion of the mixture.

i. Microorganisms as biocatalysts

Aromatic hydrocarbons (e.g., BTEX compounds (Benzene, Toluene, Ethylbenzene and Xylene) and naphthalene) belong to the large volume of petrochemicals widely used as fuels and industrial solvents. Phenols and chlorophenols are released into the environment as products and also as waste materials from different industries. Aromatic compounds are formed in large amounts by all the organisms (e.g., as aromatic amino acids, phenols or quinones). Thus, it is not surprising that many microorganisms have developed catabolic pathways to degrade aromatic compounds [13]. The induction of degradation capacity by exposing the microbes to higher levels of pollutants may, at times, result also in genetic adaptability or changes responsible for higher bioconversion rates [11].

Although many studies have reported the ability of bacteria to convert hydrocarbons with different carbon numbers, most bacteria can use only a narrow range of hydrocarbons. For example, *Bacillus stearothermophilus* is capable of growing only on n-alkanes between C15 and C17; *Geobacillus jurassicus* grows only on C6-C16; *Geobacillus thermodenitrificans* has the ability to convert long-chain *n*-alkanes (from C15 to at least C36); and *Bacillus thermoleovorans*

converts *n*-alkanes up to C23. Few strains capable of converting a wide range of hydrocarbons have been identified to date, including *Rhodococcus* strains that convert n-alkanes up to C36 [16], and a *Dietzia* strain which can use a broad range of crude oil components as the sole carbon source, including aliphatic hydrocarbons, branched alkanes, cyclo-alkanes and aromatic hydrocarbons [17].

Several fungi are known to have the ability of converting persistent pollutants. The bioconversion pathways in ligninolytic fungi have been intensively studied in the last years. Due to the irregular structure of lignin, ligninolytic fungi produce extracellular enzymes with very low substrate specificity, making them suitable for bioconversion of different compounds. The ligninolytic system consists of four main enzymatic groups, with lignin peroxidase, manganese-dependent peroxidase, phenoloxidases (lacases, tyrosinases), and H_2O_2-producing enzymes [11].

Algae are also potential hydrocarbon converters. Prokaryotic and eukaryotic photoautotrophic marine algae (i.e., cyanobacteria, green algae and diatoms) are known to metabolize naphthalene to a series of metabolites, and there are indications that cishydroxylation of naphthalene by cyanobacteria (*Oscillatoria* and *Agmenellum* spp.) involve pathways similar to those present in fungi [11].

Walker at al. [18] isolated the alga *Prototheca zopfi,* which was capable of utilizing crude oil and a mix of hydrocarbon substrate, and exhibited extensive conversion of *n*-alkanes, isoalkanes and aromatic hydrocarbons. It was also observed that nine cyanobacteria, five green algae, one red alga, one brown alga and two diatoms oxidized naphthalene. Protozoa, by contrast, have not been shown to utilize hydrocarbons [7].

ii. Biosurfactants versus surfactants

Surfactants improve solubilisation and desorption of hydrophobic organic compounds, and increase their availability (or bioavailability) [19]. Surfactants manufactured by the chemical industry are used for several applications, but they exhibit environmental problems because of their toxicity and resistance to degradation. There is currently interest in replacing these chemical surfactants with surfactants of biological origin [20]. Biosurfactants are amphiphilic compounds produced by microorganisms with pronounced surface and emulsifying activity. Microbial

surfactants comprise a diverse group of surface-active molecules which are categorized by their chemical composition and microbial origin [21]. They include glycolipids, lipopeptides, polysaccharide-protein complexes, protein-like substances, lipopolysaccharides, phospholipids, monoglycerides, diglycerides, fatty acids and neutral lipids [20, 21]. Diverse properties and physiological functions in the producer organisms are expected for different groups of biosurfactants, including enhancing the solubility of hydrophobic/water-insoluble compounds (which facilitates their uptake into the cell), heavy metal binding, bacterial pathogenesis, cell adhesion and aggregation, quorum sensing and biofilm formation [21]. Biosurfactants may substitute their synthetic counterparts in environmental applications due to their biodegradability, low toxicity and high efficiency under extreme environmental conditions. It was also reported that biosurfactants may enhance bioconversion through enzyme stimulation [22] or promote better permeability of cellular membranes in hydrocarbon-degrading microorganisms [23].

Biosurfactants are believed to increase the surface area that hydrophobic compounds can expose to the water phase, thereby facilitating the access of microorganisms to the oil phase [24]. In liquid cultures, biosurfactants have been reported to increase the uptake and assimilation of alkanes such as hexadecane [25]. However, in soils and other situations, the usefulness of biosurfactants for the uptake of alkanes is less evident [26].

Biosurfactants can isolate individual petroleum molecules, in micelles and can differentially affect the bioavailability of petroleum to the microorganisms, depending on how the biosurfactant interacts with the cell envelopes of the broad suite of bacterial species that are present in the oil-degrading community (Figure 2) [27]. The abundance of biosurfactant-producing microorganisms in petroleum conversion has mostly been studied for soils and sediments with recent oil contamination, where up to 30% of the culturable bacteria typically screen as positive for biosurfactant production [27, 28]. Those microorganisms play a keystone role in shaping the species composition and activity of petroleum-degrading consortia, by favouring the growth of those bacteria that can access to hydrocarbons that are mobilized by the predominant biosurfactants [27].

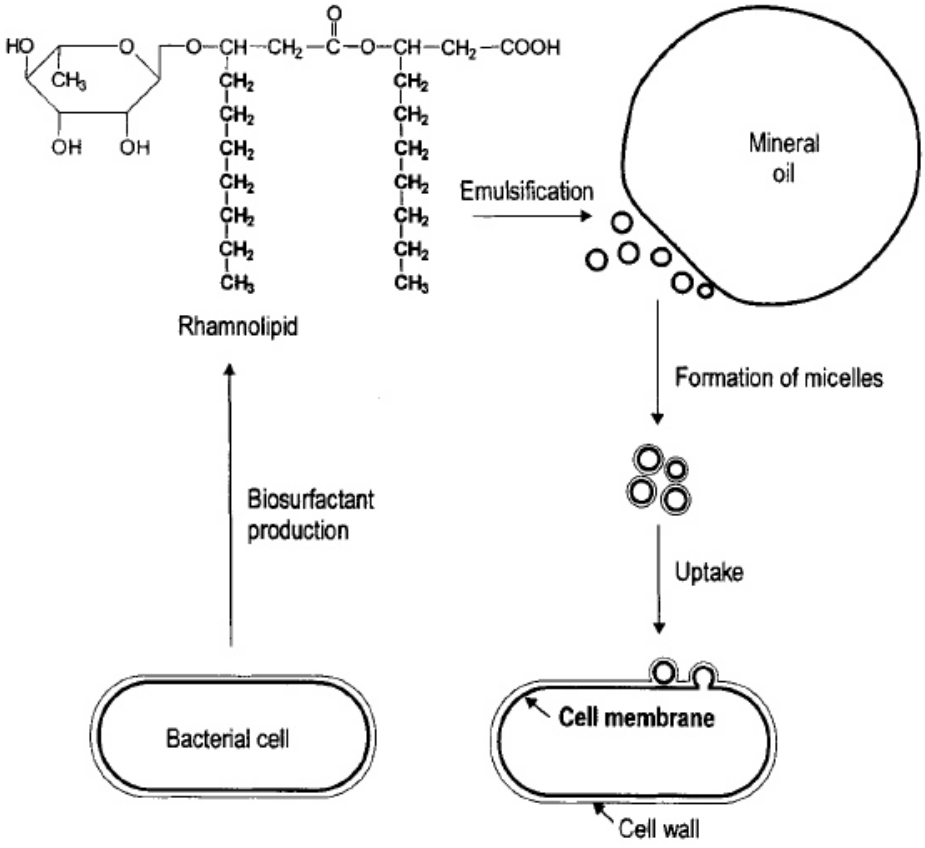


Figure 2: Representation of the role displayed by biosurfactants in the uptake of hydrocarbons. The figure shows the emulsifying effect of rhamnolipids produced by *Pseudomonas* spp. within the oil-water interphase and the formation of micelles. Adapted from Das and Chandran [7].

Viisimaa et al. [29] demonstrated that the simultaneous application of biosurfactants, microorganisms and oxidizing chemicals in moderate dosages resulted in an increased removal of aromatic compounds from selected matrixes compared to that obtained by a single process. However, further research on the effect of biosurfactants addition on the removal of various types of organic contaminants by integrated chemical–biological treatment of different types of matrixes remains to be explored.

iii. Bioemulsifiers

Microorganisms produce a wide variety of high- and low-molecular weight surface-active compounds. Low-molecular weight surface-active compounds reduce the surface and interfacial tension as their primary activity [30]. Common representatives of this group exhibit molecular masses in the range from 500 to 1500 Da. On the other hand, less-studied high-molecular weight surface-active compounds, usually referred as bioemulsifiers don't necessarily reduce surface or interfacial tension, but form stable emulsions between liquid hydrocarbons and water mixtures (Figure 3) [31, 32].

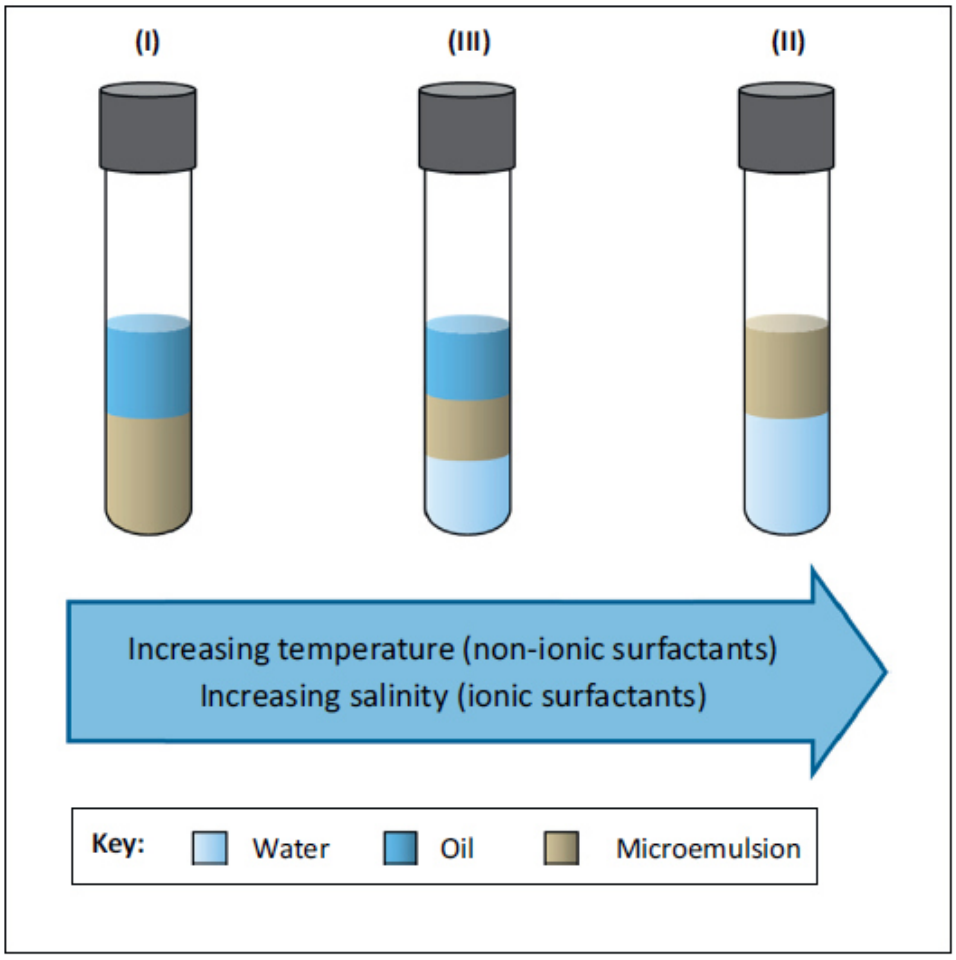


Figure 3: Winsor classification of microemulsions. Microemulsions can exist in three forms, known as Winsor type microemulsions. Type I (O/W), when the water– surfactant interaction is stronger than oil–surfactant interaction (R < 1), microemulsions solubilize oil in spherical normal micelles within the water-continuous phase. Type II (W/O), when the strength of oil–surfactant interaction is stronger than water–surfactant interaction (R > 1), microemulsions solubilize water in reverse micelles within the oil-continuous phase. Type III, when the interactions are balanced (R = 1); in this case, microemulsions are three-phase systems in which the middle phase microemulsions are in equilibrium with both excess oil and excess water phases. The Winsor R parameter represents the ratio of the total interaction energies (per unit area of interface) of the surfactant for the O and W phases and is also dependent on environmental factors. Adapted from Gudiña et al. [33].

These high-molecular weight surface-active compounds are highly efficient emulsifiers that work at low concentrations and exhibit considerable substrate specificity. They are composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins, etc. [34, 35].

The chemical diversity and functional properties of these compounds result in a broad spectrum of potential applications in sectors as diverse as agriculture, petroleum spill recovery technologies, cosmetics, environmental, food, leather, paper, pharmaceutical and textile industries, among others [30, 36].

Despite their potential applications in multiple biotechnological areas, the commercial availability of bioemulsifiers is currently limited. This is mainly due to economic obstacles to their sustainable production at industrial levels. The production of new biomolecules and the discovery of new bioemulsifier-producing strains could therefore be the key for overcoming these limitations and challenges, especially if inexpensive substrates can be used. It is important to remark that the culture media represent 10–30% of total production costs [37].

1.3. Microbial Enhanced Oil Recovery (MEOR)

Oil is an essential source of energy and one of the main factors driving the economic development of the World. Its recovery comprises a primary phase, which produces oil and gas using the natural pressure drive of the reservoir; and a secondary phase, which involves stimulating the oil wells by the injection of fluids to improve the flow of oil and gas to the well-head. While primary recovery produces 5-10% of the original oil in place, recovery efficiencies in the secondary stage range from 10% to 40%. Therefore, crude oil remaining in the reservoirs after conventional oil recovery operations makes up to two-thirds of the total oil reserves [38, 39].The main factors responsible for the poor oil recovery from producing wells are the low permeability of some reservoirs, the high viscosity of the oil and the high interfacial tension between the hydrocarbon and the aqueous phases, which result in high capillary forces that entrap oil in small pores within the reservoir. As the price of petroleum increases and petroleum reserves shrink, exploitation of oil resources in mature reservoirs is essential for meeting future energy demands. In the last years, more attention has been focused on tertiary recovery techniques, known as Chemical Enhanced Oil Recovery (CEOR), to mobilize entrapped oil [38]. The most widely used

CEOR techniques include CO_2 injection, surfactant flooding, polymer flooding, alkaline–surfactant–polymer flooding, steam injection, and the use of acids and solvents [40]. The results from the past field assays revealed that the application of these techniques typically increase oil recovery by 5–15% [41]. However, these processes are environmentally hazardous, expensive, and leave undesirable residues which are difficult to dispose of without adversely affecting the environment [38].

MEOR is an important tertiary oil recovery technology that represents a cost-effective and eco-friendly alternative to CEOR. In MEOR, selected microbial strains are used to synthesize compounds analogous to those used in CEOR processes to increase the recovery of oil from depleted and marginal reservoirs, thereby extending their life. Microorganisms can synthesize useful products by fermenting low-cost substrates or raw materials [40].

In 1926, Beckman suggested for the first time that microorganisms could be used to release oil from porous media. Between 1926 and 1940, little was done on this topic. Then, in the 1940s, Zo-Bell and co-workers (1947) started a series of systematic laboratory investigations, and their results marked the beginning of a new era of research in petroleum microbiology with application in oil recovery [42].

During MEOR processes, selected microorganisms use the crude oil from the reservoir or injected nutrients for their metabolic processes, and release some non-toxic chemicals such as biosurfactants, biopolymers, biosolvents, bioacids and gases. These biological products increase the oil sweep efficiency by changing the reservoir's physicochemical characteristics [43]. Biosurfactants, known as one of the most important bio-products, reduce the capillary forces in the reservoir rock by the reduction of oil-water interfacial tension [44, 45] (Table 1).

MEOR is a relatively inexpensive and environmentally compatible method for tertiary oil recovery and is being optimized through the development of more effective microorganisms that can subsist on inexpensive and abundant nutrients present in the oil reservoirs. However, the application of most MEOR techniques is very specific, and the choice of microorganisms is critical and field sensitive. In addition, even a few percentage of additional oil recovery can contribute an enormous amount, cumulatively and across the whole region. Thus, MEOR studies are continuously being performed on specific oil fields worldwide [41].

Another important process in MEOR is the bioconversion of heavy oil fractions by microorganisms. In this process, heavy oil fractions are converted into lighter ones, reducing the viscosity of crude oil and improving its mobility through the reservoir, which increases oil recovery. In recent years, a wide variety of microorganisms able to degrade *n*-alkanes have been described. The use of hydrocarbon degrading bacteria with the simultaneous ability to produce surface-active compounds is also a good option for MEOR [39].

Gudiña et al. [39] reported that *Bacillus subtilis* were able to convert the hydrocarbon chains higher than C27. Among them, the *B. subtilis* #311 exhibited the highest degradation with an increase in the relative weight fraction of n-alkanes lower than C19, and a decrease in the percentage of n-alkanes higher than C18. These results indicate that this isolate degraded the higher n-alkanes into lighter ones during the sandpack column assays.

MEOR can overcome the main hindrances of efficient oil recovery, such as low reservoir permeability, high viscosity of the crude oil, and high oil-water interfacial tensions, which in turn result in high capillary forces retaining the oil within the reservoir rock [40].

Continuous research and successful applications affirm the fact that MEOR can be viewed as a potent technology [46, 47] despite the existing disagreement by some groups [24]. However, successful MEOR field applications reported are specific for each well and published information to support economic advantages is lacking. MEOR is, therefore, considered as one of the promising future research areas with great preference as identified by the Oil and Gas in the 21st Century Task Force [48], may help in recovering the 377 billion barrels of oil that are unrecoverable by conventional technologies [46].

Table1: Microbial products, their role in enhanced oil recovery, and some of the effects to solve production problems[a] Adapted from Lazar et al. [42].

Microbial product	Role in enhanced oil recovery	Some of the effects
	✓ Reduce oil viscosity and improve flow characteristics ✓ Displace immobile ✓ Sweep oil in place	✓ Improved oil recovery by gases ✓ Miscible CO_2 flooding
✓ Acids (low molecular weight acids, primarily low molecular weight fatty acids)	✓ Improve effective permeability by dissolving carbonate precipitates from pores throat. Significant improvement of permeability and porosity ✓ CO_2 produced from chemical reactions between acids and carbonate reduce oil viscosity and causes oil droplet to sweel	✓ Enhanced oil flooding
✓ Solvents (alcohols and ketones that are typical cosurfactants)	✓ Dissolve in oil reduce viscosity ✓ Dissolve and remove heavy, long chain hydrocarbons from pore throat (increase effective permeability) ✓ Involved in stabilizing and lowering interf, tension that promotes emulsification ✓ Reduce interfacial tension	✓ Emulsification promotion for increased miscibility
✓ Biosurfactants	✓ Reduce interfacial tension between oil and rock/water surface which causes emulsification; improving pore scale displacement ✓ Alter wettability	✓ Microbial surfactant ✓ Flooding
✓ Biopolymers	✓ Improve the viscosity of water in waterflooding and direct reservoir fluids to previously unswept areas of the reservoir ✓ Improve the sweep efficiency of waterflood by plugging high permeability zones or water-invaded zones ✓ Control of water mobility	✓ Microbial permeability modification (selective plugging)
✓ Biomass (microbial cells)	✓ Physically displace oil by growing between oil and rock/water surface	✓ Same biopolymers

a)Formation damage; low oil relative permeability; trapped oil due to capillary forces; poor sweep efficiency channelling; unfavourable mobility ratio; low sweep efficiency; water or gas coning

2.Materials and Methods

2.1. Isolation of microorganisms from the crude oil sample

The crude oil sample obtained from a Brazilian oil field (oil well CLB) was collected in a sterile bottle and stored at room temperature until use. For isolation of microorganisms, enrichment cultures were performed in 100 ml flasks containing 50 ml of Corn Steep Liquor (CSL) (10%, v/v), Mineral Salt Solution (MSS) supplemented with 10 g/l of sucrose (MSS+) and MSS without sucrose (MSS-). Crude oil samples (1 ml) were transferred to the flasks and incubated at 37 or 50 ^{O}C, under aerobic or anaerobic conditions, for 27 days. Anaerobic cultures were prepared removing oxygen by aseptically bubbling oxygen-free nitrogen into the flasks, which were sealed with rubber stoppers. Samples (100 µl) of the enrichment cultures were periodically spread on agar plates (Luria-Bertani medium (LB) for bacterial strains and Potato Dextrose Agar (PDA) for filamentous fungi), that were incubated at 37 ^{O}C under aerobic or anaerobic conditions. Morphologically distinct colonies were re-isolated by transfer to fresh agar plates at least twice to obtain pure cultures. The bacterial strains were stored at -80 ^{O}C in LB medium containing 20% (v/v) of glycerol. The filamentous fungi were stored at 4 ^{O}C in demineralised water, and at -80 ^{O}C in a glycerol solution (80%, v/v).

The composition of MSS was (g/l): CaCl 10.0; Na_2HPO_4 5.0; NH_4NO_3 2.0; KH_2PO_4 2.0; $MgSO_4 \cdot 7H_2O$ 0.2; supplemented with 1% (v/v) of trace salt solution. The trace salt solution contained (g/l): $MnSO_4 \cdot 4H_2O$ 3.0; EDTA 1.0; $FeSO_4 \cdot 7H_2O$ 0.1; $CaCl_2 \cdot 2H_2O$ 0.1; $CoCl_2 \cdot 6H_2O$ 0.1; $ZnSO_4 \cdot 7H_2O$ 0.1; $CuSO_4 \cdot 5H_2O$ 0.01; $AlK(SO_4)_2 \cdot 12H_2O$ 0.01; H_3BO_3 0.01; $Na_2MoO_4 \cdot 2H_2O$ 0.01. The composition of LB medium was (g/l): NaCl 10.0; tryptone 10.0; yeast extract 5.0. Both media were adjusted to pH 7.0.

PDA medium was purchased from OXOID (England).

CSL was provided by COPAM (Companhia Portuguesa de Amidos, S.A., Portugal). For its use, CSL was dissolved with demineralised water (10%, v/v), and its pH was adjusted to 7.0.

2.2. Screening of biosurfactant-producing strains

The bacterial strains were grown in 25 ml of different media at 37 ^{O}C and 105 rpm, under aerobic conditions. The media used were MSS+, MSS-, Mineral Salt Medium (MSM) supplemented with 12 g/l of glucose (MSM+) and MSS- supplemented with CSL (10%, v/v)

(MSS-CSL). Regarding the medium MSS-CSL, the precipitates formed after sterilization were removed by centrifugation (9000 rpm, 30 min) and the supernatants obtained were used as culture medium. All the media were supplemented with 1% (w/v) of CLB crude oil.

The composition of MSM was (g/l): KH_2PO_4 2.3; $Na_2HPO_4 \cdot 2H_2O$ 5.03; $(NH_4)_2SO_4$ 1.06. This medium was supplemented with 1% (v/v) of a $MgSO_4 \cdot 7H_2O$ solution (40 g/l); 1% (v/v) of a $CaCl_2 \cdot 2H_2O$ solution (20 g/l); and 1% (v/v) of a trace element solution, which consisted of (grams per 200ml): $FeSO_4 \cdot 7H_2O$ 0.55; $MnCl_2 \cdot 4H_2O$ 0.396; H_3BO_3 0.06; $Na_2MoO_4 \cdot 2H_2O$ 0.006; $CoCl_2 \cdot 6H_2O$ 0.1; $ZnSO_4 \cdot 7H_2O$ 0.058; $FeSO_4 \cdot 7H_2O$ 0.55; $MnCl_2 \cdot 4H_2O$ 0.396; the trace element solution was sterilized by filtration and added to the medium. The initial pH of this medium was adjusted to 7.2.

The filamentous fungi were grown in 25 ml of MSS+ and MSS-, as well as in 100 ml of MA, BRM (basidiomycetes rich medium) and Wunder media (all of them supplemented with glucose (MA+, BRM+, Wunder+) and without glucose (MA-, BRM-, Wunder-)) at 37 ^{O}C and 105 rpm, under aerobic conditions, for 3 weeks. All the media were supplemented with 1% (w/v) of CLB crude oil. Furthermore, the fungal isolates were also grown in 100 ml of MA+ and MA- supplemented with 10% (v/v) of oil mill wastewater (OMW), at 37 ^{O}C and 180 rpm.

The composition of MA medium was (g/l): $(NH_4)_2SO_4$ 7.532; NaCl 0.152; KH_2PO_4 0.147; $MgSO_4$ 0.542; $ZnSO_4$ 8.39 × 10^{-4}; FeSO4 6.07 × 10^{-5}; glucose 10.0.

The composition of BRM medium was (g/l): NH_4NO_3 0.724; KH_2PO_4 1.0; $MgSO_4 \cdot 7H_2O$ 1.0; KCl 0.5; yeast extract 0.5; $FeSO_4 \cdot 7H_2O$ 0.001; $ZnSO_4 \cdot 7H_2O$ 0.0028; $CaCl_2 \cdot 2H_2O$ 0.033; glucose 10.0; peptone 10.0.

The composition of Wunder medium was (g/l): $(NH_4)_2SO_4$ 1.0; $MgSO_4 \cdot 7H_2O$ 0.5; KH_2PO_4 0.875; K_2HPO_4 0.125; $CaCl_2 \cdot 2H_2O$ 0.1; NaCl 0.1; $MnCl_2$ 0.05; $FeSO_4$ 0.001; peptone 2.0; glucose 10.0.

OMW was obtained from a Portuguese olive oil factory (which uses a three-phase centrifugation process for oil extraction) and stored at 4 ^{O}C until use.

In all the studies (with bacteria and fungi), samples were taken at different time points during the fermentation to determine biosurfactant production. The samples were centrifuged

(4000 rpm, 20 min, 20 ^{O}C) and the cell-free supernatants were used to measure the surface tension and the emulsifying activity, as described below.

2.3. Optimization of biosurfactant/bioemulsifier production

2.3.1. Bacterial strains

Biosurfactant-producing bacterial strains were grown in 500 ml flasks containing 150 ml of different media (LB, MSM+ and MSM+ supplemented with 10% (v/v) of OMW). Each flask was inoculated with 100 µl of a glycerol-stock of the corresponding strain. The cultures were incubated at 37 ^{O}C. The effect of the agitation rate on biosurfactant production was studied by incubating the flasks at 105 or 180 rpm. Samples were taken at different time points during the fermentation to determine biosurfactant production. The samples were centrifuged (4000 rpm, 20 min, 20 ^{O}C) and the cell-free supernatants were used to measure the surface tension and the emulsifying activity. At the end of the fermentation, the biosurfactants were recovered from the cell-free supernatants, as described below.

2.3.2. Filamentous fungi

Bioemulsifier production by filamentous fungus #28 was studied in 250 ml flasks containing 100 ml of MA+ and MA-, supplemented with 10% (w/v) of OMW, CLB crude oil, paraffin, soybean oil, gas oil or *n*-hexadecane, as well as in MA- supplemented with 20, 30, 40 or 50% of OMW. Each flask was inoculated with the stocks stored at 4 ^{O}C. The cultures were incubated at 37 ^{O}C and 105 or 180 rpm for up to 3 weeks. In all the cases, samples were taken at different time points during the fermentation to determine bioemulsifier production. The samples were centrifuged (4000 rpm, 20 min, 20 ^{O}C) and the cell-free supernatants were used to measure the surface tension and the emulsifying activity, as described below.

2.4. Biosurfactants and bioemulsifier recovery

2.4.1. Bacterial strains

To recover the biosurfactants produced by the bacterial isolates, two different techniques were evaluated: acidic precipitation [21] and adsorption on the polystyrene resin Amberlite XAD-2 (adapted from [49]). In both cases, the cell-free supernatants obtained at the end of the fermentations were used.

For the acidic precipitation, the cell-free supernatants were adjusted to pH 2.0 using HCl 6 M and left overnight at 4 ^{O}C. Afterwards, the precipitate (crude biosurfactant) was recovered by centrifugation (9000 rpm, 30 min, 4 ^{O}C). The crude biosurfactant was dissolved in a minimal amount of demineralised water by adjusting the pH to 7.0, using NaOH 1 M. Finally, the crude biosurfactant solution was freeze-dried. The product obtained was weighed and stored at -20 ^{o}C for further use.

Regarding the adsorption on the polystyrene resin Amberlite XAD-2, 50 ml of the cell-free supernatants were placed into 100 ml flasks containing 5 g of resin; subsequently, the flasks were incubated at 180 rpm for 90 minutes, to promote the adhesion of the biosurfactants to the resin. Thereafter, the supernatant was removed and 50 ml of demineralised water were added to the flasks, which were incubated at the same conditions for 30 min; this step removes those compounds that are not adhered to the resin, and was repeated three times. Afterward, the biosurfactant was released from the resin by incubation with 50 ml of methanol for 30 min at 180 rpm; this process was repeated two times to recover all the biosurfactant. The solvent was recovered and removed using a rotary evaporator at 35 ^{O}C. The product obtained (crude biosurfactant) was dissolved in a minimal amount of demineralised water and the solution was freeze-dried. The product obtained was weighed and stored at -20 ^{o}C for further use.

2.4.2. Filamentous fungi

To recover the bioemulsifier produced by the filamentous fungus #28, two different precipitation techniques were evaluated [34, 50]. In both cases, the cell-free supernatants obtained at the end of the fermentation were used. The cell-free supernatants were obtained after

centrifuging the cultures at 9000 rpm for 30 min at 4 ^{O}C, followed by filtration using a 0.45 μm pore-size filter.

Three volumes of acetone or ethanol were added to the cell-free supernatants. The mixtures were incubated overnight at -20 ^{O}C. The precipitates formed were collected by centrifugation (9000 rpm, 30min at 4 ^{O}C) and then dissolved in demineralised water. In order to confirm if the bioemulsifier was recovered using these techniques, the emulsifying activity of those solutions was calculated. Finally, the solutions were freeze-dried and the products obtained weighed and stored at -20 ^{O}C for further studies.

2.5. Surface tension measurements

Surface tension measurements of cell-free supernatants, biosurfactant solutions and bioemulsifier solutions were performed according to the Ring method, as described elsewhere [21]. A KRÜSS K6 Tensiometer (KRÜSS GmbH, Hamburg, Germany) equipped with a 1.9 cm DeNoüy platinum ring was used. To increase the accuracy of the surface tension (ST) measurements, an average of duplicates was determined. Whenever required, the cell-free supernatants were diluted 10 or 100 times with demineralised water, and the surface tension (ST^{-1} and ST^{-2}, respectively) was measured. All the measurements were performed at room temperature (20 ^{O}C).

2.6. Emulsifying activity determination

The emulsifying activity was determined by the addition of 2 ml of *n*-hexadecane to the same volume of cell-free supernatants, biosurfactant solutions or bioemulsifier solutions, in glass test tubes. The tubes were mixed with vortex at high speed for 2 minutes, and then incubated at 25 ^{O}C for 24 hours. The emulsification indexes at 24 hours (E_{24}) were calculated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm) [51]. Whenever required, the cell-free supernatants were diluted 10 times with demineralised water, and the emulsifying index (E_{24}^{-1}) was measured as described above.

2.7. Critical micelle concentration (CMC)

Critical micelle concentration is defined as the concentration of an amphiphilic compound in solution at which the formation of micelles is initiated. It is important for several biosurfactants applications to establish their CMC, as above this concentration no further effect is expected in the surface activity. Concentrations ranging from 0.001 to 5 g/l of the crude biosurfactants recovered from the different isolates were prepared in demineralised water, and the surface tension of each sample was measured by the Ring method at room temperature (20 ^{O}C), as described previously. The CMC was determined by plotting the surface tension as a function of the logarithm of biosurfactant concentration, and it was found at the point of intersection between the two lines that best fit through the pre- and post-CMC data. All the measurements were performed in duplicate.

2.8. Stability studies

The applicability of surface-active compounds can be conditioned by their stability to different environmental parameters. Therefore, the effect of pH, temperature and salinity on the activity of the bioemulsifier produced by the filamentous fungus #28 was determined. Stability studies were performed using the cell-free supernatants obtained after centrifuging the cultures at the end of the fermentation at 9000 rpm for 30 min. In order to assess the effect of salinity on bioemulsifier activity, the culture broth supernatants were supplemented with different NaCl concentrations (from 10 to 200 g/l). The emulsification indexes were measured as described above and compared with the value obtained without addition of NaCl. To evaluate the stability at high and low temperatures, the samples were incubated at 121 ^{O}C for 20 min, as well as at -20 ^{O}C and -80 ^{O}C during 5 or 10 days. The emulsification indexes obtained with those samples (at room temperature) were measured and compared with the corresponding values before each treatment. The pH stability was studied by adjusting the cell-free supernatants to different pH values (2.0-12.0) using HCl or NaOH solutions. Subsequently, the emulsification index of each sample was calculated.

2.9. Effect of OMW on the growth of the filamentous fungus #28

To study the ability of the filamentous fungus #28 of growing at different OMW concentrations, assays were performed in MA- agar plates supplemented with different OMW concentrations (from 10 to 50%, v/v). The pH of OMW was adjusted to 7.0 before use. Each plate was inoculated in the centre with 10 µl of a suspension containing spores and mycelium from the filamentous fungus #28. The plates were incubated at 37 ^{O}C and the diameter of the mycelium was measured periodically. MA- plates without OMW were used as control. Four assays were performed for each OMW concentration.

2.10. Ligninolytic enzymes determination

The production of ligninolytic enzymes (laccase, lignin peroxidise (LiP) and manganese peroxidise (MnP)) by the filamentous fungi was assayed in the cell-free supernatants obtained from fermentations performed with different culture media.

Laccase production was assessed by measuring the oxidation of 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) at 420 nm (ε_{420}=36000 M^{-1} cm^{-1}) for 3 minutes. The reaction mixture contained: 100 µl of sample, 400 µl of 5 mM ABTS and 500 µl of 0.2 M sodium acetate buffer (pH 5.0). One Unit of enzyme activity is defined as the amount of enzyme that oxides 1 µmol of ABTS in 1 minute [52].

LiP activity was monitored by measuring the oxidation of 10 mM veratryl alcohol (200 µl) to veratrylaldehyde (ε_{310}=9.3 cm^{-1} mM^{-1}) in the presence of 4.0 mM H_2O_2 (100 µl) at 310 nm. The assays were performed in 500 µl of 100 mM sodium tartrate buffer (pH 3.0) with 100 µl of sample and 100 µl of demineralised water [52].

MnP activity was assayed using $MnSO_4$ as substrate; the assay mixture contained 100 µl of sample, 200 µl of 2.5 mM $MnSO_4$, 200 µl of 0.5 mM H_2O_2 and 500 µl of 0.1 M sodium malonate buffer (pH 4.5). The oxidation of Mn^{2+} to Mn^{3+} was followed by measuring the absorbance at 270 nm (ε=11 590 M^{-1} cm^{-1}), that indicates the formation of malonate-Mn^{3+} complex [53].

For calculating the activity in units per ml was used the Equation 2

$$\frac{U}{ml} = \left(\frac{\frac{\Delta Abs}{\min}}{\varepsilon}\right) x\ 10^3\ x\ \left(\frac{Vt}{Va}\right)\ x\ Fdil \qquad \text{Equation 2}$$

Where Vt, Va and Fdil are total volume, sample volume and the dilution factor, respectively.

2.11. Mukhaizna crude oil treatments

The heavy crude oil sample used in these assays was obtained from the Mukhaizna oil field (Oman). Three *Pseudomonas* sp. strains (#1, #2 and #3) and three filamentous fungi (#21, #25 and #28), isolated from the oil sample CLB, were selected to study their applicability in improving the quality of Mukhaizna crude oil. Furthermore, other microorganisms previously isolated from crude oil samples obtained from two Brazilian oil fields (PTX-9 and PTX-11) [38] were also studied, including four *Bacillus subtilis* isolates (PX191, PX551, PX571 and PX572) and one *Pseudomonas* sp. strain (PX901).

The assays were performed in 50 or 250 ml flasks, containing the optimum culture medium for each microorganism. Different amounts of Mukhaizna crude oil (from 5 to 7 g) were used in each assay. The experiments were performed at 37 ºC and different agitation rates (105-180 rpm) for variable periods of time, according with the microorganism used. In some of the assays, the culture medium was periodically removed and replaced by fresh medium in order to guarantee the availability of nutrients to the microorganisms. Furthermore, biosurfactant and bioemulsifier production were evaluated by measuring the surface tension and the emulsifying activity of the cell-free supernatants along the assays, as described previously. At the end of the assays, the oil was recovered and its viscosity was measured.

When the culture medium was removed and replaced by new medium, in some cases it was observed the presence oil particles in the supernatants. To recover those oil fractions, the culture medium was extracted with one volume of dichloromethane or toluene. The solvent and the culture medium were mixed in a separating funnel for several minutes (at room temperature) and subsequently were allowed to separate overnight. The organic phase was recovered and the solvent was allowed to evaporate in a chemical fume hood. The oil fractions recovered were

added to the crude oil recovered at the end of the assay and the viscosity of the mixture was determined.

2.11.1. Viscosity measurements

The dynamic viscosity (η) was measured using an automated Anton Paar (model SVM 3000) rotational Stabinger viscosimeter-densimeter at 50 ^{O}C and at atmospheric pressure (approximately 0.1 MPa). The relative uncertainty in dynamic viscosity measurements is ±0.35% [39].These assays were performed at the Chemistry Department, University of Aveiro.

2.11.2. Evaluation of *n*-alkanes degradation

After the treatments performed with the different microorganisms, the *n*-alkanes degradation in the oil samples was evaluated. The oil recovered from the culture medium was diluted (20 mg/ml) in dichloromethane for gas chromatography (GC) analysis. GC analysis was performed using a CP 3800 Varian gas Chromatograph equipped with an on-column injector, FID detector, and DB-HT-SIMDIS capillary column (5 m × 0.53 mm i.d., 0.15 μm thickness) (Agilent J&W Scientific Inc., California, USA). Helium was used as the carrier gas at a constant flow rate of 18 ml/min. Injector and detector temperatures were 350 and 370 ^{O}C, respectively. The oven temperature was set at 40 ^{O}C during 5 min, raised to 350 ^{O}C at a rate of 5 ^{O}C/min, and at last kept at 370 ^{O}C during 15 min. For heating oil samples, the oven temperature was set at 40 ^{O}C during 8 min, raised to 300 ^{O}C at a rate of 5 ^{O}C/min, and at last kept at 300 ^{O}C during 15 min [39]. The *n*-alkanes degradation was evaluated by comparing the composition of the oil recovered after each treatment with the crude oil before the treatment. Each sample was analysed in duplicate. These assays were performed at the Chemistry Department, University of Aveiro.

2.12. Microbial Enhanced Oil Recovery assays

Sand-packed columns were designed to simulate the oil reservoir and used to evaluate the effect of microorganisms in oil recovery. Vertically oriented acrylic columns with a volume of 250 ml were uniformly packed with dry sand (previously sterilized). The columns were provided with a

sieve and cap fixed at the bottom. After packing the sand tightly, a top sieve and cap were fixed. The caps on both the ends of the column were provided with holes for insertion of inlet and outlet tubes. Rubber 'O' rings surrounded the caps to hermetically seal the column. The experiments were performed at 37 ^{O}C using the crude oil obtained from the oil well CLB.

A schematic representation of the process is shown in Figure 4. The column was first flooded with water at a constant flow rate of 2 ml/min. Pore volume (PV, ml), defined as the empty volume of the model, was calculated by measuring the volume of water required to saturate the column. The porosity (%) of the column was calculated as the PV divided by the total volume of the column (250 ml). In the second step, the crude oil (previously sterilized) was injected into the column in the same way, to replace the water, until there was no more water coming out from the effluent. The original oil in place (OOIP, ml) was calculated as the volume of crude oil retained in the column. Initial oil saturation (Soi, %) (Equation 3) and initial water saturation (Swi, %) (Equation 4) were calculated as follows:

$$S_{oi} = \left(\frac{OOIP}{PV}\right) \times 100 \qquad \text{Equation 3}$$

$$S_{wi} = \left(\frac{PV - OOIP}{PV}\right) \times 100 \qquad \text{Equation 4}$$

Subsequently, the sand-packed column was incubated at 37 ^{O}C overnight, and afterwards it was flooded again with water to remove the excess of crude oil, until no more crude oil was observed in the effluent. The amount of crude oil recovered, so-called oil recovered after water flooding (Sorwf, ml) was determined volumetrically. Residual oil saturation (Sor) (Equation 5) was calculated as follows:

$$S_{or} = \left(\frac{OOIP - S_{orwf}}{OOIP}\right) \times 100 \qquad \text{Equation 5}$$

Afterward, the columns were subjected to the microbial recovery processes. The columns were inoculated with the different filamentous fungi (#21, #25 and #28) supplemented with 50 ml of the medium BRM+. The filamentous fungi were previously grown in flasks containing 50 ml of BRM+, at 37 ^{O}C for 2 days; after that, the cultures were centrifuged (4000 rpm, 20 min, 20 ^{O}C) and the culture medium was removed and replaced by fresh medium (BRM+), in order to guarantee the availability of nutrients during the MEOR process.

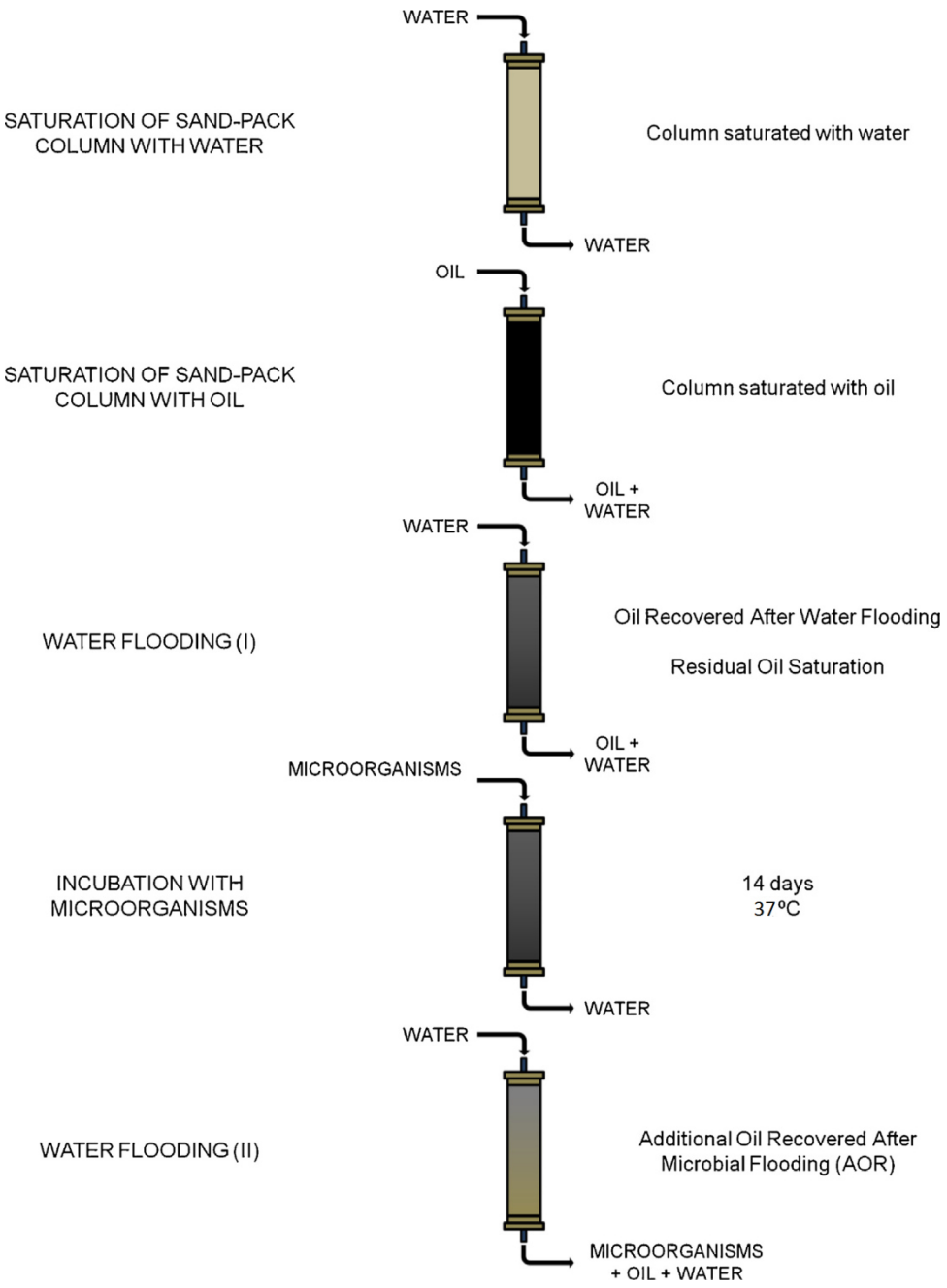


Figure 4: Schematic representation of the sand-packed column process. Adapted from Gudiña et al. [39].

After inoculation, the columns were sealed and incubated for 14 days at 37 ^{O}C. Control columns were prepared and incubated in the same way, but without addition of microorganisms. After the incubation time, the columns were flooded again with water and the volume of crude oil recovered (oil recovered after microbial flooding (Sormf, ml)) was measured volumetrically. Additional Oil Recovery (AOR, %) (Equation 6) was calculated as follows:

$$AOR = \left(\frac{S_{ormf}}{OOIP - S_{orwf}}\right) \times 100 \qquad \text{Equation 6}$$

All the experiments were performed in duplicate.

The viscosity of the oil recovered after the treatment with the different fungi was measurement as described above, and compared with the oil recovered in the abiotic control columns.

2.13. Identification of the filamentous fungus #28

The filamentous fungus #28 exhibited interesting activities. For its identification, the isolate was grown in Oatmeal Agar (OA) at 30 ^{O}C, and its macroscopic and microscopic morphology was studied.

Furthermore, a ITS (Internal Transcribed Space) region was obtained after amplification with the primers ITS 1 (5'TCCGTAGGTGAACCTGCGG3') and ITS 4 (5'TCCTCCGCTTATTGATATGC3') [54]. The sequencing was performed by STABVIDA (Portugal). The resulting sequence was compared with sequences in the GenBank database of the National Centre for Biotechnology Information (NCBI) using the nucleotide-nucleotide blast (BLASTn) network service, to determine its phylogenetic affiliations.

2.14. Analytical techniques

i. Carbohydrates concentration

Total carbohydrates were determined using the phenol-sulphuric acid method, as described by Dubois et al. [55]. Briefly, each sample (1 ml) was mixed with 20 µl of phenol solution (80%, w/v) in glass test tubes. Afterward, 2.5 ml of concentrated sulphuric acid (95-97%) were added to each tube with slight shaking. The samples were left at room temperature for 10 min, shaken again and allowed to stand at room temperature for another 20 min. Afterwards, the optical density of each sample was measured at 490 nm using a multi-detection micro-plate reader Synergy™ HT (BioTek, USA). The carbohydrates concentration was determined using a calibration curve prepared using glucose at concentrations ranging from 1 to 100 mg/l (Appendix A, Figure A1). All the measurements were performed in duplicate.

ii. Protein concentration

The protein concentration was determined according to the Bradford assay using a protein assay kit (Thermo Scientific, USA). Briefly, 30 µl of sample were mixed with 1.5 ml of Bradford reagent and incubated at room temperature for 5 min. Subsequently, the optical density at 595 nm was measured using a multi-detection microplate reader Synergy™ HT (BioTek, USA). The total amount of protein was determined using a calibration curve prepared using bovine serum albumin (BSA) at concentrations ranging from 0.1 to 1 mg/ml (Appendix A, Figure A2). All the measurements were performed in duplicate.

iii. Phenolic compounds concentration

Total phenolic compounds were determined using a colorimetric assay adapted to 96-well micro-plates. Briefly, 5 µl of the sample were mixed with 60 µl of sodium carbonate solution (7.5%, w/v) and 15 µl of Folin-Ciocalteu reagent in a micro-plate well; afterwards, 200 µl of demineralised water were added to each well and the solutions were mixed. The plate was incubated at 60 ^{O}C for 5 minutes, and subsequently allowed to cool at room temperature. The absorbance of each well was measured at 700 nm using a micro-plate reader Synergy™ HT (BioTek, USA). A calibration curve was prepared using a standard solution of gallic acid at concentrations ranging from 200 to 3000 mg/l (Appendix A, Figure A3). The phenolic content was expressed as milligram of gallic acid equivalent (mg GAE)/l. All the measurements were performed in triplicate and expressed as means ± standard deviation.

2.15. Statistical analysis

Results are presented as the mean ± standard deviation of at least two replicates. The analyses were carried out using Microsoft Office Excel software. Statistically significant differences of the conditions tested in the different assays were evaluated by a one-way ANOVA ($P < 0.05$) applying the Tukey multiple-comparisons. A significant difference was considered if $P < 0.05$. Statistical analyses were performed using GraphPad (San Diego, USA) software.

3.Results and Discussion

3.1. Isolation of microorganisms from the crude oil sample

Twenty-three isolates were obtained from the oil sample collected from the oil well CLB. Some of the isolates were characterized according to their macroscopic characteristics (colony form, colour and size). Fourteen isolates were bacterial strains (#1-#14) and the remaining nine were filamentous fungi (#20-#28) (Table 2).

Table 2: Isolates obtained from the oil sample CLB and culture media and conditions used for their isolation.

Isolate	Culture medium	Additional carbon source	Temperature (°C)	Aerobic/Anaerobic
#1	MSS	–	37	Aerobic
#2				
#3				
#4				
#5				
#6				
#7		Sucrose		
#8	CSL	–		
#9				
#10	MSS	Sucrose		
#11				
#12		-		Anaerobic
#13		Sucrose	50	Aerobic
#14		–	37	Anaerobic
#20	CSL			Aerobic
#21				
#22				
#23				
#24	MSS			
#25				
#26		Sucrose		
#27				
#28				Anaerobic

As it can be seen in Table 2, all the strains were isolated at 37 °C, except the strain #13, which was isolated at 50 °C. The strains #12, #14 and #28 were isolated under anaerobic conditions, whereas the remaining strains were isolated under aerobic conditions.

Five isolates (#1-#5) were identified as *Pseudomonas* sp. according to their macroscopic characteristics, such as, colour, distribution of the colony, smell and more ahead the type of recovery and the ability to produce certain bio-products (Figure 5).

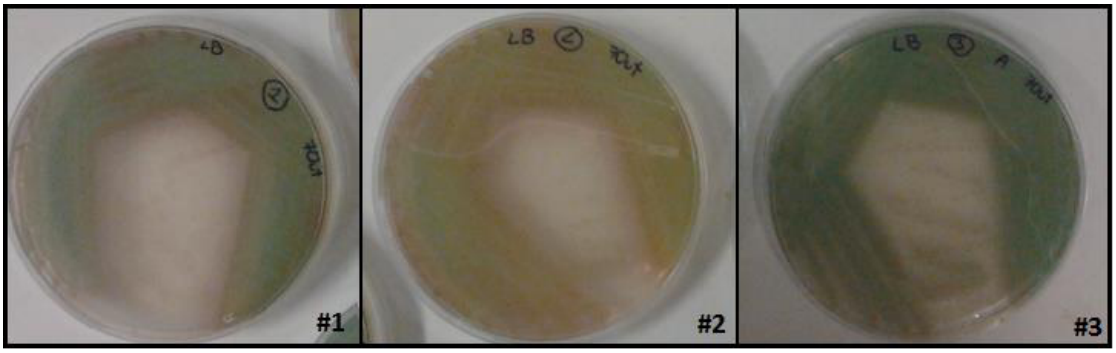


Figure 5: *Pseudomonas* sp. #1, #2 and #3 grown in LB medium at 37 ^{O}C.

3.2. Study of biosurfactant production by the bacterial strains

3.2.1. Screening of biosurfactant-producing strains

The bacterial strains (#1-#14) were screened for extracellular biosurfactant production using three different mineral media: MSS+; MSS-; and MSM+. The cultures were incubated at 37 ^{O}C and 105 rpm under aerobic conditions (seven days for isolates #1-#5 and twenty-one days for isolates #6-#14 in MSS medium; five days for isolates #1-#5 and fourteen days for isolates #6-#14 in MSM medium). Biosurfactant production was evaluated by measuring the surface tension of the cell-free supernatants along the assays. Five isolates (*Pseudomonas* sp. #1, #2, #3, #4 and #5) reduced the culture medium surface tension, and were considered potential biosurfactant producers (Table 3). The remaining isolates didn't reduce the culture medium surface tension.

Table 3: Surface tension values (mN/m) obtained with the isolates *Pseudomonas* sp. #1, #2, #3, #4 and #5 grown in MSS+, MSS- and MSM+ media for 5 or 7 days at 37 ^{O}C and 105 rpm under aerobic conditions. The surface tension values were measured at room temperature (20 ^{O}C). Results represent the average of two independent experiments ± standard deviation.

Strain	ST (mN/m)		
	MSS +	MSS -	MSM +
#1	51.8 ± 0.5	45.3 ± 0.7	35.6 ± 0.4
#2	53.9 ± 1.0	53.1 ± 1.5	52.3 ± 1.5
#3	54.0 ± 0.7	53.0 ± 1.2	45.1 ± 0.7
#4	54.0 ± 0.4	53.9 ± 0.9	51.1 ± 1.2
#5	55.0 ± 0.5	54.1 ± 0.7	43.1 ± 0.9
Control	69.4 ± 2.1	69.4 ± 2.1	66.3 ± 0.4

From the results shown in Table 3, it can be seen that the lowest surface tension value was obtained with the isolate *Pseudomonas* sp. #1 (35.6 ± 0.4 mN/m), followed by the isolates *Pseudomonas* sp. #3 and #5 (45.1 ± 0.7 and 43.1 ± 0.9 mN/m, respectively), all of them grown in MSM+. According with the morphological characteristics exhibited by the five *Pseudomonas* sp. isolates, together with the surface tension values obtained in the different media studied, it was concluded that the isolates #2/#4 and #3/#5 were the same strains. For that reason, for further studies, only the isolates #1, #2 and #3 were used.

The isolates #6-#14, which didn't reduce the surface tension of the culture medium in the previous assays, were evaluated for biosurfactant production using the medium MSS- supplemented with CSL (10%, v/v) at the same conditions described previously (37 ^{O}C and 105 rpm). As in the previous assays, no surface tension reductions were observed for any of the isolates. According with these results, it can be concluded that the isolates #6-#16 didn't produce biosurfactants in these conditions.

3.2.2. Biosurfactant production by *Pseudomonas* sp. isolates

3.2.2.1. Optimization of biosurfactant production

Biosurfactant production by *Pseudomonas* sp. #1, #2 and #3 was evaluated using different culture media. All the assays were performed at 37 ^{O}C, in 500 ml flasks containing 150 ml of culture medium. Biosurfactant production was evaluated by measuring the surface tension and

the emulsifying activity of the cell-free supernatants along the time. Whenever required, the cell-free supernatants were diluted 10 or 100 times with demineralised water, and the corresponding surface tension values and emulsifying indexes (ST^{-1}/E_{24}^{-1} and ST^{-2}/E_{24}^{-2}, respectively) were measured.

The first assays were performed using LB medium, at 180 rpm. As it can be seen in Figure 6A, in the first 24 hours of growth, a great surface tension reduction of the cell-free supernatants was observed (from 47.0 to 33.6, 33.1 and 32.8 mN/m for isolates #1, #2 and #3, respectively).

From this time point until the end of the fermentation, the surface tension values remained almost constant. The lowest surface tension value at the end of the fermentation was obtained with the isolate #1 (32.0 mN/m). A similar profile was observed for the ST^{-1} values, except for the isolate #1, were the ST^{-1} value continued decreasing until the 48 hours of growth. In this case, surface tension values between 45.0 and 47.8 mN/m were obtained for the three isolates (the surface tension of LB medium 10 times diluted is 60.0 mN/m).

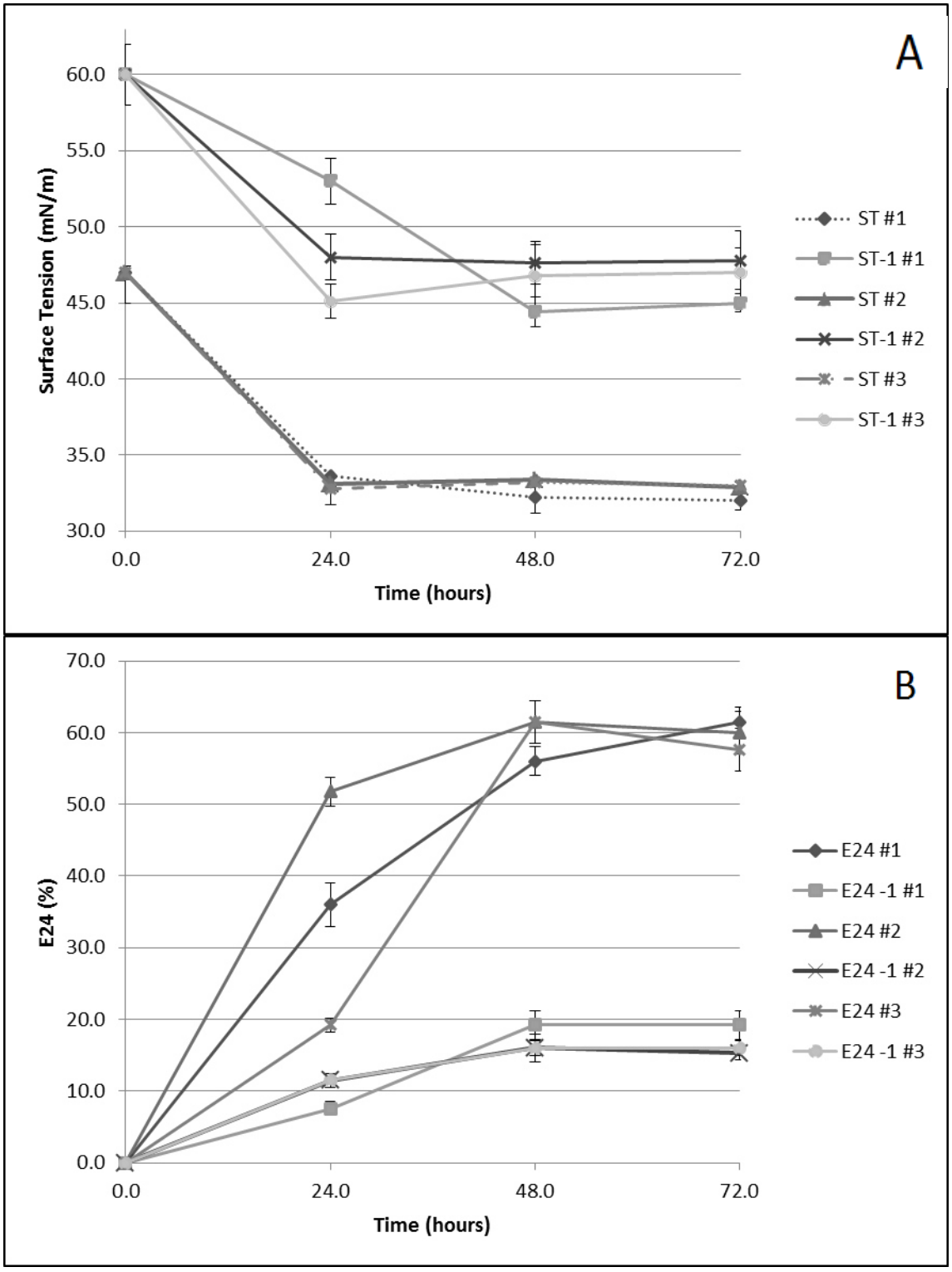


Figure 6: Evolution of surface tension (mN/m) (A) and emulsifying activity (%) (B) in cultures performed with *Pseudomonas* sp. #1, #2 and #3 in LB medium at 37 ^{O}C and 180 rpm. Results represent the average of two independent experiments ± standard deviation.

Regarding the emulsifying activity (Figure 6B), for the isolates #2 and #3, the highest value was obtained after 48 hours (61.5%), whereas the isolate #1 reached that value at 72 hours. The

emulsifying indexes of the samples E_{24}^{-1} achieved their maximum values after 48 hours (19.2% for the strain #1 and 16.0% for the strains #2 and #3), and then remained constant until the end of the fermentation (72 hours).

Biosurfactant production by these isolates was also studied using the medium MSM+, at 37 ^{O}C and 105 rpm. Isolates #1, #2 and #3 reduced the culture medium surface tension to 29.9, 30.3 and 29.6 mN/m, respectively, after 72 hours (Figure 7A), and after that the surface tension remained almost constant until the end of the fermentation (168 h). These values represent a considerable decrease when compared with the surface tension of the culture medium (66.3 mN/m). The ST^{-1} values continued decreasing until the end of the fermentation (39.8, 38.8 and 37.0 mN/m for isolates #1, #2 and #3, respectively, after 168 h).

The emulsifying indexes also reached their highest value (60.0% for all the strains) after 72 h (Figure 7B), and then remained constant until the end of the fermentation. However, the E_{24}^{-1} values continued increasing until the end of the fermentation (168 h), up to 56.0% for all the strains. These results suggest that biosurfactant production continued until the end of the fermentation.

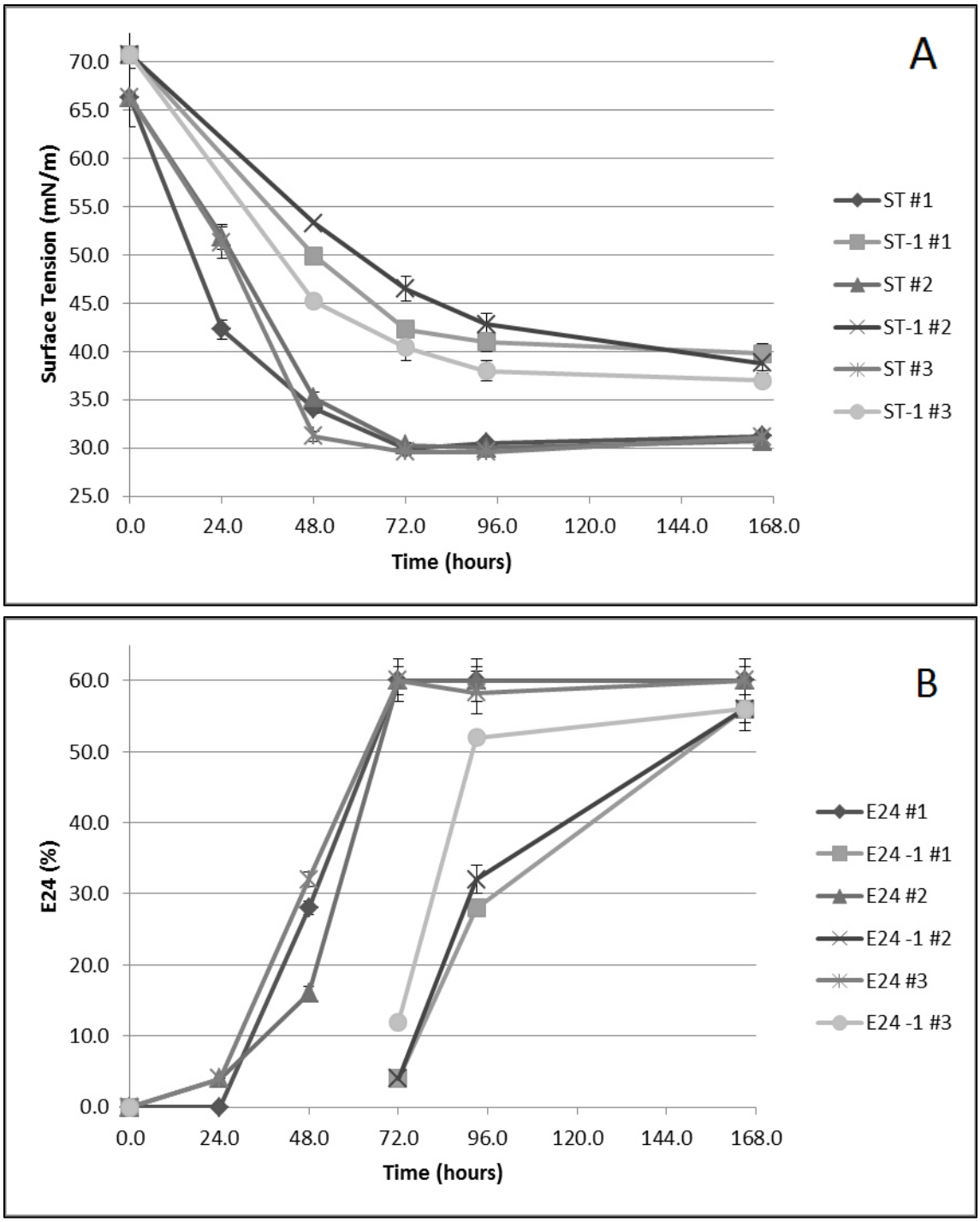


Figure 7: Evolution of surface tension (mN/m) (A) and emulsifying activity (%) (B) in cultures performed with *Pseudomonas* sp. #1, #2 and #3 in MSM+ medium at 37 ^{O}C and 105 rpm. Results represent the average of two independent experiments ± standard deviation.

To study the effect of the agitation rate on biosurfactant production, the *Pseudomonas* sp. isolates were grown in the medium MSM+ at 180 rpm, and biosurfactant production along the fermentation was evaluated as described previously. The lowest surface tension values (30.0, 28.4 and 29.3 mN/m for isolates #1, #2 and #3, respectively) were observed after 48 h (Figure 8A).

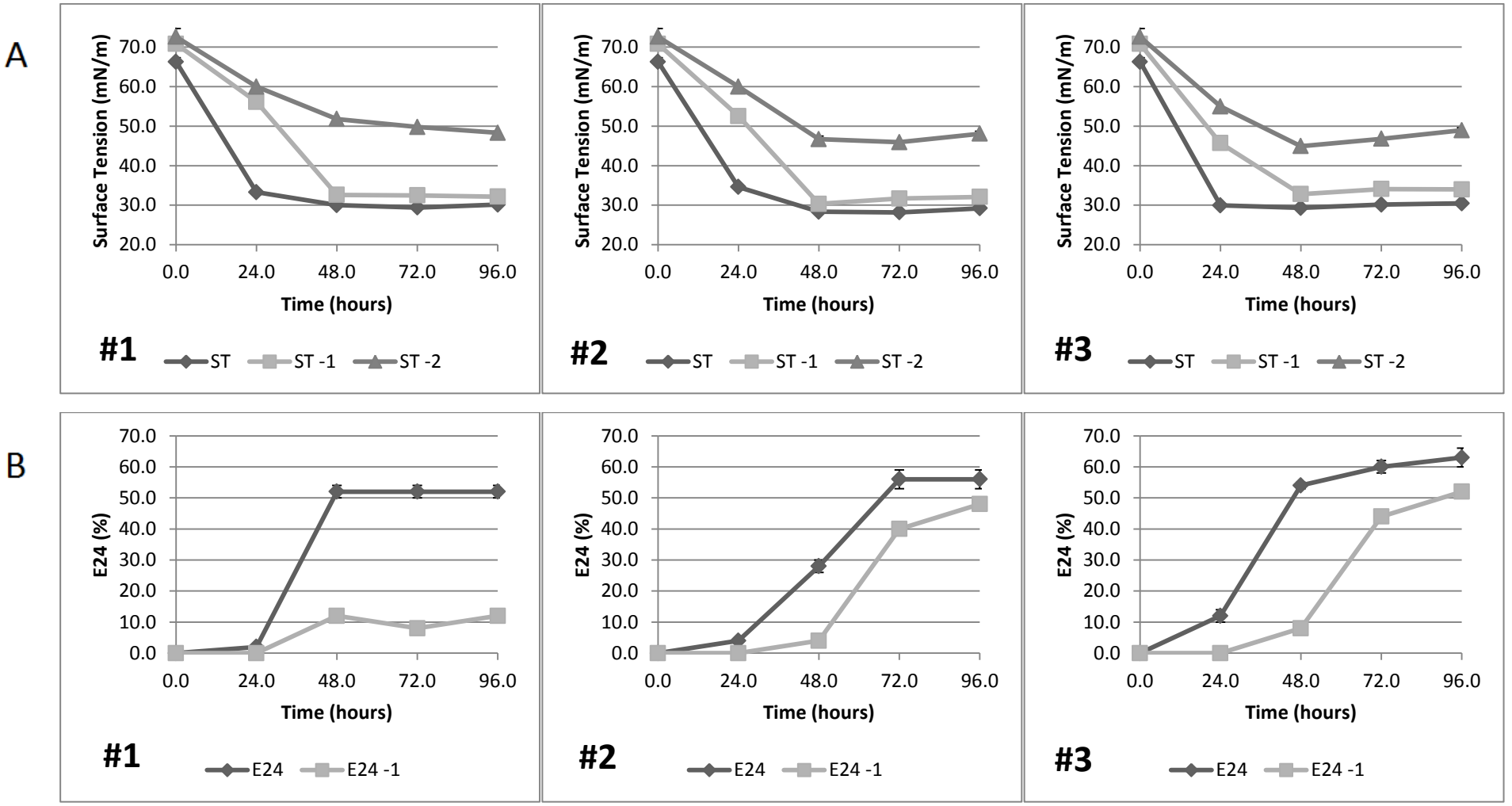


Figure 8: Evolution of surface tension (mN/m) (A) and emulsifying activity (%) (B) in cultures performed with *Pseudomonas* sp. #1, #2 and #3 in MSM+ at 37 ^{O}C and 180 rpm. Results represent the average of two independent experiments ± standard deviation.

In general, the lowest ST^{-1} and ST^{-2} values were also obtained after 48 hours for the three isolates, although with some minor exceptions (Figure 8A). Regarding the emulsifying activity, the highest values were achieved at different times with the different isolates (Figure 8B): 48 h for isolate #1 (54.0%), 72 h for isolate #2 (56.0%) and 96 h for isolate #3 (63.0%). The highest E_{24}^{-1} values (48.0 and 52.0%) were achieved with isolates #2 and #3 at the end of the fermentation (96 h), while the isolate #1 exhibited a low E_{24}^{-1} index (12.0%).

According with the ST^{-1} values obtained in assays performed with the medium MSM+ at 105 and 180 rpm, it can be concluded that a higher agitation rate results in a higher biosurfactant production for these strains.

The isolates *Pseudomonas* sp. #1, #2 and #3 exhibited significant surface tension reductions and high emulsifying indexes growing in the medium MSM+ at 37 ^{O}C and 180 rpm, which may be indicative of a high biosurfactant production. In order to try to increase biosurfactant production by these isolates, this medium was supplemented with 10% (v/v) of OMW, a liquid effluent derived from olive oil production. OMW contains many dissolved and suspended substances, including large amounts of monomeric and polymeric phenols, fatty acids, sugars, volatile acids, polyalcohols and nitrogenous compounds. The fatty acids (e.g. oleic acid) present in OMW have an inductive effect on biosurfactant production in different *Pseudomonas* isolates [56, 57].

In this case, the surface tension and the ST^{-1} values obtained after 96 and 144 hours of growth were higher when compared with the ones obtained with the medium MSM+ in the previous assay for the three isolates (Table 4). Regarding the emulsifying activity, the values obtained were lower when compared with those obtained using the medium MSM+, except for the isolate #1. From the results obtained it can be concluded that the addition of OMW to the culture medium at this concentration has a negative effect on biosurfactant production by these strains.

Contrary to the results obtained in this study, other authors reported a high biosurfactant production by different *Pseudomonas* isolates growing on media supplemented with olive oil, and suggested the possible use of other cheap waste oils as alternative substrates for a cost-effective biosurfactant production [58-60].

Table 4: Surface tension values (mN/m) and emulsifying indexes (%) obtained in cultures performed with *Pseudomonas* sp. #1, #2 and #3 in MSM+ supplemented with 10% (v/v) of OMW, at 37 ^{O}C and 180 rpm. The surface tension of the culture medium is 66.3 mN/m. Results represent the average of two independent experiments ± standard deviation.

Isolate	ST (mN/m)	ST^{-1} (mN/m)	E24 (%)
96 h			
#1	31.6 ± 0.1	35.7 ± 0.1	56.0 ± 2.0
#2	32.9 ± 0.3	38.2 ± 0.5	40.0 ± 1.0
#3	35.1 ± 0.0	46.7 ± 0.6	20.0 ± 0.5
144 h			
#1	31.7 ± 0.3	35.5 ± 0.5	56.0 ± 3.0
#2	32.3 ± 0.1	34.5 ± 0.2	36.0 ± 1.0
#3	35.4 ± 0.3	39.8 ± 0.8	24.0 ± 1.0

Biosurfactant production by different *Pseudomonas* strains has been reported by other authors. For instance, Raza and co-workers [61] reported biosurfactant production by a *P. aeruginosa* strain that reduced the surface tension up to 30 mN/m; Lotfabad et al. [60] reported biosurfactant production by *P. aeruginosa* MR01, which reduced the surface tension up to 28 mN/m, and exhibited an emulsifying index against *n*-hexadecane of 70%. Xia et al. [62] studied biosurfactant production by *Pseudomonas* sp. WJ6, that reduced the surface tension up to 29 mN/m.

The lowest surface tension values achieved with the *Pseudomonas* sp. strains herein studied (#1, #2 and #3) were 30.0, 28.4 and 29.3 mN/m, and are in agreement with the results described in the literature for other biosurfactant-producing *Pseudomonas* isolates.

3.2.2.2. Biosurfactants recovery

Depending on the nature and the composition of the biosurfactants, different methods may be required for their recovery. In this work, to recover the biosurfactants produced by the three *Pseudomonas* sp. isolates, two different techniques were evaluated: acidic precipitation and adsorption chromatography using a polystyrene resin (Amberlite XAD-2).

Acidic precipitation is one of the most simple and commonly used methods for biosurfactant recovery. However, in the case of the biosurfactants produced by *Pseudomonas* sp.

#1, #2 and #3 in LB medium, this technique allowed the recovery of only a small fraction of the biosurfactants produced, as it can be concluded from the surface tension values of the supernatants before and after acidic precipitation, which were very similar (Table 5). From the results obtained, it can be concluded that the acidic precipitation is an inefficient technique to recover the biosurfactants produced by these isolates.

Table 5: Evaluation of different methods for biosurfactant recovery. Surface tension values (mN/m) of cell-free supernatants before/after acidic precipitation and after adsorption chromatography using the polystyrene resin Amberlite XAD-2. The cell-free supernatants correspond to cultures of *Pseudomonas* sp. #1, #2 and #3 performed in LB medium at 37 ^{O}C and 180 rpm for 72h. The surface tension of LB medium is 47.0 ± 0.4 mN/m. The results represent the average of two measurements ± standard deviation.

Supernatant	Surface Tension (mN/m)		
	Pseudomonas sp. #1	*Pseudomonas* sp. #2	*Pseudomonas* sp. #3
Before acidic precipitation	32.0 ± 0.1	32.9 ± 0.0	33.0 ± 0.2
After acidic precipitation	33.0 ± 0.2	33.1 ± 0.3	34.2 ± 0.3
After adsorption chromatography	60.0 ± 1.2	64.4 ± 1.1	64.2 ± 2.9

Adsorption chromatography using the polystyrene resin Amberlite XAD-2 was evaluated as an alternative technique for biosurfactant recovery. The supernatants obtained after acidic precipitation (that, according to the surface tension values contain a considerable amount of biosurfactants) were used. In this case, a substantial increase in the surface tension values was observed (Table 5), which indicates that almost all the biosurfactants were recovered from the supernatants.

Due to the better results obtained with the adsorption chromatography, that technique was used to recover the biosurfactants produced by *Pseudomonas* sp. #1, #2 and #3 in MSM+ medium at 180 rpm. The amount of biosurfactant recovered in each case is shown in Table 6. As it can be seen, the highest biosurfactant production corresponded to the isolate #2. Although the surface tension values obtained with the three isolates at the end of the fermentation were very similar (Figure 8), the differences observed in the amount of biosurfactant produced by each isolate may be related to the properties (i.e., the CMC) or the purity of each biosurfactant, as it will be discussed later.

Table 6: Amount of biosurfactant produced by *Pseudomonas* sp. #1, #2 and #3 grown in MSM+ at 37 ^{O}C and 180 rpm for 96 hours.

Recovered biosurfactants (mg/l)		
Pseudomonas sp. #1	*Pseudomonas* sp. #2	*Pseudomonas* sp. #3
384	1030	836

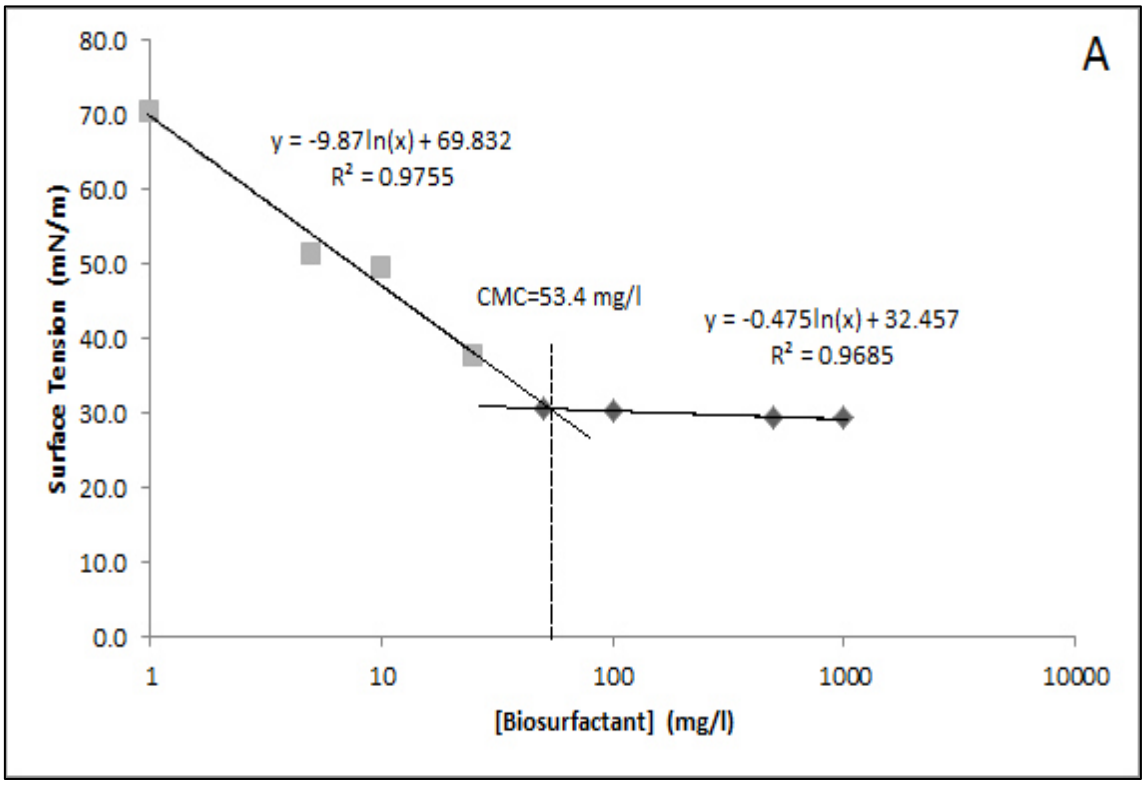
Biosurfactant recovery through adsorption chromatography using the polystyrene resin Amberlite XAD-2 proved to be more efficient than the acidic precipitation, as it can be seen from the surface tension values of the supernatants subjected to both recovery methods (Table 5). These results are in agreement with those reported by other authors regarding biosurfactants produced by *P. aeruginosa* strains [49, 63]. As it has been previously mentioned, the acidic precipitation is one of the most common methods used for biosurfactant recovery. However, the inability of recovering efficiently biosurfactants produced by *P. aeruginosa* isolates (particularly rhamnolipids) through acidic precipitation, due to the similar polarity of the product and the substrate components, has been reported by some authors [64], that suggested the use of other methods, or the combination of two methods (e.g., acidification of the culture media followed by extraction with chloroform/methanol), to recover these biosurfactants [65].

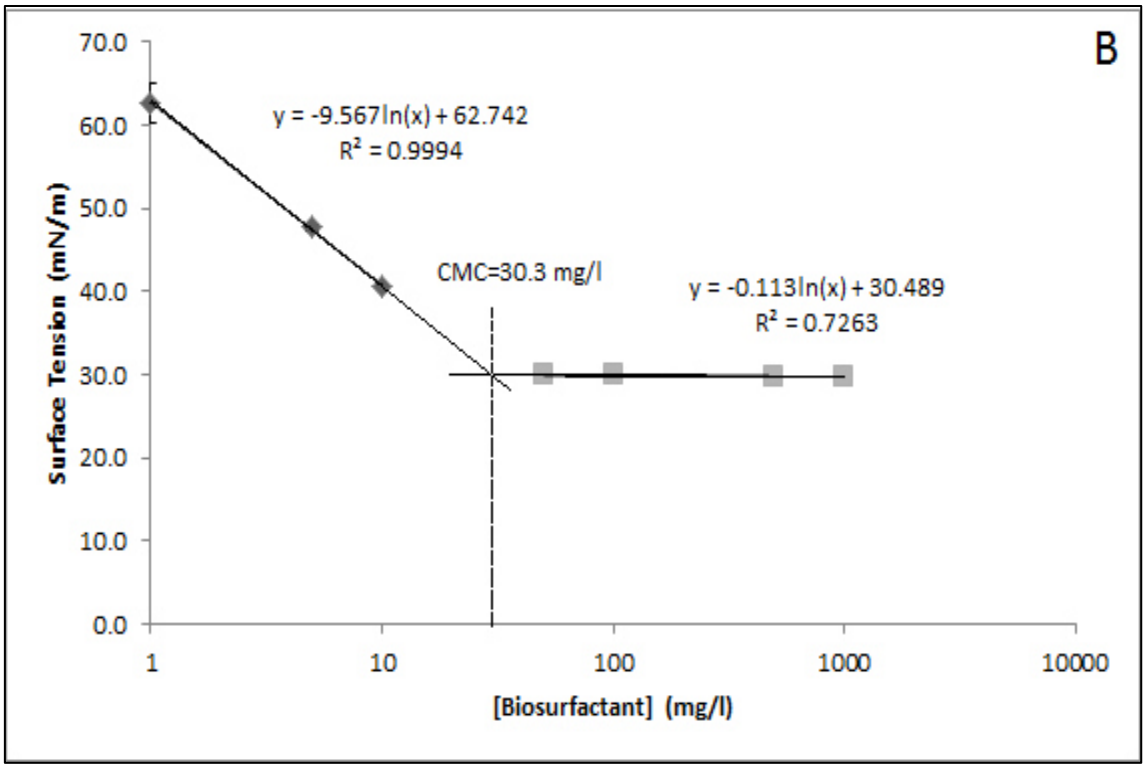
3.2.2.3. Critical micelle concentration (CMC)

One of the main properties of surfactants is their tendency to adsorb at interfaces in an oriented fashion, as a consequence of their amphipathic structure. As the surfactant concentration increases, the surface tension of the solution decreases, until a concentration where the surface tension becomes almost constant, due to the saturation of the interface with surfactant molecules. That concentration is known as the CMC, the minimum surfactant concentration at which the surfactant molecules start forming aggregates. The CMC is usually determined from the break point of the curve representing the surface tension *versus* surfactant concentration [64].

The CMC of the biosurfactants produced by *Pseudomonas* sp. #1, #2 and #3 in MSM+ medium at 37 ^{O}C and 180 rpm, and recovered through adsorption chromatography, was determined by measuring the surface tension of biosurfactant solutions prepared at different concentrations in demineralised water. The lowest surface tension values obtained for the

biosurfactants produced by *Pseudomonas* sp. #1, #2 and #3 were 30.0, 29.9 and 30.4 mN/m, respectively, and their CMC values 53.4, 30.3 and 40.4 mg/l (Figure 9). According with these results, it can be concluded that the biosurfactant produced by *Pseudomonas* sp. #2 is more efficient than the ones produced by the other two isolates, as its CMC is lower. Regarding the effectiveness, defined as the minimum value to which the surface tension can be reduced by a specific surfactant [66], from the results obtained it can be concluded that the three biosurfactants herein studied exhibit a similar performance.





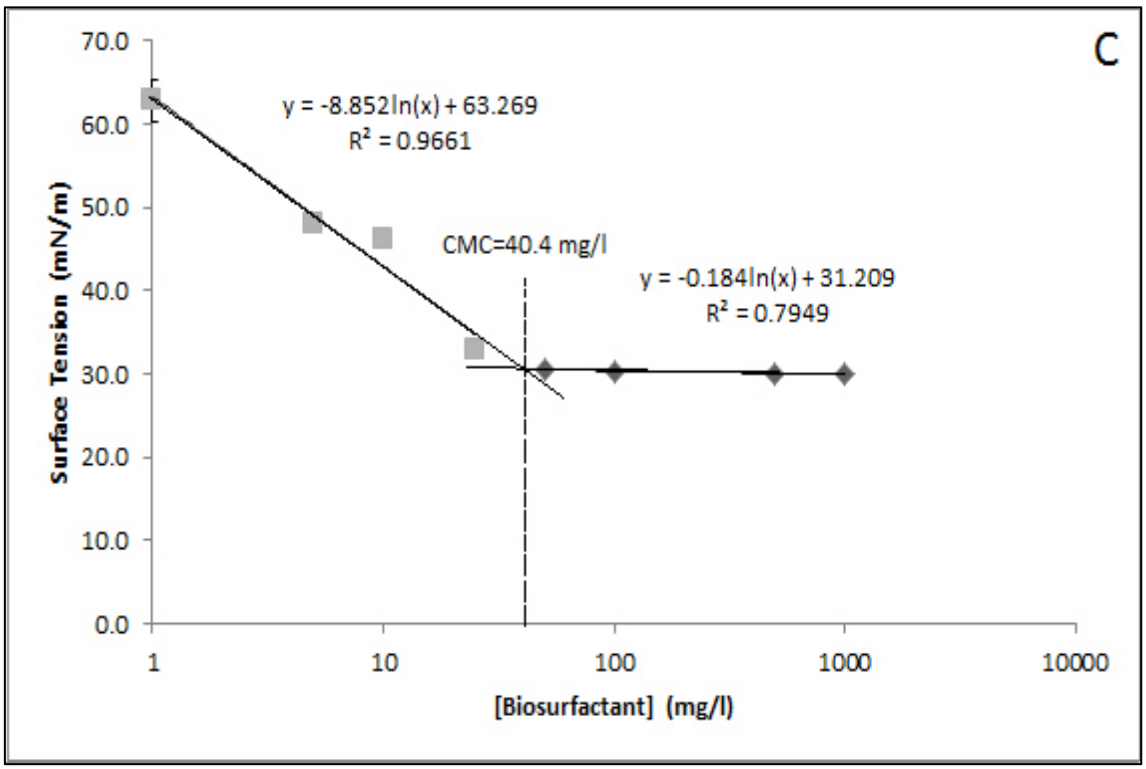


Figure 9: CMC of the biosurfactants produced by *Pseudomonas* sp. #1 (A), #2 (B) and #3 (C) in MSM+ at 37 ^{O}C and 180 rpm. The plot represents the surface tension (mN/m) *versus* the logarithm of biosurfactant concentration (mg/l). The CMC was determined from the intersection of the regression lines better describe the two parts of the curve, below and above the CMC. Results represent the average of two measurements and error bars represent the standard deviation of the mean values.

CMC values between 5 and 200 mg/l have been reported for biosurfactants (rhamnolipids) produced by different *P. aeruginosa* strains, depending on the different congeners present in the mixture and their proportions [67]. Sánchez et al. [49] reported a surface tension reduction from 55 to 36 mN/m, with a CMC of 71.5 mg/l, for biosurfactants produced by a *P. aeruginosa* strain. Benincasa et al. [68] reported a minimum surface tension value of 24 mN/m and a CMC of 120 mg/l for biosurfactants produced by *P. aeruginosa* LBI. As it can be seen, the CMC values obtained for the biosurfactants produced by the isolates *Pseudomonas* sp. #1, #2 and #3 were similar or lower than the ones reported by other authors.

3.3. Study of biosurfactant production by the filamentous fungi

3.3.1. Screening of biosurfactant-producing strains

Three different filamentous fungi (#21, #25 and #28) were isolated from the crude oil sample obtained from the oil well CLB. The remaining isolates were considered replicas of these according with their morphology. Extracellular biosurfactant production was evaluated through the measurement of the surface tension and the determination of the emulsifying activity in the cell-free supernatants.

In a first assay, the fungi were grown in 25 ml of MSS+ and MSS- media, at 37 ^{O}C and 105 rpm, for 3 weeks. No significant surface tension reductions or emulsifying indexes were observed, meaning that they didn't produce biosurfactants in these conditions.

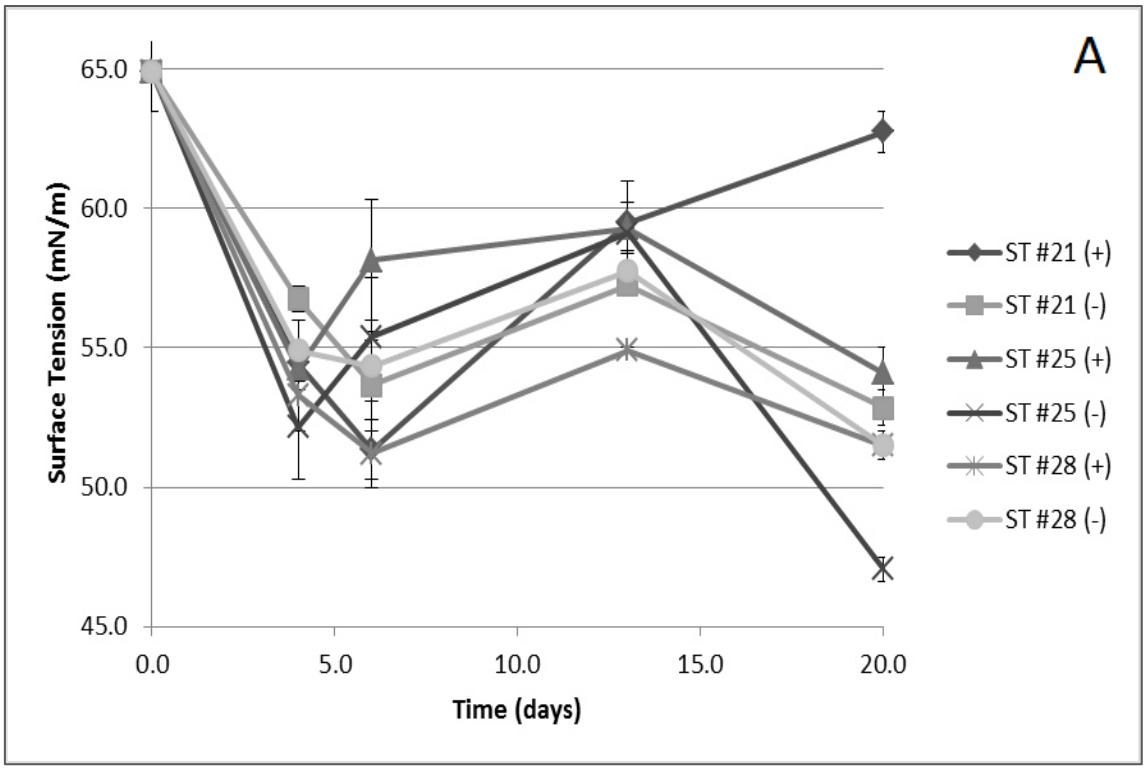
Afterwards, the fungi were grown in 100 ml of MA, BRM and Wunder media, at 37 ^{O}C and 105 rpm for 3 weeks. All the media were prepared with glucose (MA+, BRM+, Wunder+) and without glucose (MA-, BRM- and Wunder-). As it can be seen from the results obtained (Table 7), in all the cases decreases in the surface tension values were observed when compared with the culture media, although most of them were irrelevant. Furthermore, in some cases, emulsifying activity was observed. Emulsifying indexes of 24.0 and 18.0% were obtained for the fungi #21 and #25, respectively, grown in the medium BRM+; and the fungus #28 exhibited an emulsifying index of 28.0%, both in BRM+ and Wunder+ media.

Table 7: Study of biosurfactant production by the filamentous fungi #21, #25 and #28. Surface tension (mN/m) and emulsifying indexes (%) were measured in the cell-free supernatants obtained after growing the three isolates at 37 ^{O}C in the different media for 3 weeks. The surface tension values of the different media are 69.8, 69.2 and 67.0 mN/m for MA (+/-), BRM (+/-) and Wunder (+/-) media, respectively.

Microorganism	Medium	Surface Tension (mN/m)	E_{24} (%)
#21	MA +	54.4	4.0
	MA -	55.0	0.0
	BRM +	49.3	24.0
	BRM -	58.0	0.0
	Wunder +	53.3	8.0
	Wunder -	59.1	0.0
#25	MA +	50.1	13.8
	MA -	60.0	0.0
	BRM +	47.8	18.0
	BRM -	57.3	0.0
	Wunder +	46.8	8.0
	Wunder -	57.6	0.0
#28	MA +	61.0	0.0
	MA -	65.0	0.0
	BRM +	50.8	28.0
	BRM -	62.1	0.0
	Wunder +	55.1	28.0
	Wunder -	59.8	0.0

As in the assays performed with the *Pseudomonas* sp. strains, the possible inductive effect of OMW on biosurfactant production by the three filamentous fungi was studied. The isolates were grown in 100 ml of MA+ and MA- media supplemented with 10% (v/v) of OMW. The assays were performed at 37 ^{O}C and 180 rpm for 20 days. As it can be seen from the results obtained (Figure 10A) the surface tension was reduced from 65.0 mN/m to values between 51.4 and 58.2 mN/m after 6 days of growth. The lowest surface tension value (47.1 mN/m) was obtained with the isolate #25 grown in MA- medium after 20 days of growth.

Regarding the emulsifying activity, the filamentous fungi #21 and #25 achieved a maximum value of 8.0 and 4.0%, respectively, from the 6th day of culture until the end of the fermentation (Figure 10B). However, the highest activities were obtained with the isolate #28: 44.0% at the 20th day of fermentation with the medium MA-, and 24.0% at the 6th day of fermentation with the medium MA+ (Figure 10B).



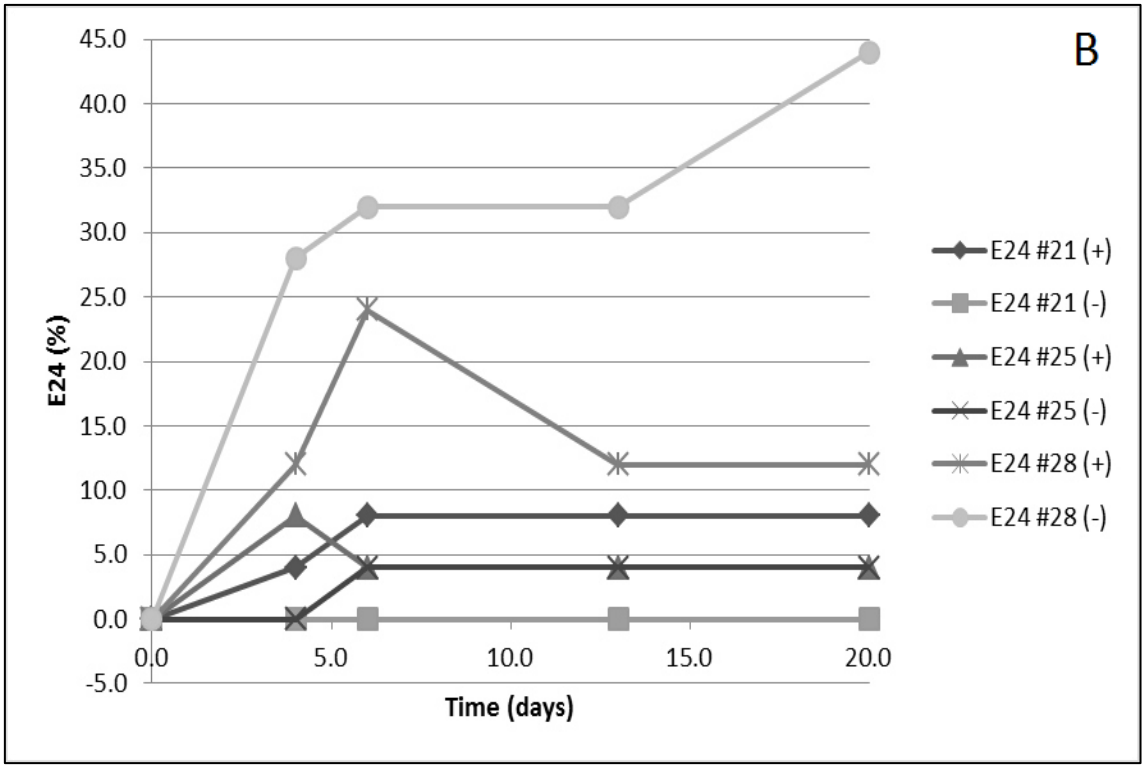


Figure 10: Evolution of surface tension (mN/m) **(A)** and emulsifying activity (%) **(B)** in cultures performed with the filamentous fungi #21, #25 and #28 grown in MA+ and MA- media supplemented with 10% (v/v) of OMW. The cultures were performed at 37 ^{O}C and 180 rpm. Results represent the average of two independent experiments ± standard deviation.

The results obtained with the isolate #28 grown in MA- medium supplemented with 10% (v/v) of OMW suggest the production of a bioemulsifier by this filamentous fungus. Bioemulsifiers are surface-active compounds synthesized by microorganisms which are more effective in forming and stabilizing oil-in-water or water-in-oil emulsions, but don't necessarily reduce surface or interfacial tension [35].

3.3.2. Study of bioemulsifier production by the isolate #28

3.3.2.1. Optimization of bioemulsifier production

Due to the promising bioemulsifier production observed with the isolate #28 in medium MA- supplemented with 10% (v/v) of OMW, other substrates (CLB crude oil, paraffin, soybean oil, gas oil and *n*-hexadecane) were evaluated for their inductive effect on bioemulsifier production by this isolate. The cultures were performed in flasks containing 100 ml of the culture media MA+ and MA-, supplemented with 10% (w/v) of the different substrates, and were incubated at 37 ^{O}C and 105 rpm for 3 weeks. Surface tension and emulsifying activity were measured along the fermentation, and the results obtained after 21 days of growth are shown in Table 8.

Regarding the surface tension values obtained in these assays, only slight decreases were observed. The lowest surface tension value (51.2 mN/m) was obtained with the medium MA+ supplemented with *n*-hexadecane after 7 days of growth (*data not shown*). Regarding the emulsifying indexes, the highest values were observed at the end of the fermentation (21 days); the media MA- and MA+ supplemented with OMW offered the best results (40.0 and 28.0%, respectively), followed by MA+ supplemented with paraffin (20.0%) and MA+/MA- supplemented with *n*-hexadecane (16.0%) (Table 8).

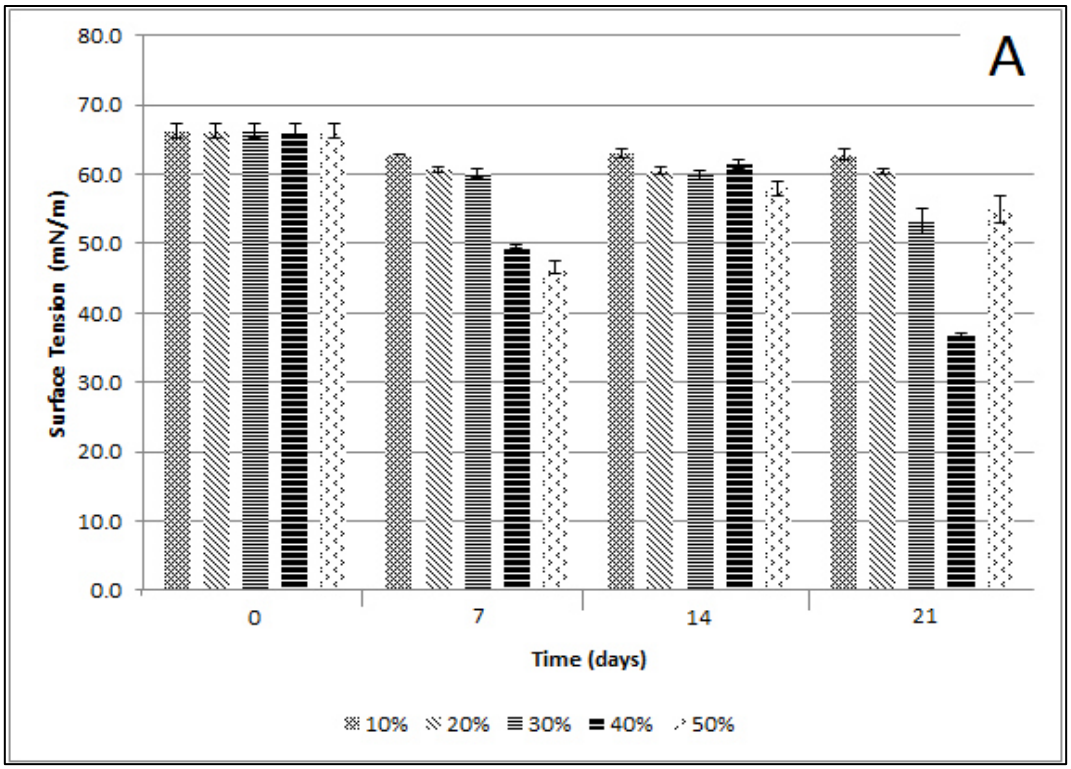
References regarding bioemulsifier production by filamentous fungi are scarce. Luna-Velasco et al. [35] described the production and properties of a bioemulsifier synthesized by *Penicillium* sp. grown in a medium supplemented with phenanthrene; the highest emulsifying activity (60.0 ± 4.0%) was obtained in the cell-free supernatants after 5 days of growth. Camargo-de-Morais et al. [36] studied the production of a glycolipid bioemulsifier by a *Penicillium citrinum* isolate growing in mineral medium with olive oil (1%, v/v) as the carbon source; in this case, the bioemulsifier production was growth-associated, and reached its maximum at 60 hours of growth. Similar results have been reported for *Curvularia lunata* IM2901 [31] and *Pleurotus ostreatus* D1 [69]. However, bioemulsifier production has been more extensively studied in bacteria and yeasts [32,

70-72]. To the best of our knowledge, this is the first description of bioemulsifier production by a filamentous fungus in a medium supplemented with OMW.

Table 8: Study of bioemulsifier production by the filamentous fungus #28. Surface tension values (mN/m) and emulsifying indexes (%) measured in the cell-free supernatants obtained after growing the isolate at 37 ^{O}C in MA+/- media supplemented with the different substrates for 21 days. The surface tension value of MA (+/-) medium is 69.8 mN/m.

	ST (mN/m)	E24 (%)
OMW +	53.6 ± 0.2	28.0
OMW -	62.4 ± 0.6	40.0
n-hexadecane +	52.0 ± 0.0	16.0
n-hexadecane -	58.5 ± 0.5	16.0
Gas oil +	52.1 ± 0.7	4.0
Gas oil -	57.5 ± 0.5	0.0
Paraffin +	53.9 ± 0.4	20.0
Paraffin -	71.2 ± 1.4	0.0
Soybean oil +	54.1 ± 0.2	4.0
Soybean oil -	62.3 ± 0.3	0.0
CLB crude oil +	71.5 ± 0.2	0.0
CLB crude oil -	71.1 ± 0.1	0.0

As it was demonstrated in the previous assays, the filamentous fungus #28 produces a bioemulsifier growing in culture media (MA+ and MA-) supplemented with 10% (v/v) of OMW. In order to try to optimize bioemulsifier production by this isolate, different assays were performed using the medium MA- supplemented with OMW at different concentrations (between 10 and 50% (v/v)) (Figure 11). The cultures were performed at 37 ^{O}C and 180 rpm. Regarding the surface tension (Figure 11A), after 7 days of growth, the lowest values were obtained with the media containing 40 and 50% of OMW (49.6 ± 0.2 and 46.5 ± 0.9 mN/m, respectively). However, after 14 days, the surface tension values in all the media were around 60.5 mN/m. In the medium containing 40% of OMW, the surface tension decreased again after 21 days of growth, reaching its minimum value: 36.7 ± 0.5. mN/m. Also in the media supplemented with 30 and 50% of OMW a slight decrease in the surface tension values was observed at the end of the fermentation.



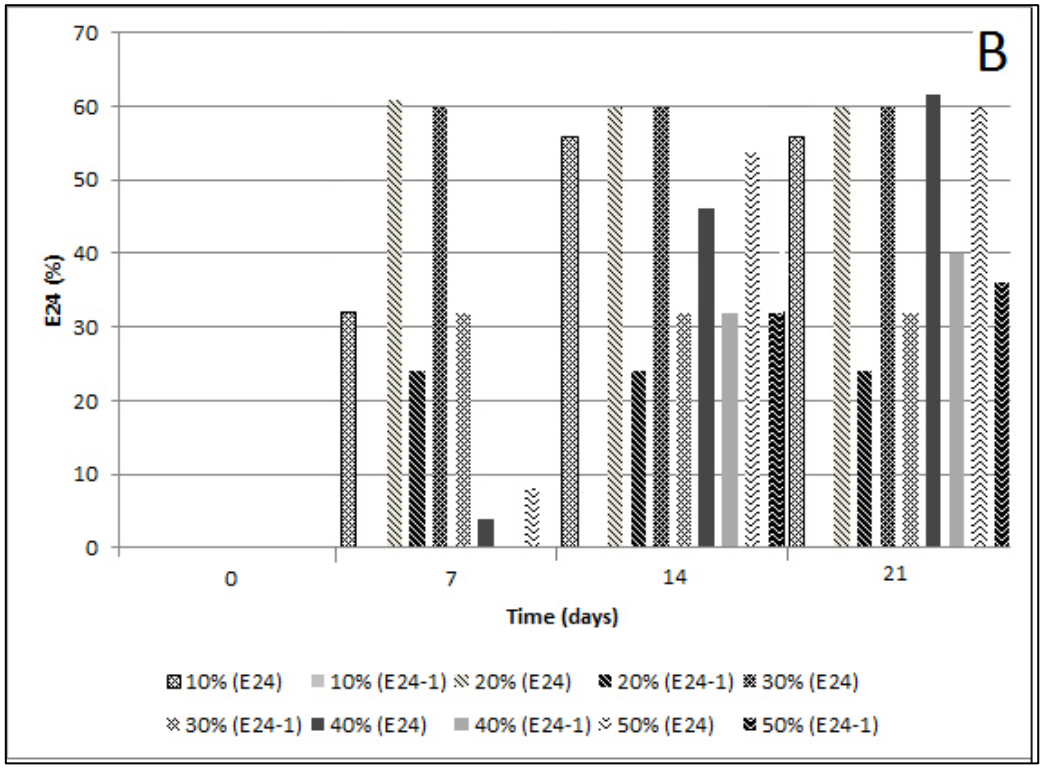


Figure 11: Surface tension values (mN/m) (A) and emulsifying indexes (%) (B) obtained in cultures performed with the filamentous fungus #28 grown in MA- medium supplemented with different OMW concentrations (between 10 and 50%). The cultures were performed at 37 ^{O}C and 180 rpm. Results represent the average of two independent experiments ± standard deviation.

Regarding the emulsifying activity, in order to study the evolution of bioemulsifier production along the time, the emulsifying indexes were calculated for the cell-free supernatants without dilution and 10 times diluted with demineralised water. As it can be seen from Figure 11B, the bioemulsifier production profile depends on the concentration of OMW present in the culture medium. In the medium containing 10% of OMW, the emulsifying index obtained at the 7th day was 32.0%, increasing up to 56.0% at the 14th day, and then remained constant until the end of the fermentation; in this case, no emulsifying activity was observed in the 10 times diluted supernatants. However, in the media containing 20 and 30% of OMW, the emulsifying indexes obtained with the cell-free supernatants without dilution (60.0% in both cases) and 10 times diluted (24.0 and 32.0%, respectively) remained constant from the 7th day of fermentation until the 21th day. Regarding the media containing 40 and 50% of OMW, there is a clear increase in the emulsifying activity along the time, suggesting that the bioemulsifier is produced continuously along the fermentation. The highest emulsifying activities were obtained with the medium supplemented with 40% of OMW after 21 days of growth: 62.0% and 40.0% in the cell-free supernatant without dilution and 10 times diluted, respectively. The fact that in the media containing the highest OMW concentrations more time is required for bioemulsifier production, suggests that the OMW contains compounds that may inhibit its production (i.e., phenolic compounds, which exhibit phytotoxic and antimicrobial activities [73]).

3.3.2.2. Bioemulsifier recovery

Two different techniques were assayed to recover the bioemulsifier produced by the filamentous fungus #28: acetone and ethanol precipitation. For these assays, cultures were performed using the medium MA- supplemented with 10, 20 and 30% (v/v) of OMW, at 37 ^{O}C and 180 rpm for 3 weeks. The emulsifying indexes obtained in these assays were very similar to those shown in Figure 11. At the end of the fermentation, the cell-free supernatants were subjected to precipitation with acetone or ethanol. In order to study if these techniques allowed the recovery of the bioemulsifier, the precipitates obtained were dissolved in the same volume of demineralised water than volume of the cell-free supernatant used to perform the precipitation, and their emulsifying activities were measured (Table 9).

Table 9: Emulsifying indexes (%) obtained with the products recovered from the cell-free supernatants after precipitation with acetone or ethanol. The cell-free supernatants used were obtained from cultures performed with the filamentous fungus #28 in MA- medium supplemented with 10, 20 and 30% (v/v) of OMW, at 37 ^{O}C and 180 rpm for 3 weeks.

%OMW (v/v)	Acetone		Ethanol	
	E_{24}	E_{24}^{-1}	E_{24}	E_{24}^{-1}
10	32	0	36	0
20	48	24	48	32
30	62	36	60	32

From the emulsifying indexes shown in Table 9, it can be concluded that both recovery techniques offered similar results with the three media assayed. Comparing the emulsifying indexes obtained with the precipitates with those obtained using the cell-free supernatants (Figure 11), it can be seen that, in the media containing 10 and 20% of OMW, some emulsifying activity was lost; however, regarding the medium containing 30% of OMW, almost the same emulsifying activity was observed with the products obtained through precipitation (both with acetone and ethanol) when compared with the cell-free supernatants, suggesting that almost all the bioemulsifier was recovered.

In order to quantify the amount of bioemulsifier produced, the isolate #28 was grown in MA- medium supplemented with 30% (v/v) of OMW, at 37 ^{O}C and 180 rpm for 14 days. The cell-free supernatants obtained at the end of the fermentation were subjected to precipitation with acetone; the precipitate obtained was dissolved in a minimal amount of demineralised water and freeze-dried. At the end of the process, the product obtained was weighed. The amount of crude bioemulsifier recovered was 10.6 ± 4.1 g/l. The freeze-dried products obtained were dissolved in demineralised water at different concentrations (from 1 to 15 g/l), and their emulsifying activities were calculated. Although in the preliminary assays, this technique allowed the recovery of the bioemulsifier, with the freeze-dried products obtained it wasn't possible to obtain stable emulsions in most of the cases. That can be probably due to the stability of the bioemulsifier after exposition at low temperatures, as it will be discussed later.

3.3.2.3. Bioemulsifier stability

The applicability of a bioemulsifier in several fields depends on its stability at different salinities, temperatures and pH values. The effect of different environmental factors on the activity of the bioemulsifier produced by the filamentous fungus #28 was studied using the cell-free supernatants obtained at the end of the fermentation (21 days) in medium MA-supplemented with 30% of OMW.

The effect of salinity on emulsifying activity was studied by measuring the emulsifying indexes on cell-free supernatants supplemented with different NaCl concentrations (ranging from 10 to 200 g/l) (Figure 12). With the highest NaCl concentrations tested (150 and 200 g/l), emulsifying indexes of 54.0 ± 2.0 and 58.0 ± 2.0%, respectively, were obtained, whereas in the control (without addition of NaCl) the emulsifying index was 56.0%. The results obtained show that this bioemulsifier is very stable, even at high salinities. These results were analysed by one-way ANOVA followed by a multiple comparison test, Tukey's Multiple Comparison Test. The differences observed between the emulsifying indexes at different NaCl concentrations were found to be not statistically significant (p-value < 0.0001).

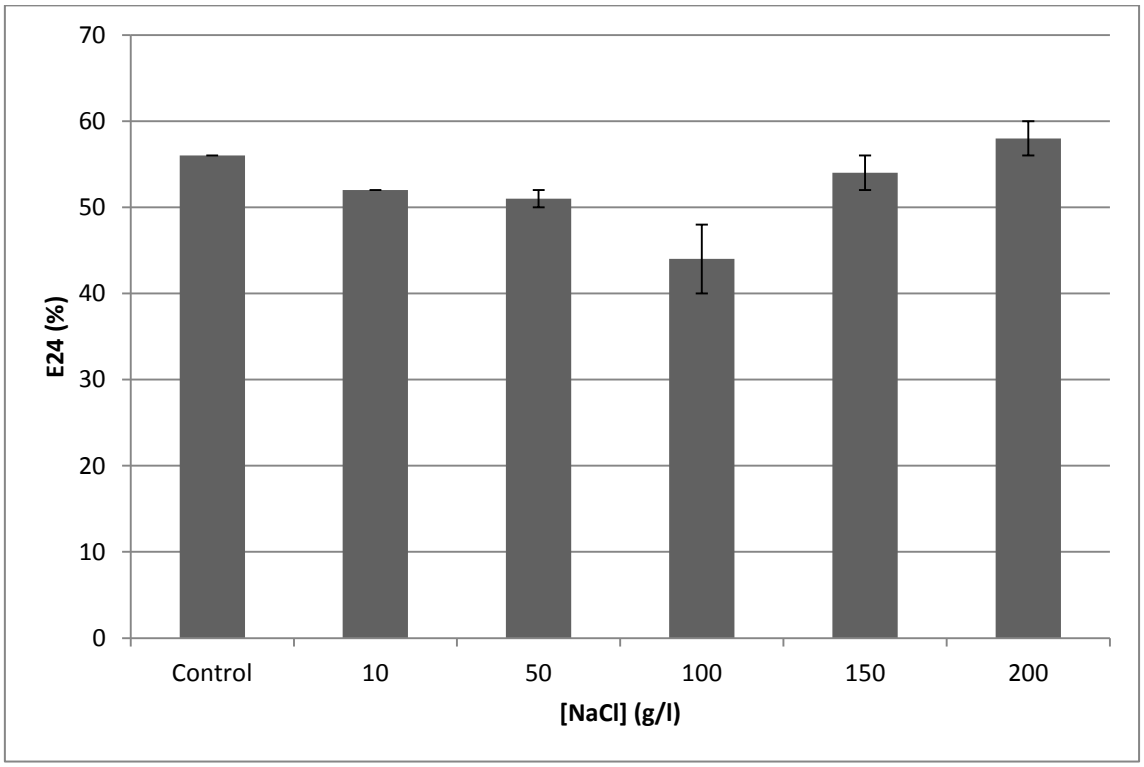


Figure 12: Effect of salinity on the activity of the bioemulsifier produced by the filamentous fungus #28 in MA-medium supplemented with 30% (v/v) of OMW. The emulsifying indexes were performed at room temperature. Results represent the average of two independent assays and error bars represent the standard deviation.

To study the stability of the bioemulsifier at high and low temperatures, the culture broth supernatants were incubated at 121 ^{O}C for 20min, and at -20 or -80 ^{O}C for 5 and 10 days. The emulsifying indexes were measured before and after each treatment (Table 10). From the results obtained, it can be concluded that the incubation at high temperatures has a negative effect on its activity, as the emulsifying indexes were reduced from 56.0 to 16.0%. Also the incubation at -20 ^{O}C exhibited a negative effect, reducing the emulsifying indexes up to 46.0%. The negative effect of incubations at -80 ^{O}C was also considerable, and increases with the incubation time. The instability of this bioemulsifier after exposition to low temperatures can explain the reduced activity of the freeze-dried products.

Table 10: Effect of high and low temperatures on the activity of the bioemulsifier produced by the filamentous fungus #28. The emulsification indexes (%) were performed using the supernatants obtained from cultures of the filamentous fungus #28 grown on MA- medium supplemented with 30% of OMW, after exposition to different temperatures for different time intervals. The emulsifying index before the treatments (control) was 56.0%. Results represent the average of three independent experiments ± standard deviation.

Treatment		E24 (%)
Temperature (^{O}C)	Time	
- 80	10 days	34.0 ± 2.0
- 80	5 days	48.0 ± 0.0
- 20	10 days	46.0 ± 2.0
- 20	5 days	46.0 ± 2.0
121	20 minutes	16.0 ± 8.0

To study the stability at different pHs, samples of the cell-free supernatants obtained at the end of the fermentation (pH 6.5) were adjusted to different pH values and, subsequently, their emulsifying activities were calculated. As it can be seen from the results obtained (Figure 13), the emulsifying activity was quite stable at different pH values (except at pH 10). The highest emulsifying indexes were obtained at pH 2 and 4 (60.0%). For the other pH values, emulsifying indexes around 50% were obtained, except at pH 10, where, surprisingly, no emulsifying activity was observed. The differences observed between the emulsifying indexes obtained at pH 4 and pH 7, as well as at pH 10 compared with the other pH values, were found to be statistically significant (p-value = 0.0046).

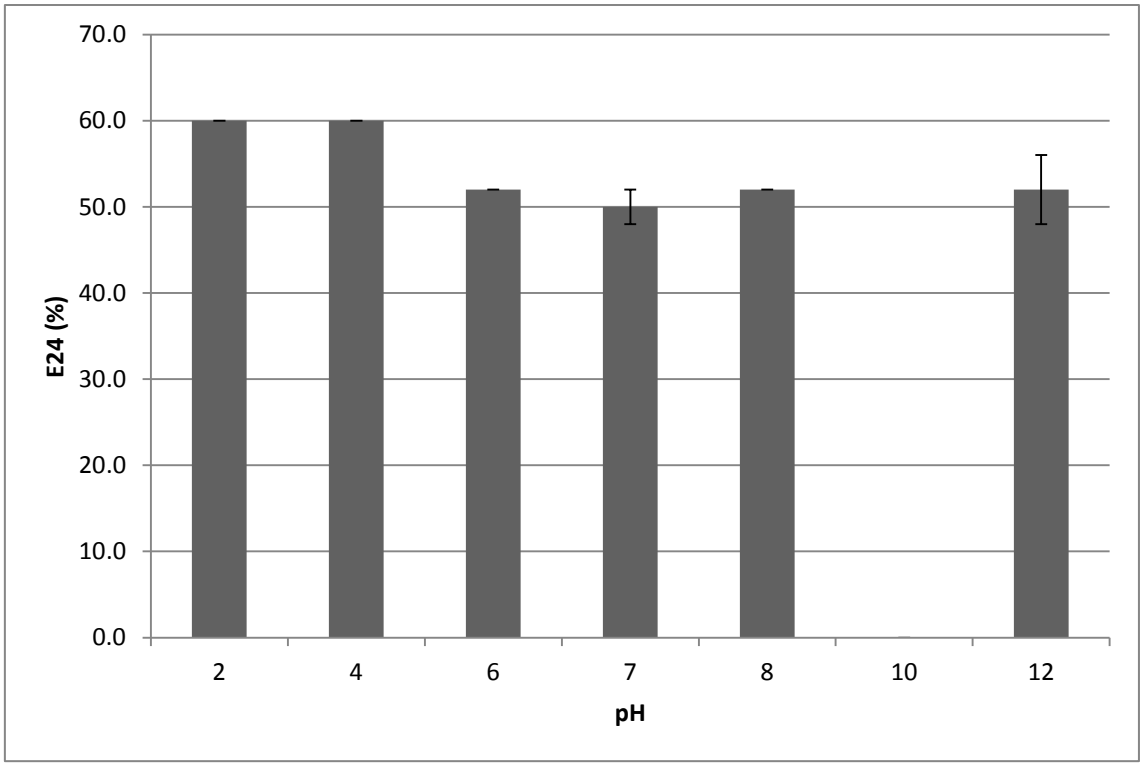


Figure 13: Effect of pH on the activity of the bioemulsifier produced by the filamentous fungus #28 in MA- medium supplemented with 30% (v/v) of OMW. The emulsifying indexes were performed at room temperature. Results represent the average of two independent assays and error bars represent the standard deviation.

The stability of the bioemulsifier produced by the filamentous fungus #28 at different pH values, salinities and temperatures is an important factor for its applicability in different fields.

This bioemulsifier resulted more stable at high NaCl concentrations when compared with other previously described. Camacho-Chab et al. [30] reported a maximum emulsifying activity for the emulsifier produced by *Microbacterium* sp. MC3B-10 at a NaCl concentration of 3.5 mg/ml, whereas at 10 mg/ml, the emulsifying activity reached its minimum value. Also the bioemulsifier produced by an *Aeromonas* spp. strain maintained its emulsifying activity only at NaCl concentrations up to 5 mg/ml [74]. Similar results were reported by other authors [35, 37, 75].

Regarding the effect of temperature, the bioemulsifier produced by *Pseudomonas nitroreducens* TSB.MJ10 was stable at temperatures between 20 and 90 ^{O}C [32] For temperatures below 20 ^{O}C and above 90 ^{O}C a negative effect in the emulsifying activity was observed. The activity of the bioemulsifier produced by *Streptomyces* sp. MAB36 was negatively affected by incubation at temperatures between 30 and 65 ^{O}C [75].

Emulsifiers produced by microorganisms have been widely studied regarding their stability in order to find novel applications in which extreme conditions are commonly used. Colin et al. [37] reported a high stability of bioemulsifiers produced by *Amycolatopsis tucumanensis* DSM 45259 at alkaline conditions, specifically at pH values ranging from 6 to 12. Ilori et al. [74] also reported that the activity of a bioemulsifier produced by an *Aeromonas* spp. strain was stable over a wide range of pH values (from 5 to 9) with a maximum activity at pH 8.0. Luna-Velasco et al. [35] reported that the bioemulsifier produced by a *Penicillium* sp. isolate remained active over a broad pH range (from 3 to 9), with a maximum activity at pH 8.0.

The composition of a bioemulsifier is determined by the nature of the producer microorganism, the composition of the culture medium and the culture conditions. That variable composition confers specific physico-chemical properties and several applications to the different bioemulsifiers [35].

3.3.2.4. OMW: toxicity and degradation of phenolic compounds by the filamentous fungus #28

To evaluate the toxicity of OMW against the filamentous fungus #28, MA- agar plates supplemented with 10, 30 and 50% (v/v) of OMW were prepared. 30µl of a mycelium suspension of this isolate were placed in the centre of each plate. The plates were incubated at 37 ^{O}C, and the growth was calculated by measuring the diameter of the mycelium after 4 and 8 days (Figure 14).

As it can be seen from the results obtained (Figure 14), after 4 days, a similar growth was observed in the medium without OMW and the one containing 10% of OMW (almost 14 mm). However, higher OMW concentrations exhibited a negative effect on growth: in the medium supplemented with 30% of OMW, the diameter measured at the 4th day was 6.4 mm. However, after 8 days, the profile observed was different. In this case, the highest growth was observed in the medium containing 10% of OMW, followed by the medium supplemented with 30% of OMW, both of them higher than the medium without OMW. In the medium containing 50% of OMW, no visible growth was observed, neither after 4 or 8 days. According with these results, it seems that OMW doesn't have a negative effect on the growth of this isolate at concentrations up to 30%. Martirani et al. [76] studied the capability of the white-rot fungus *Pleurotus ostreatus* to survive in

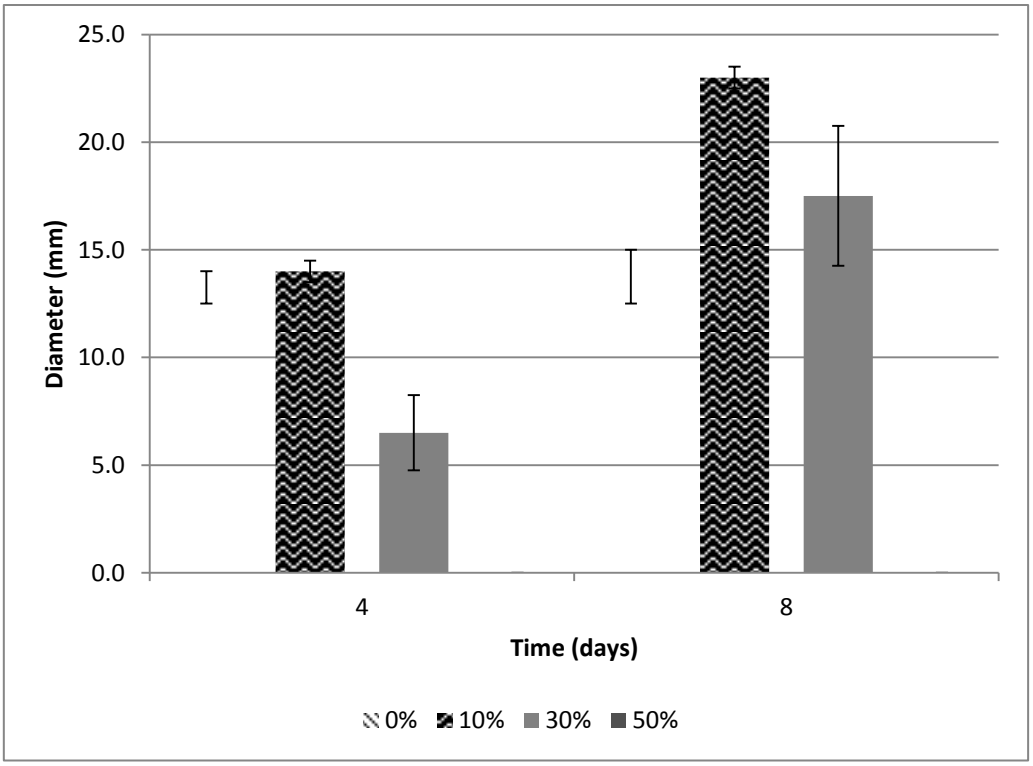


Figure 14: Diameter of the mycelium of the filamentous fungus #28 grown in plates of MA- medium supplemented with 10, 30 and 50% of OMW at 37 ^{O}C. MA- medium without OMW was used as control (0%). Results represent the average of four independent experiments ± standard deviation.

the presence of OMW, and they found that its growth was inhibited at OMW concentrations higher than 20%. In our case, the higher growth observed in the media supplemented with 10 and 30% of OMW after 8 days when compared with the control may be due to some extra nutrients provided by the OMW. To try to study this phenomenon, the carbohydrate and protein concentrations, as well as the concentration of phenolic compounds in the OMW used in this work were determined (Table 11). As it can be seen, the OMW contains about 10 g/l of carbohydrates and 0.5 g/l of protein, which can contribute to the higher growth observed in some of the media supplemented with OMW when compared with the control.

Several factors influence the composition (qualitative and quantitative) of OMW, such as the type of olives used and their maturity, the climacteric conditions, the region, the farming methods and, specially, the technology used for oil extraction [77].

Table 11: Concentration of carbohydrates, protein and phenolic compounds (g/l) in the OMW used in this study.

Parameter	Concentration (g/l)
Carbohydrates	10.25
Protein	0.46
Phenolic Compounds	1.50

As previously mentioned, the toxicity of OMW is attributed to the presence of phenolic compounds, and its direct disposal may pollute both land and aquatic environments [78]. Phenols present in OMW are unstable and tend to polymerize during storage into condensed high-molecular-weight polymers that are difficult to degrade. For that reason, the research on the treatment of this wastes is mostly focused on the degradation of this class of compounds [79]. In order to study if the filamentous fungus #28 exhibits the ability of degrading the phenolic compounds present in OMW, their concentration was evaluated along the time in cultures performed in MA- medium supplemented with 30% of OMW. The cultures were performed in flasks, at 37 ^{O}C and 180 rpm (Figure 15).

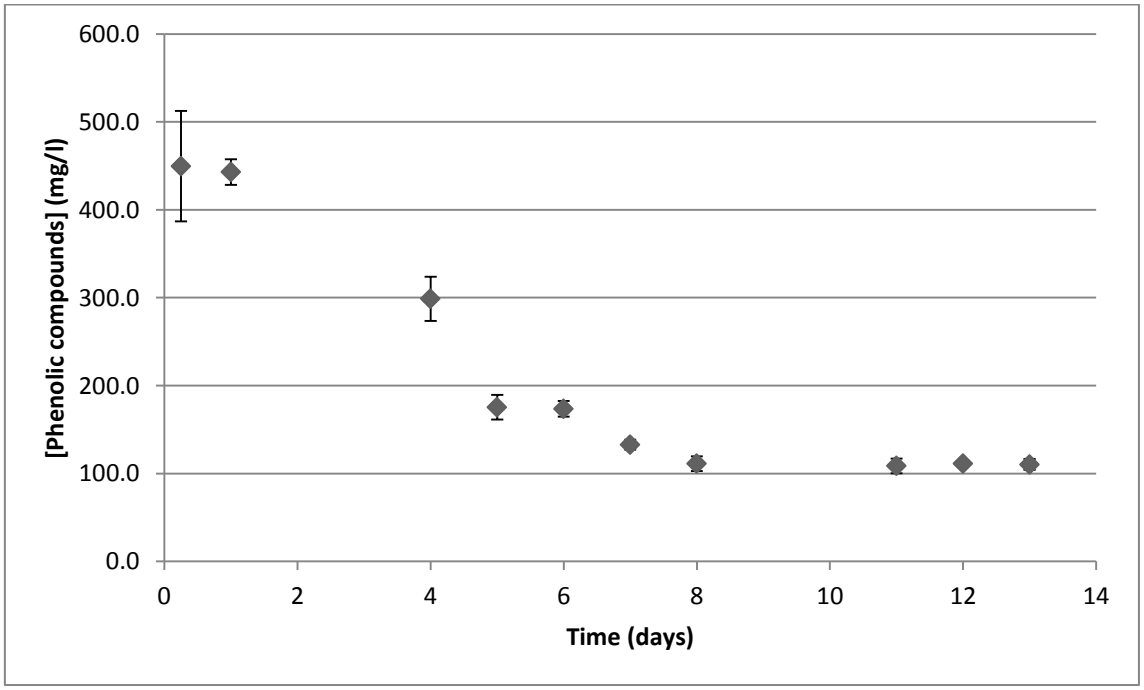


Figure 15: Evolution of phenolic compounds concentration along the time in cultures performed with the filamentous fungus #28 grown in MA- medium supplemented with 30% of OMW. The cultures were performed at 37 ^{O}C and 180 rpm. Results represent the average of three measurements ± standard deviation.

As it can be seen in Figure 15, the initial concentration of phenolic compounds in the medium (449.6 ± 62.8 mg/l) was reduced by 75% after 8 days of growth (111.3 ± 8.3 mg/l), and from this time point until the end of the fermentation (13 days) remained constant. These results demonstrate the ability of this isolate of growing and producing a bioemulsifier in culture media supplemented with OMW and, at the same time, degrading great part of the phenolic compounds present, constituting a potential alternative approach in the treatment of OMW.

Martirani et al. [76] demonstrated that *Pleurotus ostreatus* removes phenolic compounds and detoxifies OMW (diluted to a concentration of 10%) without addition of other nutrients, reducing the concentration of phenolic compounds by 90% after 100 hours of incubation. However, worst results were reported by other authors. *Y. lipolytica* ACA-DC 50109 growing on OMW-based media reduced the phenolic content of the residue by ~15% (w/w) [80]. Scioli and Vollaro [81] cultivated *Y. lipolytica* ATCC 20255 on low phenolic content OMW, and despite a noticeable biomass and lipase production, the phenolic content wasn't reduced at all. Lanciotti et al. [82] used undiluted OMW containing 0.7 g/l of phenolic compounds to grow different *Y. lipolytica* strains, and some of the strains couldn't reduce the concentration of phenolic compounds.

According with the results reported by Crognale et al [83], OMW can be considered an interesting substrate for different biotechnological applications (e.g. production of different metabolites); furthermore, its characteristics can be improved through different bio-treatments, for its use, for example, as a potential fertiliser. However, the applicability and reliability of fermentation processes using OMW depend, not only on the adaptability and versatility of microbial strains, but also on the waste manipulations required, such as addition or removal of specific compounds. In the last years, the valorisation of OMW for the production of added-value products, including single cell protein, ethanol, citric acid or methane, among others, using different microorganisms, has been attempted [83-87].

The results herein obtained demonstrated that the filamentous fungus #28 is a good alternative for OMW treatment, with the advantage that, simultaneously, a bioemulsifier, that can be useful for several industrial applications, is produced.

3.3.2.5. Identification of the filamentous fungus #28

Due to the interesting properties exhibited by the filamentous fungus #28, more studies were performed for its identification. First of all, its macroscopic and microscopic morphology was studied (Figures 16 and 17).

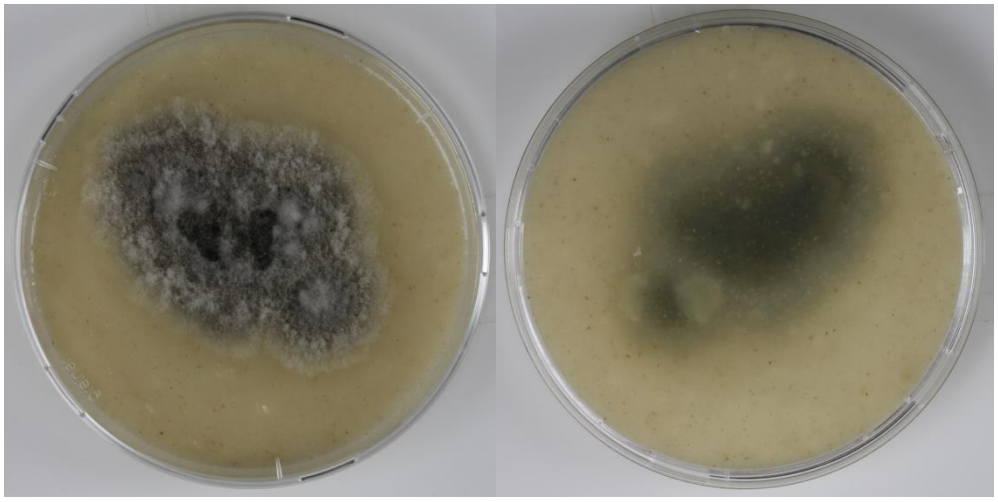


Figure 16: Macroscopic view (front and reverse) of the filamentous fungus #28 grown in Oatmeal Agar for 7 days at 30 ºC. Picture provided by Micoteca da Universidade do Minho (MUM).

The colonies grown in Oatmeal Agar (OA) at 30 ºC are fast growing, greyish-white with a greyish-black reverse (Figure 16). The colonies have expanding flat, moist with depressed, cobweb-like aerial mycelium, olivaceous grey to blackish. The optimum temperature for growth is 30-37 ºC.

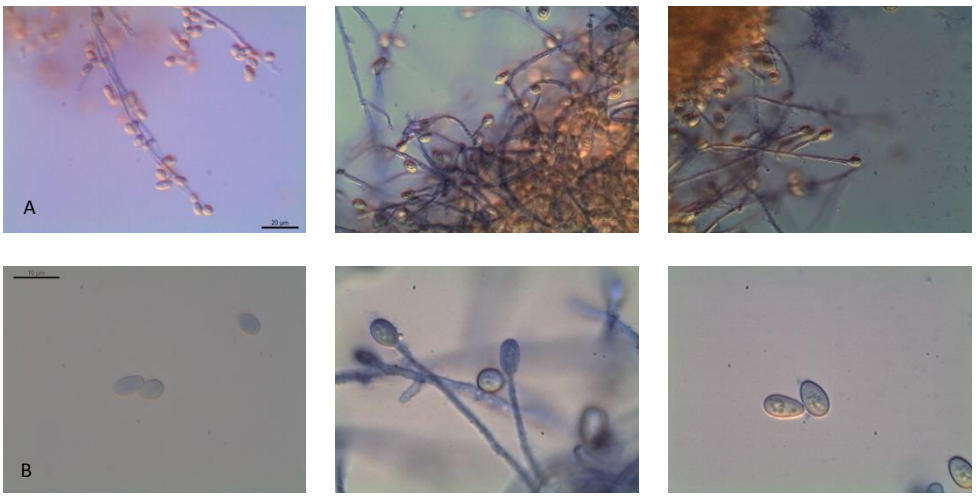


Figure 17: Microscopic pictures of the filamentous fungus #28 after 7 days of growth at 30 ºC. (A): 40x10; (B): 100x10. Pictures provided by Micoteca da Universidade do Minho (MUM).

From the microscopic analysis, numerous single-celled, pale-brown, broadly clavate to ovoid conidia (4-9 x 6-10 mm), rounded above with truncate bases were observed. Conidia are borne singly or in small groups on elongate, simple or branched conidiophores or laterally on hyphae (Figure 17).

For a molecular characterization, the ITS region was amplified using the oligonucelotides ITS1 and ITS4. The 610 bp fragment obtained was sequenced and compared with sequences in the GenBank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) using the nucleotide-nucleotide blast (BLASTn) network service. Multiple sequence alignments were carried out using ClustalW and a consensus neighbour-joining tree was designed using the Molecular Evolutionary Genetics Analysis (MEGA) Software version 5.1. The sequence showed 99% similarity with *Pseudallescheria boydii*, *Pseudallescheria apiosperma* and *Scedosporium apiosperma*. The phylogenetic tree obtained is shown in Figure 18.

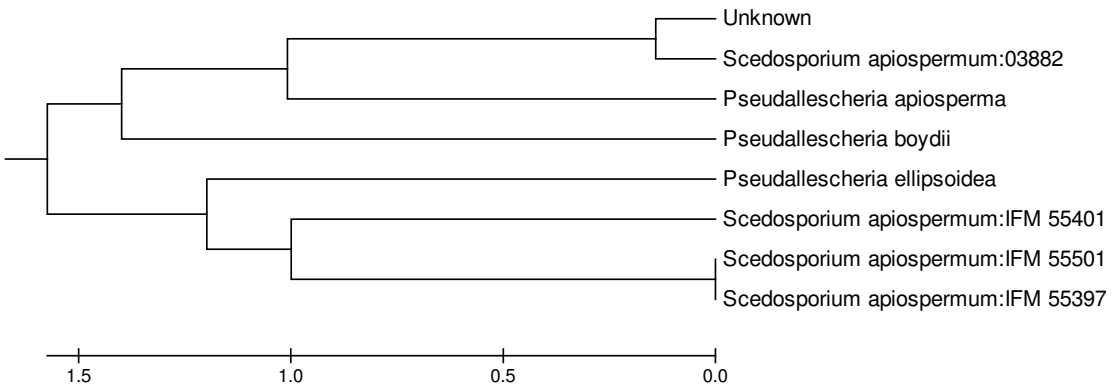


Figure 18: Phylogenetic neighbour-joining tree based on the sequence of the ITS region from the filamentous fungus #28 (*unknown*). The tree was constructed using sequences of comparable regions of the ITS regions available in the GenBank database of the National Center for Biotechnology Information (NCBI).

Significant taxonomic developments have taken place in *Pseudallescheria* during the last years. The teleomorph is currently referred to as *Pseudallescheria* spp; however as all the species of *Pseudallescheria* have *Scedosporium* spp. anamorphs, it is presumptive to use the teleomorph name to describe this fungus without seeing cleistothecia (ascocarps). By observation of the Figure 18, the data reported that the filamentous fungus #28 is an anamorph, namely *Scedosporium* spp.

However, to distinguish between *Scedosporium boydii* and *Scedosporium apiosperma*, it will be necessary to obtain the sequences of the tubulin (BT2 and TUB) and calmodulin (CAL) genes, as the information provided by the sequence of the ITS region is not enough to separate both species [88].

3.4. Screening of enzyme-producing microorganisms

Enzyme production (laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP)) by the filamentous fungi #21, #25 and #28 was evaluated in the cell-free supernatants obtained along the fermentation from cultures performed in the media MA (+/-), BRM (+/-) and Wunder (+/-). The cultures were performed at 37 ^{O}C and 105 rpm for 21 days. According with the results obtained, none of those enzymes was produced in these media.

The production of those enzymes in the filamentous fungus #28 was also evaluated in the medium MA- supplemented with 30% of OMW along the time. However, as in the previous assays, none of the enzymes was detected.

It has been reported that, in some cases, the degradation of phenolic compounds and the biodegradation of OMW by filamentous fungi is associated to the production of ligninolytic (and other) enzymes. Polymeric phenols present in OMW have a lignin-like structure, being their most recalcitrant fraction, and are mainly responsible for the typical colour of OMW [89, 90]. Lignin is a heterogeneous group of tridimensional phenol polymers which, together with cellulose and hemicellulose, constitute fibrils in the plant cell walls. Tissues containing lignin are difficult to degrade, because of its complex irregular structure. Martirani et al. [76] reported decolourisation of OMW by *Pleurotus ostreatus* and the treatment of OMW with purified phenol oxidase. Other authors reported that LiP has a crucial role in this process [91-93]. *Phanerochaete flavido-alba* displayed high decolourisation potential under nitrogen-limiting conditions after flushing with pure oxygen; MnP was the main enzymatic activity detected, together with small amounts of laccase [94]. In the presence of OMW, *Pleurotus* sp. strains [95] and *Lentinus edode* [96] showed enhanced laccase activity. *Lentinula edodes* also promoted a high decolourisation of OMW, although no evident correlation was found between this process and the enzymatic (laccase and MnP) activities [97]. Furthermore, an extensive removal of monomeric phenols from OMW by immobilized laccase has been reported [98].

3.5. Treatments to improve the quality of Mukhaizna crude oil

Among the different microorganisms isolated from the oil sample CLB, three *Pseudomonas* sp. strains (#1, #2 and #3) and three filamentous fungi (#21, #25 and #28) were selected to study their applicability in improving the quality of Mukhaizna crude oil. The *Pseudomonas* sp. isolates were selected due to their ability of producing biosurfactants, which can be useful to increase the availability of the oil to the microorganisms. The fungi were selected because they usually produce ligninolityc enzymes that can be useful to degrade some recalcitrant compounds present in the crude oil. Furthermore, other microorganisms previously isolated from crude oil samples obtained from two Brazilian oil fields (PTX-9 and PTX-11) [38] were assayed.

3.5.1. Treatments performed with *Pseudomonas* sp. strains isolated from the oil sample CLB

The *Pseudomonas* sp. strains #1, #2 and #3, isolated from the oil sample CLB, were identified as biosurfactant producers in the previous assays. The treatments with these microorganisms were performed in 50 ml flasks containing 30 ml of MSS+ or MSS- media and 5 g of Mukhaizna crude oil. The assays were performed at 37 ^{O}C and 105 rpm for 25 days (medium MSS+) or 30 days (medium MSS-). At the end of the assays, the oil was recovered and its viscosity and density were measured. Furthermore, the surface tension and the emulsifying activity of the cell-free supernatants were measured to evaluate the production of biosurfactants by these isolates in these conditions (Table 12).

Table 12: Viscosity (η) and API degree of Mukhaizna crude oil after treatment with *Pseudomonas* sp. #1, #2 and #3 in MSS+ (25 days) and MSS- (30 days) at 37 ^{O}C and 105 rpm. Surface tension values and emulsifying indexes of cell-free supernatants obtained at the end of the treatment. The API degree of Mukhaizna crude oil is 15.2^{O}, and its viscosity at 50 ^{O}C 2430 mPa.s. The surface tension of MSS (+/-) is 69.4 ± 2.1 mN/m.

Isolate	MSS -				MSS +			
	η 50^{O} C (mPa.s)	API^{O}	ST (mN/m)	E24 (%)	η 50^{O} C (mPa.s)	API^{O}	ST (mN/m)	E24 (%)
#1	4458	14.3	52.8 ± 0.3	32.0	4436	14.8	54.9 ± 1.2	15.3
#2	4621	14.3	55.8 ± 0.5	16.0	4564	14.3	55.0 ± 0.7	19.2
#3	4450	14.0	55.0 ± 0.8	16.0	4359	13.9	56.0 ± 1.6	12.0

As it can be seen in Table 12, in all the cases the API degree measured after the different treatments was lower than the corresponding to the original oil, and its viscosity increased. In consequence, the quality of the oil wasn't improved. In assays performed in the beginning of this project, it was shown that the incubation of Mukhaizna crude oil with water resulted in an increase in its viscosity, and consequently, the API degree was reduced, due to the incorporation of water in the oil (*data not shown*). That can explain why, in all the assays performed, where the crude oil is in contact with the culture medium for several days, the viscosity of the oil increased and its API degree decreased.

The surface tension of the culture media was slightly reduced in all the assays, and in all the cases some emulsifying activity was detected. However, these results suggest a lower biosurfactant production when compared with other assays performed with these isolates, as described previously.

Other set of experiments was performed with these isolates in 250 ml flasks containing 100 ml of MSM- or MSM supplemented with 6.0 g/l of glucose ($MSM_{1/2}$), and 7 g of Mukhaizna crude oil. These treatments were performed for 30 days. The main difference compared with the previous assays was that every 7 days, the culture medium was removed and replaced by fresh medium in order to guarantee the availability of nutrients to the microorganisms. In the assays performed with the medium $MSM_{1/2}$, the supernatants recovered every 7 days exhibited a dark colour, due to the presence of oil fractions released from the oil by the microorganisms (Figure 19).

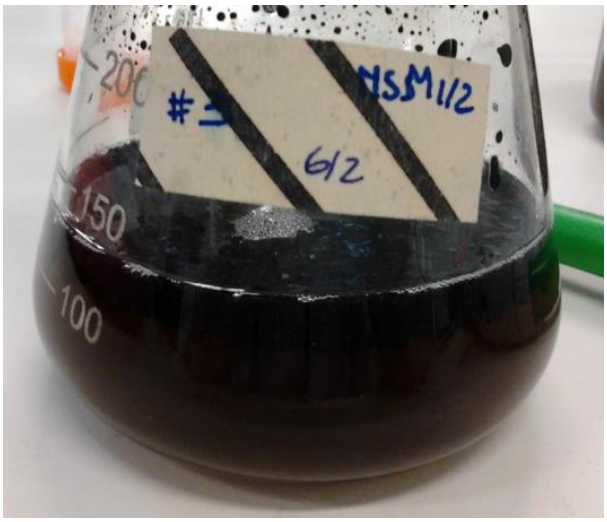


Figure 19: Treatment performed with *Pseudomonas* sp. #3 with $MSM_{1/2}$ and Mukhaizna crude oil.

At the end of the assays, the oil was recovered and its viscosity and density were measured (Table 13). As it can be seen, regarding the treatments performed using the medium $MSM_{1/2}$, the API degree measured after the different treatments decreased when compared with the original oil, and its viscosity increased. The surface tension of the culture medium recovered at the end of the assays was reduced to values as low as 30-31 mN/m, and a high emulsifying activity (60.0%) was observed in all the assays, which suggests a considerable biosurfactant production in these conditions. As shown in Figure 20, the emulsifying indexes obtained in these assays were oil-in-water emulsions, contrary to the emulsifying indexes obtained with these isolates in the previous assays, which were water-in-oil emulsions. That can be due to the release of crude oil particles to the supernatant. An oil-in-water emulsion (type I emulsion) is formed when the water–surfactant interaction is stronger than the oil–surfactant interaction (R < 1) [33].

Table 13: Viscosity (η) and API degree of Mukhaizna crude oil after treatment with *Pseudomonas* sp. #1, #2 and #3 in $MSM_{1/2}$ at 37 ^{O}C and 180 rpm during 30 days. Surface tension values and emulsifying indexes of cell-free supernatants obtained at the end of the treatment. The API degree of Mukhaizna crude oil is 15.2^{O}, and its viscosity at 50 ^{O}C 2430 mPa.s. The ST and $ST^{-1}$of $MSM_{1/2}$ is 66.3 ± 0.54 and 70.8 ± 4.0 mN/m, respectively.

Isolate	η 50^{O} C (mPa.s)	API^{O}	ST	ST^{-1}	E24 (%)
			(mN/m)	(mN/m)	
#1	-	11.4	30.5 ± 0.2	44.6 ± 1.2	60.0
#2	9764	12.6	31.4 ± 0.8	43.2 ± 0.9	60.0
#3	14371	11.8	31.6 ± 0.6	44.2 ± 1.0	60.0

The treatments using the medium MSM- were not effective. The surface tension values were high (~61.0 mN/m) and the emulsification indexes were very low (≤ 4.0%). Furthermore, in those treatments, it wasn't possible to measure the API degree or the viscosity of Mukhaizna crude oil at the end of the assays.

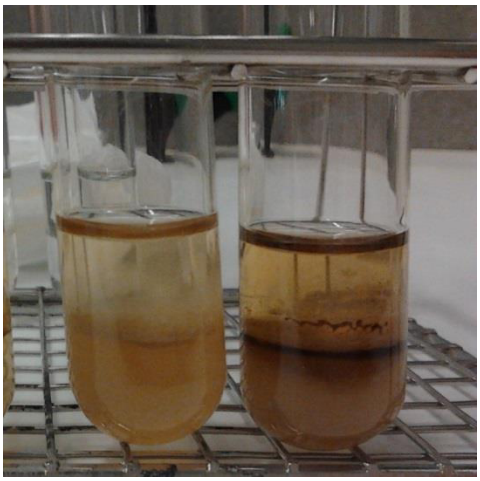


Figure 20: Oil-in-water emulsions formed with the supernatants obtained after the treatment of Mukhaizna crude oil with *Pseudomonas* sp. #1 in $MSM_{1/2}$ medium at 37 ^{O}C and 180 rpm during 30 days.

3.5.2. Treatments performed with the filamentous fungi isolated from the oil sample CLB

The treatments with the filamentous fungi isolated from the oil sample CLB (#21, #25 and #28) were performed in 50 ml flasks containing 30 ml of MSS+ and 5 g of Mukhaizna crude oil, at 37 ^{O}C and 105 rpm for 25 days. As it can be seen in Figure 21, the fungi #21 and #25 exhibited a good growth, making difficult the separation of the oil and the fungi at the end of the assays.

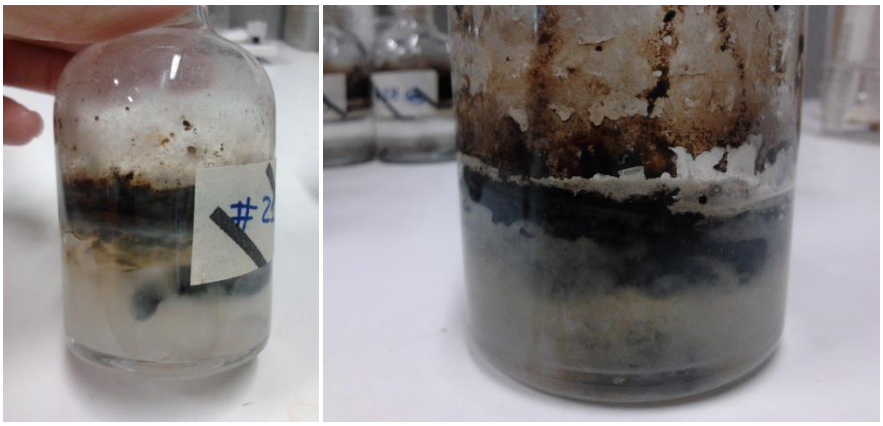


Figure 21: Treatments performed with Mukhaizna crude oil and the filamentous fungi #21 and #25, after 25 days of incubation with the medium MSS+ at 37 ^{O}C and 105 rpm.

At the end of the assays, the oil was recovered and its viscosity and density were measured (Table 14). Regarding the filamentous fungi #21 and #25, it wasn't possible to analyse

the oil due to the difficult in separating it from the fungi. In the case of the fungus #28, as it exhibited a lower growth when compared with the other two, it was possible to recover the oil and analyse it; however, its API degree was reduced and its viscosity was increased when compared with the original oil. Regarding the cell-free supernatants obtained at the end of the treatments, their surface tension values were high, and the emulsifying indexes very low, which indicate that biosurfactants were not produced.

Table 14: Viscosity (η) and API degree of Mukhaizna crude oil after treatment with the filamentous fungi #21, #25 and #28 in MSS+ medium at 37 ^{O}C and 105 rpm for 25 days. Surface tension values and emulsifying indexes of cell-free supernatants obtained at the end of the treatments. The API degree of Mukhaizna crude oil is 15.2^{O}, and its viscosity at 50 ^{O}C 2430 mPa.s. The ST of MSS+ is 69.4 ± 2.1 mN/m.

Isolate	η 50 ^{O}C (mPa.s)	API^{O}	ST (mN/m)	E_{24} (%)
#21	-	-	58.8 ± 1.2	4.0
#25	-	-	56.3 ± 0.8	4.0
#28	3371	14.3	58.1 ± 1.1	8.0

3.5.3. Treatments performed with *B. subtilis* and *Pseudomonas* sp. strains isolated from the oil samples PTX-9 and PTX-11

Four *B. subtilis* (PX191, PX551, PX571 and PX572) and one *Pseudomonas* sp. (PX901) strains isolated from the oil samples PTX-9 and PTX-11 in a previous project were assayed to increase the quality of Mukhaizna crude oil. The assays were carried out in 50 ml flaks containing 30 ml of MSS+ or MSS- media and 5 g of Mukhaizna crude oil, at 37 ^{O}C and 105 rpm, for 25 days. At the end of the assays, the oil was recovered and its viscosity and density were measured (Table 15). As it can be seen, in all the cases, the API degree measured after the different treatments was lower than the corresponding to the original oil, and its viscosity was higher. Regarding the cell-free supernatants, the lowest surface tension values were obtained with the isolates PX191, PX551 and PX572 in MSS+ medium; however, those values were higher than the obtained in previous assays performed with these isolates (*data not shown*), which suggests a low biosurfactant production.

Table 15: Viscosity (η) and API degree of Mukhaizna crude oil after treatment with *B. subtilis* PX191, PX551, PX571, PX572 and *Pseudomonas* sp. PX901 with the media MSS+ or MSS- at 37 ^{O}C and 105 rpm for 25 days. Surface tension values of cell-free supernatants obtained at the end of the treatments. The API^{o} of Mukhaizna crude oil is 15.2, and its viscosity at 50 ^{O}C 2430 mPa.s. The ST of MSS+ is 69.4 ± 2.1 mN/m.

Isolate	MSS -			MSS +		
	η 50 ^{O}C (mPa.s)	API^{o}	ST (mN/m)	η 50 ^{O}C (mPa.s)	API^{o}	ST (mN/m)
PX191	4079	14.5	53.4 ± 1.1	3787	14.5	41.5 ± 0.8
PX551	3761	14.4	52.0 ± 0.8	4712	14.5	40.0 ± 0.5
PX571	4207	14.3	58.4 ± 0.7	4881	13.8	53.0 ± 1.5
PX572	3892	14.6	54.3 ± 1.1	3880	14.4	36.2 ± 0.4
PX901	4236	14.5	56.1 ± 0.8	3790	14.5	45.0 ± 1.2

Other set of experiments was performed with these isolates in 250 ml flasks containing 100 ml of $MSM_{1/2}$ and 7 g of Mukhaizna crude oil, at 37 ^{O}C and 180 rpm for 30 days. Every 7 days, the culture medium was removed and replaced by fresh medium, in order to guarantee the availability of nutrients to the microorganisms. The supernatants recovered every 7 days contained oil fractions, as it can be seen from their dark colour, suggesting that the microorganisms released some oil fractions (Figure 22). The *n*-alkanes degradation of these oil fractions was evaluated through gas chromatography (GC). However, the chromatograms of the oil recovered after the treatments and the original oil were very similar, suggesting that no significant changes occurred in the *n*-alkanes composition.

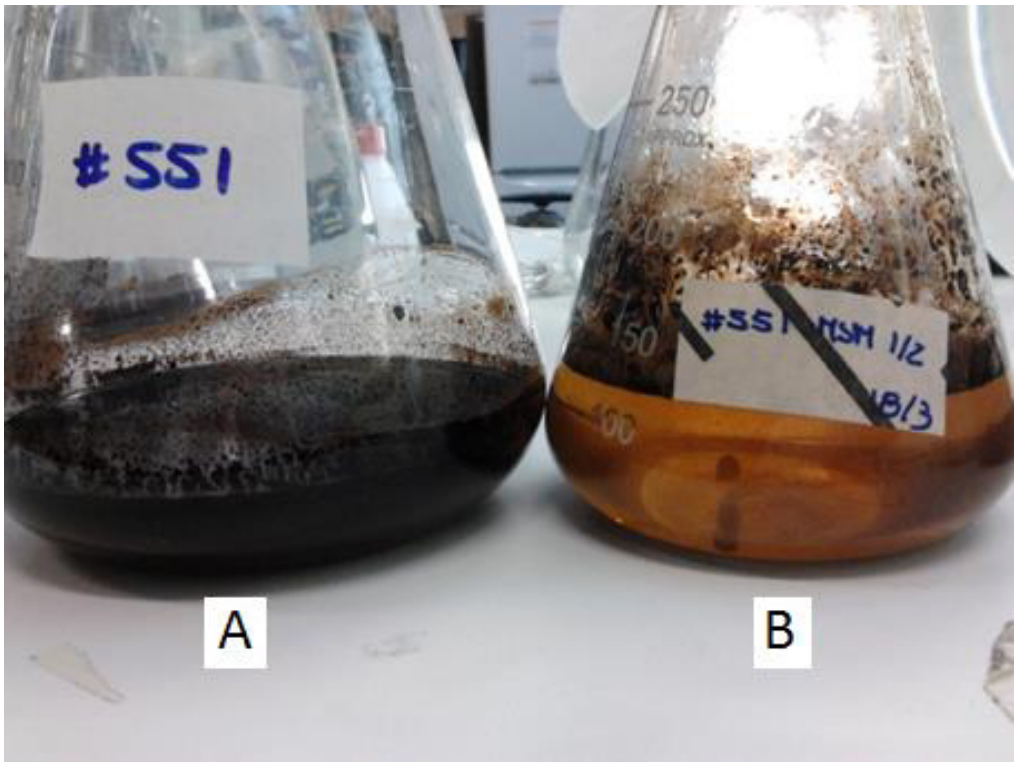


Figure 22: Treatments performed with Mukhaizna crude oil and *B. subtilis* PX551 during 30 days in $MSM_{1/2}$ at 37 ^{O}C and 180 rpm. Culture medium removed (A) and new culture medium (B).

At the end of the assays, the oil was recovered for its analysis. However, it wasn't possible to measure its viscosity or density, which suggests that the quality of the oil wasn't improved. Regarding the surface tension of the supernatants, no significant reductions were observed when compared with the culture medium, and no emulsifying activity was observed (Table 16), which suggests the absence of biosurfactants.

Table 16: Surface tension values and emulsifying activities measured in the supernatants obtained after the treatment of Mukhaizna crude oil with *B. subtilis* PX191, PX551, PX571 and PX572 for 30 days in medium $MSS_{1/2}$ at 37 ^{O}C and 180 rpm. The surface tension of $MSS_{1/2}$ is 69.4 ± 2.1 mN/m.

Isolate	ST (mN/m)	E_{24} (%)
PX191	62.2 ± 0.1	0.0
PX551	46.3 ± 0.7	0.0
PX571	66.3 ± 1.2	0.0
PX572	65.7 ± 0.7	0.0

In conclusion, none of the treatments performed with the different microorganisms tested (bacteria and filamentous fungi) improved the quality of this oil, although some of them exhibited a considerable growth, produced biosurfactants and degraded some of the oil fractions; by the contrary, in all the cases its viscosity was increased and its API degree reduced.

3.6. Additional biotech applications

3.6.1. Microbial Enhanced Oil Recovery

Laboratory studies on MEOR typically use sand-packed columns, which provide a suitable bench-scale approach to evaluate oil recovery for several reasons: it is an economic model; a battery of columns can be set up simultaneously; and they simulate the oil recovery operations usually conducted in reservoirs [99]. In this work, a sand-packed column model was used to study the effect of three filamentous fungi (#21, #25 and #28), isolated from the crude oil sample CLB, on the mobilization of entrapped oil. Furthermore, the additional oil recovered was characterized by measuring its density and viscosity.

The pore volume of the columns was 116.8 ± 8.1 ml, which results in a porosity of 46.7 ± 3.2%. The remaining parameters are shown in Table 17. After the water-flooding process, between 31.1 ± 4.7 and 37.4 ± 3.0% of the OOIP remained entrapped into the columns. The oil recovery assays were performed by inoculating the water-flooded columns with the different filamentous fungi supplemented with the culture medium BRM+. Furthermore, a negative control was performed in the same conditions but without addition of microorganisms. Subsequently, all the columns were incubated at 37 ^{O}C for 14 days. After the incubation time, the columns were subjected to water-flooding again, and the additional oil recovered (AOR) was measured. The results obtained are shown in Table 17.

Table 17: Summary of the results obtained in the sand-packed column assays performed with the filamentous fungi #21, #25 and #28. Results represent the average of two independent experiments ± standard deviation.

	#21	#25	#28	Control
OOIP (ml).	89.4 ± 0.6	88.8 ± 5.2	94.1 ± 6.8	91.1 ± 6.8
S_{oi} (%)	80.9 ± 0.9	79.3 ± 0.9	69.5 ± 5.3	80.5 ± 6.7
OOIP-S_{orwf} (ml)	33.4 ± 2.4	31.2 ± 2.1	31.6 ± 4.3	28.6 ± 5.8
S_{or} (%)	37.4 ± 3.0	35.1 ± 1.8	33.4 ± 2.1	31.1 ± 4.7
S_{ormf} (ml)	7.8 ± 0.4	9.4 ± 0.6	7.5 ± 0.0	6.1 ± 1.3
AOR (%).	23.3 ± 0.6	30.6 ± 0.2	24.2 ± 3.3	21.4 ± 0.8

As it can be seen in Table 17, in the control assays, 21.4 ± 0.8% of the entrapped oil was recovered. In the assays performed with the isolates #21 and #28, the percentage of entrapped oil recovered wasn't much higher (between 23.3 ± 0.6 and 24.2 ± 3.3%). However, in the assays performed with the isolate #25, up to 30.6 ± 0.2% of the entrapped oil was recovered.

Enhanced oil recovery in laboratory assays has been reported by several authors after growing *in situ* different microorganisms. Sarafzadeh et al. [100] reported an additional oil recovery of 10.3% using an *Enterobacter cloacae* isolate; and *Bacillus subtilis* strains allowed recoveries between 14.6-16.9% [39]. However, it is important to take into consideration that the properties of the crude oil and the substrate used in the different studies are very different, which makes difficult the comparison of those data. In our case, the best result was obtained with the filamentous fungus #25, which allowed an additional oil recovery (compared with the control) of 9.2%.

Additional oil recoveries can be attributed to different factors. One of them is biosurfactant production by the microorganisms used, which reduce the interfacial tension of the oil-sand-water system, reducing the capillary forces that entrap the oil in the reservoir and increasing oil mobility. However, in the previous studies performed with these fungi, they didn't show the ability of producing biosurfactants under these conditions, and neither biosurfactant production was observed in the fluids recovered from the sand-packed columns. Other possibility to increase oil recovery is the conversion of the heavy oil fractions, which reduces the viscosity of the oil and, consequently, increases its mobility.

The viscosity of the oil recovered at the end of the assays was measured, and it was observed an increase, when compared with the original oil (Table 18); the increases observed in the oil viscosity can be also explained, as in the case of the Mukhaizna crude oil, by the incorporation of water in the oil. These results suggest that this is not the cause for the additional oil recovery obtained in these assays. In this case, the additional oil recovery can be due to the selective plugging of oil depleted channels in the column [101]. During the first water-flooding process, the injected water uses preferential channels, displacing the oil. When the culture media and the fungi are injected into the column, they will use also those preferential (oil depleted) channels. However, if the filamentous fungi introduced into the column are able of growing in those oil depleted channels, blocking them, when a new water-flooding process takes place, the water will be directed to other ways (oil-rich channels) that were bypassed in the first water-

flooding. As a result, the oil present in those channels can be displaced, increasing oil production.

Table 18: Viscosity and API degree of crude oil before passing through the column (control), after water-flooding, and after the incubation with the filamentous fungi (AOR).

Crude oil	η 50 ºC (mPa.s)	API º
Control	14.4	30
After water-flooding	29.0	26
AOR	56.0	28

Although the selective-plugging theory can explain the additional oil recovered in the assays performed with the filamentous fungi, it requires that those fungi can grow under the limited oxygen conditions that prevail in the sand-packed columns. To study this possibility, the filamentous fungi were grown in 25 ml of BRM (+/-) medium incubated under aerobic (Ae), anaerobic (An) and microaerophilic conditions (Ae/An), at 37 ºC for 12 days; at the end of the assays, the growth, expressed as dry weigh, was calculated for each fungi and condition (Table 19).

Table 19: Dry weigh (mg/L) obtained for the filamentous fungi after growing in BRM (+/-) at 37 ºC during 12 days. The cultures were incubated in three different conditions: aerobic (Ae), microaerophilic (Ae/An) and anaerobic (An).

	BRM	#21	#25	#28
Ae	+	1808	2980	3020
	-	1552	2688	2724
Ae/An	+	1080	1156	608
	-	168	208	516
An	+	328	548	0
	-	184	0	8

As it can be seen in Table 19, the fungi #21 and #25 exhibited growth in all the conditions tested, although the highest growth was observed under aerobic conditions. However, the isolate #28 couldn't grow under anaerobic conditions. Regarding the anaerobic and microaerophilic conditions, the isolate #25 exhibited the highest growth, which is in accordance with the AOR values obtained in the sand-packed columns.

4.General Conclusions and Recommendations

4.1. Conclusions

The aim of the present work was the application of biotech strategies to improve the quality of heavy crude oils using different microorganisms. To reach these purposes several subjects were studied and different applications were tested. The main conclusions drawn from this work are the following:

- ✓ Fourteen bacterial strains and nine filamentous fungi were isolated from the oil sample collected from the oil well CLB;

- ✓ The isolates *Pseudomonas* sp. #1, #2 and #3 were identified as biosurfactant producers, and exhibited significant surface tension reductions and high emulsifying indexes growing in the medium MSM+ at 37 ^{O}C and 180 rpm;

- ✓ The addition of OMW to the culture medium (10%, v/v) had a negative effect on biosurfactant production by *Pseudomonas* sp. #1, #2 and #3;

- ✓ Regarding the biosurfactants produced by the *Pseudomonas* sp. isolates, their recovery through adsorption chromatography using the polystyrene resin Amberlite XAD-2 proved to be more efficient than the acidic precipitation;

- ✓ One filamentous fungus isolated from the oil sample (isolate #28) was found to produce a bioemulsifier;

- ✓ The filamentous fungus #28 was identified as *Scedosporium* spp. using a combination of morphological and molecular studies;

- ✓ The isolate #28 showed the ability of growing and producing a bioemulsifier in culture media supplemented with OMW at different concentrations; the optimum conditions were established in a medium containing 30% (v/v) of OMW, at 37 ^{o}C and 180 rpm.

- ✓ Growing in a medium containing 30% of OMW, the isolate #28 reduced the concentration of phenolic compounds by 75% after 8 days;

- ✓ Acetone and ethanol precipitation showed to be efficient to recover the bioemulsifier in preliminary assays, although further optimization of the recovery process will be necessary;

- ✓ The bioemulsifier was quite stable at different pH values and salinities; however, incubation at high and low temperatures had a negative effect in its activity;

- ✓ The production of ligninolytic enzymes (laccase, LiP and MnP) by the filamentous fungi #21, #25 and #28 wasn't observed;

- ✓ Mukhaizna crude oil treatments performed with the different microorganisms didn't improve its quality, and all of them resulted in an increase in its viscosity and a decrease of its API degree;

- ✓ MEOR assays performed with the filamentous fungi #21, #25 and #28 resulted in additional oil recoveries between 1.9 and 9.2%.

4.2. Recommendations

The results achieved in this work showed that microorganisms isolated from the oil sample CLB have the capacity of producing biosurfactants and bioemulsifiers. Furthermore, strategies with the microorganisms of interest have been applied in the treatment of heavy oil and MEOR. However, further research should be done as suggested bellow:

- ✓ Since the results achieved in the production of biosurfactants by *Pseudomonas* sp. #1, #2 and #3, using a defined medium were very promising, it would be interesting to study the fermentation with low-cost substrates to reduce the production costs. Nevertheless, other strategies involving different variables of the production process can also be explored, as, for example, the supplementation of the low-cost media with trace elements, Fe2+, Mn2+ and Mg2+, incubation at different pH and agitation rates;

- ✓ Another point of interest is the biochemical composition of biosurfactants and bioemulsifiers. In general, they have been characterized as multi-components biosurfactants. Therefore, further purification and characterization steps are required, mainly in the case of the bioemulsifier produced by the filamentous fungus #28, for which information on their structures is still scarce. This characterization of biosurfactants, in terms of structure, would help to better understand their interaction, thus facilitating the prediction of their effects and activities;

- ✓ Regarding the bioconversion assays of heavy crude oil, different combinations can be tested: consortia of different crude-oil degrading microorganisms; combination of crude-oil degrading microorganisms and biosurfactant-producing microorganisms and/or combination of crude-oil degrading microorganisms and enzymes (or enzyme-producing microorganisms). It will be necessary to establish if the different microorganisms can work together, or if, for example, one microorganism produces compounds which inhibit the growth or the activity of other microorganisms (in that case they could not be used together). And the same can be said for the enzymes;

- ✓ Finally, studies in MEOR with filamentous fungi #21, #25 and #28, different nutrient media can be tested. The *Pseudomonas* sp #1, #2 and #3 strains may also be tested for *in situ* and *ex situ* applications.

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6.Appendices

Appendix A – Calibration curves

Calibration curves were performed to carbohydrate, protein and phenolic compounds. In Figure A.1 it is represented the relationship between concentration (mg/l) of one carbohydrate (glucose) and optical density (490 nm).

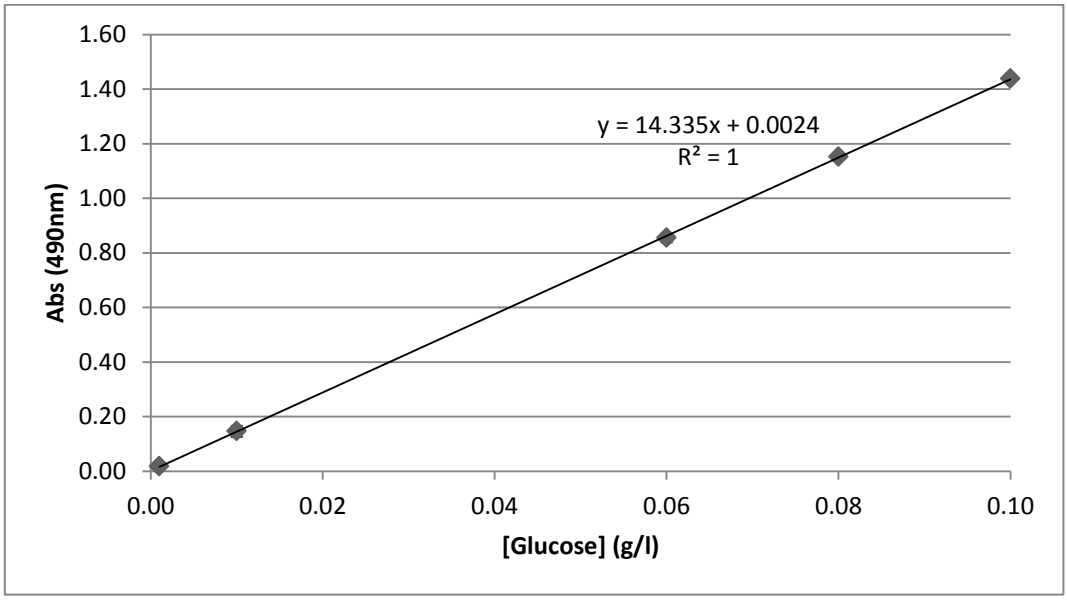

Figure A.1: Calibration curve of carbohydrates concentration.

Calibration curve of protein (Figure A.2) reflect the relationship between BSA concentration (mg/ml) and optical density (595 nm).

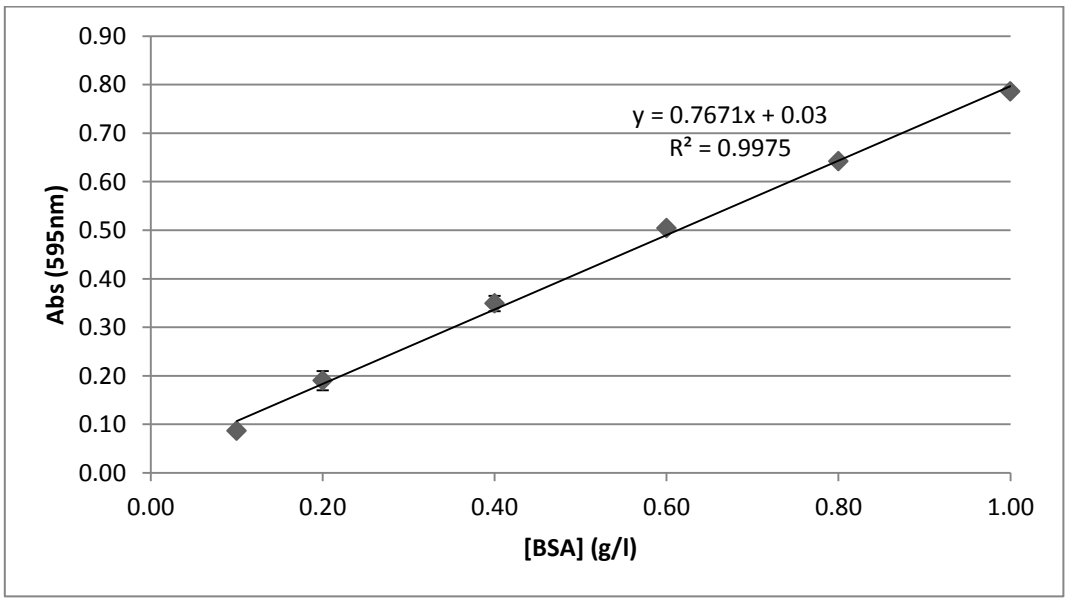

Figure A.2: Calibration curve of proteins concentration.

In Figure A.3 it is represented the relationship between concentration (mg/l) of phenolic compounds (gallic acid) and optical density (700 nm).

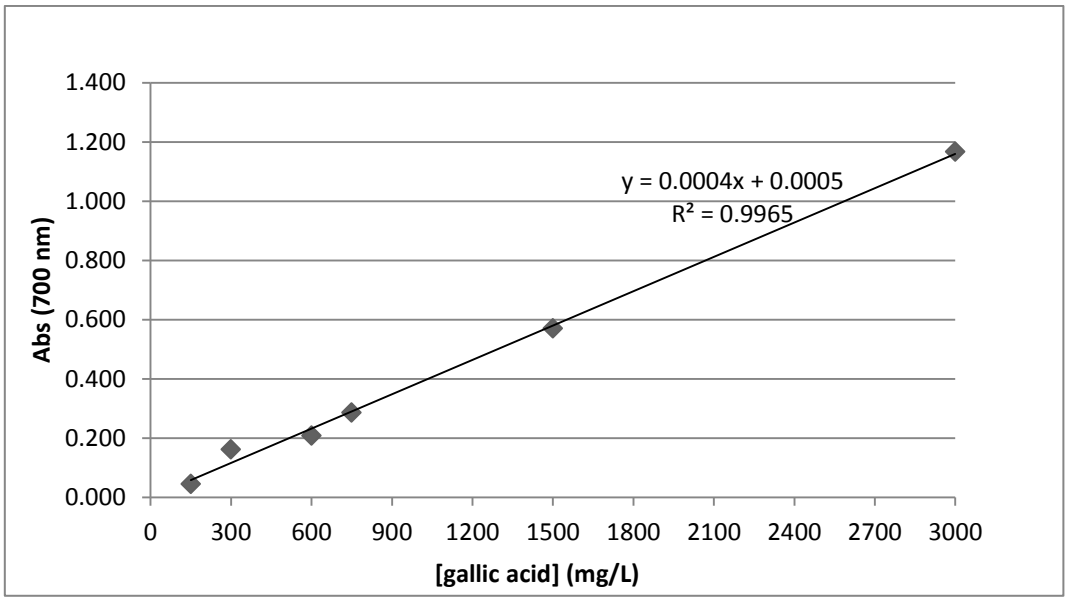

Figure A.3: Calibration curve concentration of phenolic compounds.