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Development of efficient Microbial Enhanced Oil Recovery processes at high pressure

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e co-orientação do
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There are many ways to write the history of petroleum, for a geologist everything began millions years ago, for an economist began sensibly at 50 years. For me, it started with the beginning of this project.

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ABSTRACT

Crude oil is expected to remain the main source of energy in the near future. Under these circumstances, and considering the decrease of oil reserves, it is necessary to explore mature reservoirs to ensure the growing oil demand. Current technologies recover only 30 to 50% of the oil present in the reservoirs. Microbial enhanced oil recovery (MEOR) uses microorganisms and their metabolites, to recover the entrapped oil, and it is expected that this technology can increase oil recovery by 30%.

In this study, *Bacillus subtilis* #573 isolated from a Brazilian oil well with a temperature of 40°C and a pressure of 32.4 bar was studied for its applicability in MEOR. The aim of this work was to study the potential of this isolate for MEOR applications under the reservoir conditions, namely under high pressures. For this purpose, five different methods were used, namely the culture in serum bottles, shaken flasks, non-pressurized bioreactor and in pressurized reactor, and finally in sand-pack columns. This allowed the study of the growth and production of biosurfactants under atmospheric pressure and under high pressures. The results showed that the isolate was able to grow and produce biosurfactants (reducing the surface tension up to 26 mN/m) under aerobic conditions, oxygen-limiting conditions, and pressures and temperature up to 47bar and 50°C, respectively. The biosurfactants produced in the different conditions were extracted, purified and characterized, being constituted by different surfactin isoforms (C_{12}, C_{13}, C_{14}, C_{15} and C_{16}). Furthermore, the expression of the gene *srfA* involved in the synthesis of surfactin) was studied in order to assess the impact of high pressure in its expression: The results obtained suggest a higher relative expression at high pressure. Biosurfactant production was studied at different temperatures and pressures, and it was concluded that this isolate presents a high versatility with respect to the range of temperatures and pressures in which biosurfactant is observed. Under 37°C and 46 bar it was possible to obtain a positive additional oil recovery of 14.5% in a sand-pack column assay. In conclusion, *B. subtilis* #573 is a potential candidate for use in advanced oil recovery processes under the high pressures studied.

RESUMO

É expectável que o petróleo permaneça como a principal fonte de energia num futuro próximo. Nesse sentido, e tendo em conta o decréscimo das reservas existentes torna-se necessária a exploração de reservatórios maduros para satisfazer a demanda crescente de petróleo. As tecnologias atuais permitem recuperar apenas 30 a 50% do petróleo presente nos reservatórios. A recuperação avançada de petróleo com recurso a microrganismos (MEOR) é expectável que permita recuperar até 30% do petróleo retido nos reservatórios.

Neste estudo, utilizou-se a estirpe *Bacillus subtilis* #573 isolada de um poço de petróleo brasileiro com uma temperatura e pressão de 40°C e 32.4 bar, respetivamente. O objetivo foi estudar o potencial deste isolado para aplicações de MEOR nas condições dos reservatórios, nomeadamente sob altas pressões. Para o efeito, foram utilizados 5 métodos diferentes, nomeadamente o cultivo da bactéria em garrafas, em matrazes, em biorreator não pressurizado e em reator pressurizado, e por último em colunas de areia. O que permitiu o estudo do crescimento e da produção de biossurfactantes sob pressão atmosférica e sob altas pressões. Os resultados mostraram que o isolado foi capaz de crescer e produzir biossurfactantes (reduzindo a tensão superficial até 26 mN/m) sob condições aeróbicas, condições limitantes de oxigénio, e com pressões até 47 bar e temperaturas de 50°C. Os biossurfactantes produzidos nas diferentes condições foram extraídos, purificados e caracterizados, sendo constituídos por diferentes isoformas de surfactina (C_{12}, C_{13}, C_{14}, C_{15} and C_{16}). Adicionalmente, conduziu-se uma análise da expressão do gene *srfA* (gene envolvido na síntese de surfactina) por forma a aferir o impacto das condições extremas do poço na sua expressão, sendo que estes dados sugerem uma expressão relativa superior em reator pressurizado. A produção de biossurfactantes foi estudada a diferentes valores de pressão e temperatura e concluiu-se que este isolado apresenta uma elevada versatilidade no que diz respeito à gama de temperaturas e pressões, nas quais, uma boa produção de biossurfactantes pode ser atingida. A 37°C e 46 bar foi possível obter uma recuperação adicional de petróleo de 14.5% em modelo de coluna de areia. Em conclusão, o *B. subtilis* #573 é um potencial candidato para utilização em processos de recuperação avançada de petróleo sob as pressões elevadas estudadas.

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LIST OF ABBREVIATIONS

ANOVA – Analysis of variance

AOR - Additional oil recover

CCD – Central composite design

cDNA – Complementary deoxyribonucleic acid

gDNA – Genomic deoxyribonucleic acid

CEOR - Chemical enhanced oil recovery

CMC - Critical micelle concentration

DOE - Design of experiments

EEOR – Enzymatic enhanced oil recovery

EOR - Enhanced oil recovery

FTIR- Fourier-transform infrared spectroscopy

GC content - Guanine-cytosine content

GEMEOR - Genetically engineered microbial enhanced oil recovery

HPLC – High performance liquid chromatography

LC-MS- Liquid chromatography–mass spectrometry

MEOR - Microbial enhanced oil recovery

MSS – Mineral salt solution medium

OD – Optical density

OOIP - Original oil in place

PBS – Phosphate-buffered saline

PV - Pore volume

qRT-PCR – Quantitative real-time polymerase chain reaction

RNA – Ribonucleic acid

rpm – Rotations per minute

Soi - Initial oil saturation

Sor - Residual oil saturation

Sormf - Oil recovered after microbial flooding

Sorwf - Oil recovered after water flooding

LIST OF ABBREVIATIONS (CONT.)

ST – Surface tension

$ST^{1/5}$ - Surface tension diluted 5 times

$ST^{1/10}$ - Surface tension diluted 10 times

$ST^{1/100}$ - Surface tension diluted 100 times

Swi - initial water saturation

MOTIVATION AND AIMS OF THE PROJECT

According to the latest International Energy Agency report, global energy needs have been rising slower than in the past, but they will still grow by 30% until 2040. A strong World economy is expected to sustain solid increases in demand for oil. Indeed, a global economy growing at an average rate of 3.4% a year together with a population that expands from 7.4 billion to more than 9 billion by 2040, are key drivers for the increasing oil demand.

Due to the increased depletion of natural resources, the oil industry has made great efforts to maximize oil extraction from mature and abandoned reservoirs, using tertiary recovery techniques known as Enhanced Oil Recovery (EOR). Currently, oil production technologies recover only between 10 and 40% of the oil originally present in the reservoirs. In the last years attention has been focused on Chemical Enhanced Oil Recovery (CEOR), a technology that uses chemical compounds to mobilize entrapped oil. However, these processes are often expensive and present some environmental hazardous. Microbial Enhanced Oil Recovery (MEOR) represents a cost-effective and eco-friendly alternative as it uses selected microorganisms to synthesize compounds analogous to those used in CEOR to increase oil recovery that can be used *in situ* or be produced *ex situ* using non-renewable resources.

The overall goal of this project was to achieve a better understanding of the growth and the metabolism of a potentially useful microorganism isolated in our group for application in MEOR at high pressures (similar to those existing in the oil reservoirs), in order to improve the recovery of additional oil, which will greatly benefit the oil industry. For this purpose, a number of specific aims were pursued, namely: Study of the effect of different combinations of pressure and temperature on the growth and biosurfactant production by *Bacillus subtilis* #573; Analysis of the expression of one gene related with surfactin production; Assessment of additional oil recovery in sand-pack column at high pressures.

The thesis is organized in six chapters as follows: In chapter 1 - State of the Art, a theoretical introduction on the topic of thesis is presented; in this chapter several sub-themes are approached from the perspective of the need for oil, the phases of oil

recovery which includes an approach of microbial enhanced oil recovery; in this chapter is also developed the theme of high pressures. In chapter 2 – Materials and Methods, all the materials and methods that allowed to reach the results presented in this thesis are presented. In chapter 3 – Results and Discussion, the results obtained to attain the proposed aims are presented as well as a discussion of them. In chapter 4 – Conclusions and Future Perspectives the conclusions of the work are presented as well as future work that must be developed for an additional support of the presented results. In chapter 5 – References, it is possible to consult the works and articles quoted throughout this thesis as well as supplementary material in in chapter 6 – Appendixes.

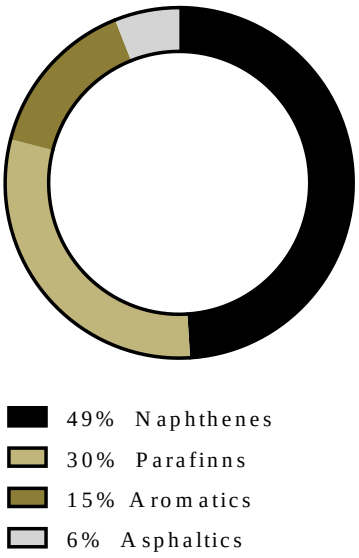
CHAPTER 1

STATE OF THE ART

1.1. THE ERA OF OIL IS NOT YET OVER

Petroleum or crude oil is a dark brown liquid, flammable and toxic, that occurs naturally in porous rocks or fractured geological formations. It is a complex mixture of hydrocarbons of different molecular weights and other organic compounds. Table 1 illustrates the relative percentages of oil components. Its composition generally includes paraffins, naphthenes, aromatic hydrocarbons, and asphaltics, as well as other chemical compounds (although to a less extent) that contain nitrogen, oxygen or sulphur and vestigial amounts of heavy metals such as copper, nickel and iron. Although the hydrocarbon composition varies among different reservoirs, the proportion of the different components is similar [5], [6].

Table 1. Average composition of hydrocarbons and chemical composition expressed as percentage (adapted from, [2], [3])

Hydrocarbons weight (%)	Element	weight (%)
	Carbon	78-89
	Hydrogen	7,5-14
	Nitrogen	0,1-3,3
	Oxygen	0,1-3
	Sulphur	0,5-10
	Metals	<0,1

According to the latest International Energy Agency report [7], global energy needs rise slower than in the past but still will grow by 30% until 2040. This increase is equivalent to adding another China and India to today's global energy demand. A strong World economy is expected to sustain solid increases in demand for oil. A global economy growing at an average rate of 3.4% a year, a population that expands from 7.4

billion to more than 9 billion by 2040, and an urbanization process that adds a city of 24.2 millions to the urban population of the World every four months are key drivers for oil demand [8].

This global economic growth is pushing more people into the middle class in developing countries and higher incomes means a demand for ever-increasing consumer goods and services. A large group of chemicals derived from crude oil and natural gas is crucial to the manufacture of many products that meet growing demand. Examples include personal care items, food preservatives, fertilizers, furniture, paints and lubricants for automotive and industrial purposes. The use of oil to produce petrochemicals, an increased consumption for trucks, aviation and shipping is enough to keep the oil demand on a rising trajectory to 105 million barrels per day by 2040 [7].

Crude oil is a key fuel for global transportation, as it accounts about 94% of the total energy used for transport, and, in turn transport is an important factor in our globalized economy. Consequently, the behaviour of oil liquids production is vital in economic terms due to its role on connecting the flows of goods and services worldwide [9]. This is the resource which availability has been most studied, and which future production evolution has reached the largest consensus among the research community [10]. The debate in the research community is centred in the estimation of the amount of resources that can be potentially recoverable, plus those quantities already produced and therefore, on the specific strategies to estimate the production [10].

Although efforts are being developed to introduce renewable energy sources into the global market, the society is still highly dependent on oil, which is still an essential source of energy for global economic development [11]. Oil is expected to remain the main energy resource in the near future, even with declining discoveries of new oil reservoirs and the increasing use of renewable energies [2].

1.2. OIL RECOVERY

As previously mentioned, oil is a crucial source of energy and raw material to obtain various chemicals used in the manufacture of lubricants, solvents, fertilizers, pesticides, plastics, pharmaceuticals and many other value-added products [11]. Consequently, an efficient and productive oil extraction that meets the current needs of the society is important.

With the advances in science, particularly in the field of biotechnology and petroleum engineering, concise steps are being taken to introduce a new set of economically viable and environmentally friendly oil recovery methods [12].

At the beginning of the exploration of an oil field, the pressure trapped inside is high enough to drive the oil to the surface. This stage of the lifetime of a reservoir is called the primary recovery. The primary recovery is the less expensive stage; however only about 5 to 10% of the reservoir´s original oil in place (OOIP) is collected [13]. As the natural pressure of the reservoir decreases, it is necessary to introduce energy so that the oil continues to be directed to the surface. That can be done through water or gas injection, which contribute to increase the internal pressure. In this second stage (the so-called secondary recovery), other mechanical and physical methods are generally used, such as pumping and gas lift, to increase the extraction of oil. The secondary recovery stage can increase oil recovery to about 10 to 40% of the OOIP [2][13][14]. Together these two traditional stages of oil recovery allow only for the extraction of 20-40% of the OOIP, thus leaving a large percentage of oil entrapped in the reservoirs [15][16]. Most of this entrapped oil is unrecoverable by traditional methods due to several reasons, such as the high viscosity of the oil, the low permeability of the reservoirs and the high interfacial tension between the hydrocarbon, the aqueous phase and the reservoir rocks, which results in high capillary forces that entrap the oil in small pores inside the rock that constitutes the reservoir [17].

1.3. TERTIARY OIL RECOVERY: CHEMICAL *VERSUS* MICROBIAL ENHANCED OIL RECOVERY

As previously mentioned, given the increasing demand of energy expected for the next years, the oil industry has been making great efforts to maximize oil extraction from mature and abandoned reservoirs, using tertiary recovery techniques known as Enhanced Oil Recovery (EOR). EOR processes, in particular the Chemical Enhanced Oil Recovery (CEOR), uses a number of chemicals such as surfactants, polymers, acids, gases and solvents that lead to an increased oil production form mature oil fields [17]. Surfactants reduce the interfacial tension between the oil/water and oil/rock interfaces, alter the rock's wettability and decrease the capillary forces that prevent the oil from moving through the rock pores. Polymers fill the oil reservoir pores, directing the flow of the injected water to oil rich channels, thus mobilizing the residual oil. Polymers are also used during the water-flooding process, to increase the viscosity of the injected water. Gases and solvents decrease the viscosity of crude oil thereby promoting their flow. Gases also play a role in increasing the reservoir pressure. Acids are used to expand the permeability through the porous rock network [2].

The use of CEOR strategies can increase the crude oil recovery by 30% [20]. However, CEOR is economically unattractive, since some of the products used are derived from petroleum. Furthermore it is potentially environmentally hazardous, generating undesirable wastes that are difficult to remove [2][21].

In the recent years, a strong interest in biotechnology by the oil industries has emerged, in order to find innovative solutions that promote the production of crude oil using more environmental friendly and cheaper processes [12], [22]. Among them, Microbial Enhanced Oil Recovery (MEOR) arises as a response to the main disadvantages of CEOR [23], by focusing in the use of microorganisms and microbial products that are analogous to those used in CEOR, such as biosurfactants, biofilms, biopolymers, acids and solvents [14][19]. Microorganisms can synthesize useful products by fermenting low-cost raw materials; these bio-products exhibit low toxicity and are biodegradable, which does not impose an environmental constraint for their use in the petroleum production processes [19]. It is also noteworthy that these bio-products are economically more attractive because their price is not directly influenced by global

geopolitical aspects, that are usually associated with the fluctuation in the price of crude oil.

Tertiary oil recovery through MEOR technology is one of the most promising processes to recover a substantial proportion of the unrecoverable oil retained in mature oil fields [24] and can imminently be established as an important industrial instrument, since efforts are made and goals are reached in scientific research [12].

As previously discussed, MEOR encompasses the use of microorganisms, with a greater focus on those selected by bio-prospecting the oil wells. Currently, the research in MEOR is focused on developing both *ex situ* and *in situ* approaches, which can meet the need for more eco-friendlier oil extraction processes, and also to overcome the main challenges of oil extraction, which include high viscosity and size of petroleum components. The *ex situ* MEOR is based on the CEOR approach, as the bio-products of interest are produced outside the reservoir (*i.e*. in bioreactors) and subsequently introduced in the oil wells with the objective of increasing oil extraction. The production of metabolites *ex situ* allows a more specific control since specific bio-product compositions can be selected and injected into the reservoir [12]. On the other hand, selected microorganisms isolated in the laboratory can be injected directly into the oil reservoir in order to produce the desired metabolites *in situ*. Despite the greater control of the *ex situ* method, the production of metabolites exhibits excessive costs, leading to a concern of the petroleum industry to move forwards with their use [25][26]. Although both *in situ* and *ex situ* approaches have potential, *in situ* operations can be more favourable for the oil industry [2][13].

1.4. MEOR

1.4.1. STRATEGIES

A major challenge in applying is the need of using microorganisms able of growing under anaerobic conditions, due to lack of oxygen in most oil reservoirs. One possible alternative is the introduction air, as an oxygen source into the oil reservoirs in order to use aerobic microorganisms; however, economically is not feasible and this can lead to material corrosion and equipment damage, and the presence of oxygen as electron acceptor can lead to an imbalance in the microbial community [19].

The bio-products and microorganisms used in this type of strategies must be able to overcome and thrive in the severe conditions of the reservoir (extreme pH, temperature, salinity and pressure), and therefore despite the apparent advantages of *in situ* MEOR there are still many improvements to be studied [13][27]. Each reservoir exhibits unique characteristics; therefore, different bacterial consortia and different combinations of bio-products must be studied for each particular application [13].

1.4.1.1. BIOSURFACTANTS

The mechanisms by which microorganisms are helpful in oil recovery are not fully understood and it is generally accepted that several microbial processes are synergistically associated to increase oil recovery [28].

Among the useful microbial metabolites for MEOR, biosurfactants are the ones that have gained the most pronounced interest from the scientific community because they are more eco-friendlier than chemical surfactants [19][29]. Moreover, lower concentrations are required to attain similar results [2][13]. In addition, biosurfactants are biodegradable, stable at different pH values and high temperatures. These amphipathic molecules produced by microorganisms reduce the surface and interfacial tensions, altering the adsorption at immiscible interfaces, emulsifying crude oil and increasing the mobility of bacterial cells [13][30].

There are several types of biosurfactants, including glycolipids, fatty acid biosurfactants, lipopeptides, emulsifying proteins and particulate biosurfactants [31].

The lipopeptides and glycolipids, are extremely important in MEOR because they can reduce the interfacial tension between water and crude oil to values as low as 0.1 mN/m [6]. For application in MEOR, biosurfactants should have a strong interfacial activity, tolerance to wide range of pH values and temperatures, good solubility in water, high emulsification capacity and low critical micelle concentration (CMC) [32].

Species belonging to genus *Bacillus* produce different lipopeptide biosurfactants, including surfactin and lichenysin, which have been widely reported. Different strains of *Bacillus subtilis* are surfactin producers [33] and *Bacillus licheniformis* strains have been reported to produce different types of lichenysin [34][35]. Several authors reported the isolation of lipopeptide biosurfactant-producing *Bacillus* strains from oil reservoirs [2] [36]–[39].

Surfactin is indeed one of the most powerful known biosurfactants. This secondary metabolite was first found in cultures of *B. subtilis* in 1968 by Arima and co-workers [40], and it was named surfactin due to its exceptional surfactant activity [33]. As depicted in Figure 1, the chemical structure of surfactin comprises a peptide ring of seven amino acids (L-aspartic acid, L-leucine, L-glutamic acid, L-leucine, L-valine and two D-leucines) linked to a long hydrophobic fatty acid chain. A natural diversity of structures occurs, giving rise to homologues that differ from each other in the length (12 to 16 atoms of carbon) and the ramification of the fatty acid chain, and to isoforms, characterized by some differences in the peptidic sequence [40]. Because of its unique structure, surfactin is not only highly surface-active and capable of lowering the surface tension of water from 72 mN/m to 27 mN/m [2][33], but it is also stable at high temperatures (even at 120° C [2][41]). Hence, exhibiting great potential for application in MEOR [2][41].

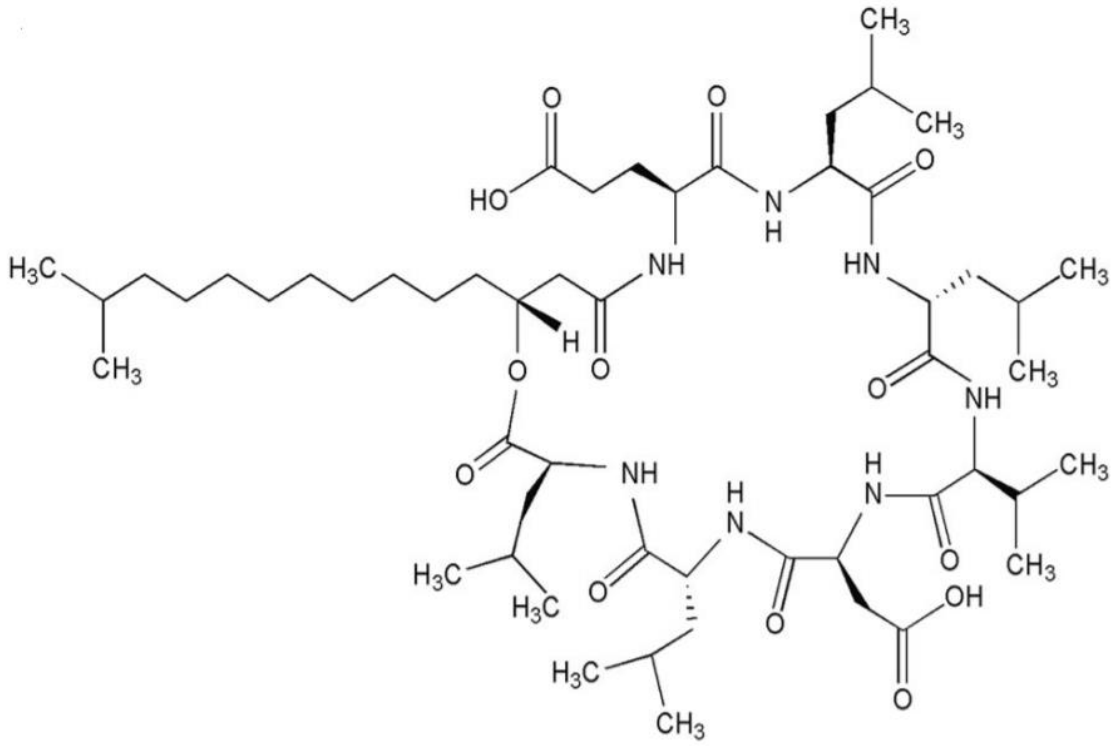


Figure 1. Chemical structure of surfactin. In the cyclic ring seven amino acids are arranged and connected with a fatty acid (β-hydroxy) which chain length varies from 12 to 16 carbon atoms.

1.4.1.2. MICROBIAL BIOMASS AND SELECTIVE PLUGGING

One of the main focus of MEOR is to mobilize the trapped oil in the reservoirs to high permeability regions, in order to facilitate its recovery. During the secondary recovery, when water is injected into the reservoir, it flows preferentially through high permeability channels, whereas the oil trapped in low permeability regions remains inaccessible [42]. One of the strategies to direct the injected water to the low permeability zones is the injection of nutrients and microorganisms into the reservoir. The injected microorganisms grow in the high permeability channels of the reservoir, blocking those preferential zones and redirecting the injected water to oil rich areas. As an alternative, polymers can be used instead of stimulating the bacterial growth *in situ* to achieve the same objective [43].

Microorganisms have a tendency to grow in the porous media, forming a biofilm, which prevents the introduction of additional oil into those areas [44]. The blockage of these channels alters the flow of water into the pore space [42][45][46]. This selective obstruction, known as microbial selective plugging, can result in the increase of the oil production by directing the flow of water to previously inaccessible areas of the reservoir [47].

1.4.1.3. BIOPOLYMERS

As previously mentioned, the production of microbial biomass is not the only way to alter the permeability of the reservoir. The production of biopolymers can influence and aid in the selective blocking of the rock pores. Several microorganisms, when growing inside the oil reservoirs produce biopolymers [12]. Many of these biopolymers are exopolysaccharides which function is to enhance cell adhesion and protect the bacterial cells from desiccation and predation [48].

Some microorganisms of the genera *Bacillus*, *Xanthomonas* and *Aureobasidium* are known to produce biopolymers that are helpful in the recovery of petroleum [13], namely xanthan gum, levan, pullulan and curdlan, among others. Xanthan gum is one of the most versatile biopolymers due to its tolerance to high temperatures and salinities, making it useful for application in MEOR [49]. Although curdlan is not as effective as xanthan gum, it can also increase oil production [13].

1.4.1.4. GASES, ACIDS AND SOLVENTS

Microorganisms can also produce gases, acids and solvents, which are important metabolites in MEOR strategies. Tertiary oil recovery strategies include re-pressurizing the oil reservoirs [50], which may force oil production, similar to what happens in the primary recovery. For the *in situ* application, microorganisms that can generate gases (such as methane, carbon dioxide and hydrogen) by fermentation of carbohydrates can be stimulated into the oil reservoir [13]. In addition to increasing the pressure, gases can dissolve in the oil reducing its viscosity and favouring its flow [19].

The production of extracellular acids by injected microorganisms helps to recover additional oil by dissolving parts of the carbonate rock [13][19]. Contrary to the selective plugging strategies or the alteration of the rock wettability (that aim to change the rock properties), the use of bio-acids is based on the dissolution of parts of the carbonate rock [19]. A similar effect is achieved using solvents produced by microorganisms, such as acetone and ethanol [51].

1.4.1.5. DEGRADATION OF HYDROCARBONS

Some microorganisms present in petroleum reservoirs break down the alkyl chains of heavy crude oil, wich makes the oil less viscous and easier to recover, increasing its value[2]. The ability of a microorganism to degrade crude oil is one of the most interesting strategies in MEOR. Whether by *in situ* stimulation of the bacteria already present in the reservoir or by injection of strains of hydrocarbon-degrading bacteria isolated in the laboratory, promising results have been reported by several authors [2][52][53][54].

1.4.1.6. GEMEOR AND EEOR

Most of the microorganisms studied and used in MEOR strategies are native microorganisms, selected by bio-prospecting the microbial communities of the reservoirs. However, in order to overcome the limitations of those microorganisms, a new trend emerged in the last years, known as Genetically Engineered Microbial Enhanced Oil Recovery (GEMEOR). GEMEOR uses genetic engineering tools to improve those microorganisms robustness and ability to overcome the extreme conditions of the reservoirs [12][55]. Another recent perspective is the use of enzymes in the tertiary oil recovery processes (EEOR – Enzymatic Enhanced Oil Recovery). Recent studies suggest that an enzymatic consortium with biosurfactants alters the dynamics of the oil-rock-water interface, altering the wettability and capillary number, ultimately reinforcing oil recovery [56].

1.4.2. LABORATORY STUDIES: *BACILLUS SUBTILIS*

B. subtilis is a very well characterized Gram-positive bacteria, being generally recognized as safe (GRAS). It has been used in many biotechnological applications as a producer of microbial metabolites [57], and as previous mentioned it is a target organism for MEOR. Table 2 summarizes some studies reported in the literature where useful microorganisms for MEOR applications are used.

Table 2. Laboratorial studies using different microorganisms with potential for MEOR applications.

Microorganisms	**Growth Conditions**	**Metabolites Produced**	**Oil Recovery (AOR, %)**	***In situ /Ex situ***	**Ref.**
B. subtilis BR-15	Aerobic	Surfactin	Sand-pack column (66%)	*Ex situ*	[36]
B. subtilis RI4914	Aerobic	Surfactin, Acids, Bio-polymers	Sand-pack column (up to 88%)	*Ex situ*	[58]
B. subtilis B20	Aerobic	Biosurfactants, Bio-polymers	Core flood experiments (10%)	*Ex situ*	[59]
B. subtilis R1	Aerobic	Surfactin	Sand-pack column (33%)	*Ex situ*	[39]
B. subtilis B30	Aerobic	Surfactin	Core flood experiments (17–31%)	*Ex situ*	[38]
B. subtilis BS-37	Aerobic	Surfactin	Sand-pack column (9–14%)	*Ex situ*	[41]
B. subtilis 20B	Aerobic	Biosurfactants	Sand-pack column (30%)	*Ex situ*	[37]

Table 2. (continued)

Microorganisms	Growth Conditions	Metabolites Produced	Oil Recovery (AOR, %)	*In situ /Ex situ*	Ref.
Mixture of strains, includes *B. subtilis*	Oxygen limited	Surfactin, Acids, Bio-polymers	Core flood experiments (7–13%)	*In situ*	[54]
Consortium NJS-4	Oxygen limited	Biosurfactants, Acids, Gas	Sand-pack column (27%)	*In situ*	[60]
Consortium TERIL146	Oxygen limited	Acids, Gas	Sand-pack column (8%)	*In situ*	[61]
Heterotrophic nitrate reducing bacteria	Oxygen limited	Biosurfactants, Gas	Sand-packed bioreactors (11–18%)	*In situ*	[62]
Consortium A7	Oxygen limited	Biosurfactants, Acids; Gas	Not specified (12%)	*In situ*	[63]
B. stearothermophilus SUCPM#14	Oxygen limited	Biosurfactants	Core flood experiments (11–22%)	*In situ*	[64]
B. licheniformis BNP29	Oxygen limited	Biosurfactants	Core flood experiments (9–22%)	*In situ*	[65]
B. subtilis strains	Oxygen limited	Surfactin	Sand-pack column (6–24%)	*In situ*	[66]
Consortium TERIL 63	Oxygen limited	Solvents, Acids, Gas	Core flood experiments (16%)	*In situ*	[67]

Table 2. (continued)

Microorganisms	Growth Conditions	Metabolites Produced	Oil Recovery (AOR, %)	*In situ /Ex situ*	Ref.
Pseudomonas stutzeri Rhl	Oxygen limited	Biosurfactants	Core flood experiments (16%)	*In situ*	[68]
Enterobacter sp.	Oxygen limited	Biosurfactants	Core flood experiments (6%)	*In situ*	[16]
Pseudomonas aeruginosa 709	Oxygen limited	Biosurfactants	Core flood experiments (7%)	*In situ*	[69]

(AOR, %) – Additional Oil Recovery

1.4.3. FIELD STUDIES

The selected microbial consortia injected into the reservoirs will have to compete with the indigenous microorganisms. The injected microorganisms will probably be surpassed by the indigenous species, more adapted to the severe conditions of the reservoirs [17]. Several studies have established that the indigenous anaerobic, thermophilic and halotolerant populations are more appropriate for application in MEOR. It is expected that the indigenous microorganisms will remain metabolically active in the reservoir, since this is their native environment [16]. However, nutritional factors play an important role in microbial growth for the development of biological processes in MEOR strategies [67].

Gao and collaborators [70] demonstrated that the concentration and composition of nutrients are the main factors that affect the microbial proliferation in an oil well, suggesting that the continuous supply of nutrients with adequate concentration and composition in the pore spaces is the critical factor for the final oil recovery achieved.

The oil reservoirs worldwide represent very complex biological systems for which laboratory simulations of microbial activities become very challenging. While it may be possible to demonstrate advantageous effects in laboratory conditions with microbial cultures, in the reservoir these are likely to be out-competed by the better adapted indigenous species [13]. MEOR field trials using various approaches have been extensively reviewed by several authors [12][13][19][37][71]. These field trials are an important tool to determine and document the efficacy of microbial processes and validate the laboratorial studies and models [13]. Some microbial enhanced oil recovery field trials are presented in Table 3 which includes the reservoir name, microorganisms used, microbial strategy and obtained effects in oil recovery.

Table 3. Microbial enhanced oil recovery field trials

Reservoir	**Microbial systems**	**Microbial strategy**	**Duration**	**Incremental of oil production**	**Ref.**
Lisbon oil field, USA	*Clostridium acetobutylicum*	Gas production	6 months	250%	[12] [72]
Bebee oil field, USA	*Bacillus* strains	Biosurfactant production	60 days	37%	[73]
Romanian oil fields, Romania	*Clostridium* and *Bacillus* strains	Microbial flooding	5 months	100–200%	[19]
Piedras Coloradas oil fields, Argentina	Hydrocarbon degrading facultative microorganisms	Hydrocarbons degradation	12 months	26–110%	[74]
Bokor Offshore field, Malaysia	Sulphate Reducing Bacteria	Hydrocarbons degradation	5 months	47%	[75]

Table 3. (continued)

Reservoir	Microbial systems	Microbial strategy	Duration	Incremental of oil production	Ref.
Vyngapor oil fields, Russia	Indigenous microorganisms and Lactobacteria	Oil-displacing metabolites	7 months	2268.6 tons	[76]
Alton oil field, Australia	Indigenous microorganisms	Stimulation of indigenous microorganisms	12 months	40%	[54]

1.5. HIGH PRESSURE

Pressure is a physical parameter that influences the evolution and distribution and proliferation of microorganisms [77]. The ability to respond and adapt to changes in dozens of MPa is thought to be restricted to organisms inhabiting high pressure environments [78]. Deep sea environments and oil reservoirs incurring high pressures, are inhabited by a high diversity of microorganisms, known as piezophiles (organisms adapted to high pressures), which often require these high pressures for optimal growth [79]. On contrary, the growth of microorganisms adapted to atmospheric pressure, such as the mesophile *Escherichia coli*, progressively becomes compromised as the pressure increases, and ceases completely around 50 MPa [80]. Usually, as a result of the exposure to pressures greater than 100 MPa, microorganisms start suffering lethal injuries [81]. The influence of high pressure on biomolecules is essentially described by the thermodynamic principle of Le Châtelier and Braun, which states that a molecular system will counteract an increase in pressure occupying a smaller volume [82].

A piezophile strain, *Photobacterium profundum* SS9 is used as a model microorganism in adaptation studies because of its hyper-responsiveness to pressure changes [78]. Lipids, which are more compressible than proteins, are particularly

sensitive to the effect of pressure [83]. High pressures expand the thickness of the lipid bilayer by reducing the "kinking" of the acyl chains [84]. In addition to lipids, certain membrane proteins also have a relevant role in the growth of piezophiles at high pressure [78]. It is expected a significant effect of pressure on microorganisms, which inevitably culminates in pleiotropic cellular defects and phenotypes, compromising DNA replication, transcription, translation, protein functionality, and membrane integrity [85].

Dissociation of protein complexes at pressures under 100 MPa may play a decisive role in inhibiting the growth of mesophile *E. coli*, as many of these complexes are involved in essential cellular processes such as replication, transcription and translation. In this context, DNA replication has been found to be one of the most pressure sensitive processes of macromolecule synthesis [80].

Transcriptomic and proteomic studies have shown that ribosome disruption and inhibition of translation are critical aspects of growth arrest and survival of mesophile *E. coli* under pressure. *E. coli* and *Lactobacillus sanfranciscensis*, for example, have been shown to respond mainly to high pressure shock (<100 MPa) by strong upregulation of rRNA genes, ribosomal proteins and translation-associated proteins [86].

The cells have to adapt specifically to certain pressures; some strains have their optimal growth at high pressures and only grow slightly under atmospheric pressure [86][87]. Park and Clark [88] stated that many microorganisms can live under high or low pressures if a certain time of adaptation is given, such as the depressurizing a reservoir sample in a controlled and slow manner.

High temperatures and salinity have an adverse impact on the rates of microbial growth and production of metabolites in oil reservoirs *in situ* [16]. However, it is thought that the reservoir pressure is not a limiting factor for the survival and proliferation of indigenous microorganisms [89][90]. In addition, high pressure influences the redox potential of gases such as carbon dioxide and increases gas solubility, which may affect the redox potential of gases participating as electron acceptors and donors, such as hydrogen or CO_2 [91].

Fida and collaborators [81], studied the link between bioremediation of toxic solvents and enhanced oil recovery processes by stimulating resident bacteria of oil reservoirs. In this study, the authors reported the use of the indigenous microorganism *Thauera sp.* TK001, to improve oil recovery. *In situ* incubation for 14 days resulted in an additional 17.0 ± 6.7% of residual from low pressure bioreactors and an additional 18.3% of residual oil in high pressure bioreactors (27.2 bar). The increase in oil production in high pressure bioreactors, when compared to the low pressure bioreactors where the mechanism might be due to the formation of N_2 and CO_2, has suggested other mechanisms than gas production, plugging or increased oil emulsion contributed to MEOR.

Yue and co-workers [92] studied the oil displacement mechanism by indigenous communities under reservoirs conditions (100 bar and 60°C) and state that, under high pressure conditions, endogenous microbial community grows slowly and less bacteria can adapt to the environment.

Zhao and collaborators [69] used two strains of biosurfactant-producing bacteria (*Bacillus amyloliquefaciens* 702 and *Pseudomonas aeruginosa* 709) that were isolated from Xinjiang oil field, China. Biosurfactant production by those strains was evaluated under aerobic, anaerobic and high pressure (100 bar) conditions at laboratorial scale. Although both strains can grow under anaerobic or high pressure, only the strain 709 can efficiently produce biosurfactant under these conditions.

It is important to note that although most of the studies on MEOR strategies do not report the effects of high pressure, some authors affirm that pressure is a negligible parameter for the proliferation of microorganisms in the oil reservoirs [70][88] while others report the opposite [92]. The effects of high pressure on metabolism and metabolite production are still unknown and controversial. However, some studies show that pressure exhibits a significant effect on biomolecules [79][84]. Therefore, it is crucial to better understand the effect of high pressures on the indigenous microorganisms growth and metabolism.

In summary, the success of MEOR strategies depends to a great extent on the ability of microorganisms to tolerate and produce relevant metabolites under the severe

conditions of the oil reservoirs [16]. Current advances in genetic engineering hold a great promise as it is possible for MEOR applications through the improvement of their robustness [13].

CHAPTER 2

MATERIALS AND METHODS

2.1 MICROORGANISM AND CULTURE MEDIA

The strain *B. subtilis* #573, previously isolated from a crude oil sample retrieved from a Brazilian oil field, [2], was used in this study. This isolate produced extracellular biosurfactants with high surface and emulsifying activities at 40°C under aerobic and anaerobic conditions, in culture media with or without hydrocarbons, at atmospheric pressure. Additionally, this isolate degraded the large alkyl chains of hydrocarbon mixtures under anaerobic conditions [2], causing a reduction in viscosity and an increase in the commercial value of petroleum. These characteristics make this isolate a good candidate for applications in MEOR as previously reported by Gudiña and collaborators [2].

Stocks of *B. subtilis* #573 were prepared in LB medium supplemented with 20% (v/v) glycerol and stored at -80 °C. The composition of LB medium was (g/L): NaCl 10.0; tryptone 10.0; yeast extract 5.0. The pH was adjusted to 7.0.

The culture medium used in this work was the Mineral Salt Solution (MSS). This medium was previously selected as the most appropriate medium for biosurfactant production by *B. subtilis* #573 [2]. The MSS medium consisted of (g/L): NaCl 10.0; sucrose 10.0; Na_2HPO_4 5.0; NH_4NO_3 2.0; KH_2PO_4 2.0; $MgSO_4 \cdot 7H_2O$ 0.2; pH 7.0. This medium contains nitrate (ammonium nitrate) to act as electron acceptor as an alternative to oxygen, which can be used by the microorganism when growing under anaerobic or oxygen-limiting conditions, as demonstrated in a previous work [2]. In some cases, the MSS medium was supplemented with 1% (v/v) of trace salt solution (TSS). TSS consisted of (g/L): $MnSO_4 \cdot 4H_2O$ 3.0; EDTA 1.0; $FeSO_4 \cdot 7H_2O$ 0.1; $CaCl_2 \cdot 2H \cdot O$ 0.1; $CoCl_2 \cdot 6H_2O$ 0.1; $ZnSO_4 \cdot 7H2O$ 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.

Whenever required, samples of the different cultures were plated on LB agar to analyze the morphology of the microorganism grown at different pressures, and to verify the presence of contaminations. The composition of LB agar was the same described for the LB liquid medium supplemented with 20 g/L of agar.

2.2 BIOSURFACTANT PRODUCTION IN SERUM BOTTLES

A control experiment was performed on serum bottles (50 mL capacity) containing 25 mL of MSS medium. The experiment with 21 serum bottles (was performed in triplicate) was followed up for 7 days. Each bottle was inoculated with 0.25 mL (1%) of a pre-culture of *B. subtilis* # 573 in aerobic conditions (grown for 24h in the same culture medium at 37°C and 200 rpm) and after inoculation the bottles were closed with metal caps to create a system with oxygen-limiting conditions. Throughout the experiment, performed under oxygen-limiting conditions at 37°C, atmospheric pressure and without agitation, 3 bottles were opened per day to evaluate the growth and biosurfactant production.

The bacterial growth was determined by measuring the optical density (OD) of the samples at 600 nm using a spectrophotometer (Biotek, Synergy HT, USA). Each sample was measured in triplicate, and biomass concentration (g dry weight/L) was determined using a calibration curve (Annex 1). When necessary, the samples were diluted with demineralised water to adjust the OD to the linear range of absorbance of the calibration curve (0 to 0.7 absorbance units).

Subsequently, the surface tension of each sample (containing the cells) was measured as described below. After that, the samples were centrifuged at 4000 rpm for 10 min, and the surface tension of the cell-free supernatants was measured.

2.3 BIOSURFACTANT PRODUCTION IN SHAKEN FLASK

The experiments were performed in shaken flaks (500 mL capacity) containing 200 mL of MSS medium. Each flask was inoculated with 2 mL (1%, v/v) of a pre-culture of *B. subtilis* #573 (grown for 24h in the same culture medium at 37°C and 200 rpm). Thereafter, the flaks were incubated at the same conditions as the pre-culture. The cultures were maintained until the maximum biosurfactant production was achieved (according to the surface tension values). Samples (10 mL) were taken along the fermentation to evaluate the bacterial growth and biosurfactant production.

2.4 BIOSURFACTANT PRODUCTION IN BIOREACTOR

A 2 L bioreactor (BIOSTAT A Fermentor, B. Braun Biotech International GmbH, Germany) equipped with agitation and temperature control was used to study biosurfactant production by *B. subtilis* #573 under oxygen-limiting conditions. The experiments were performed using 1 L of MSS medium, at 100 rpm, without pH control and at different temperatures. In order to maintain oxygen-limiting conditions, the fermentations were performed without air injection, and the exhaust valve of the bioreactor was kept closed to avoid the exchange of gases with the exterior. The bioreactor was inoculated with 20 mL (2%, v/v) of a pre-culture of *B. subtilis* #573 grown for 24 h in MSS medium at 37°C and 200 rpm. The effect of different temperatures (35, 37, 45, 50, 51, 52 and 55°C) on growth and biosurfactant production was studied. In order to evaluate cell growth and biosurfactant production, samples (10 mL) were taken during the fermentation. Cell growth and biosurfactant production were analyzed as in the assays performed in shaken flasks.

2.5 BIOSURFACTANT PRODUCTION IN PRESSURIZED REACTOR

To study the effect of high pressure on the growth and biosurfactant production by *B. subtilis* #573 a 1.9 L pressurized reactor (Parr Instruments Company, Moline, Illinois, USA) equipped with a Parr PID temperature and stirring controller (model 4848) was used. The MSS medium (1.6 L) was placed inside the reactor vessel and sterilized *in situ* (121°C for 30 min). After that, the temperature of the reactor was adjusted to the desired value, and the reactor was inoculated with 32 mL (2%, v/v) of a pre-culture of *B. subtilis* #573 grown for 24h in MSS medium at 37°C and 200 rpm. Subsequently, the reactor was pressurized to the desired pressure by injecting N_2. All the experiments were performed at 50 rpm, without pH control and under semi-anaerobic conditions. Different combinations of pressure and temperature were studied. Samples (10 mL) were taken throughout the experiments to analyze the bacterial growth and biosurfactant production.

2.6 EXPERIMENTAL DESIGN

The Design of Experiments (DoE) provides the experiment plan definition related to the parameters chosen in virtual modeling. The user can use his/her know-how to set the parameters range and to evaluate the most suitable configuration. According to the DoE approach, a reduced number of experiments is required to elaborate the final optimum condition. In this work, a central composite design (CCD) was implemented to model the effect of pressure and temperature on biosurfactant production by *B. subtilis* #573 using the software STATISTICA 10.0 from Statsoft Inc. (2010). CCD is a factorial or fractional factorial design with center points that is augmented with a group of axial points (or star points) that allow the estimation of the curvature. A 2^2 full-factorial central composite design with 4 axials (star configuration), and 2 central points was used, resulting in 10 experiments. The experimental ranges and the results obtained are shown in Table 4. Two replicates at the central point of the design were performed to allow the estimation of the pure error.

The relationship between the factors and each response variable was modeled by fitting the second-degree polynomial equation given by Eq. (1), and the quality of the fitted models was validated by analysis of variance (ANOVA).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \quad (1)$$

Where X_1 and X_2 are the independent parameters, β_0, β_1, β_2, β_{12}, β_{11} and β_{22} are the regression coefficients, and Y is the response function. The objective of performing a CCD was to minimize the surface tension values, that is, to maximize the biosurfactant production.

Table 4. Design matrix for the central composite factorial design.

Experiment	Temperature (°C)	Pressure (Bar)	Temperature (°C)	Pressure (Bar)
	X_1	X_2	X_1	X_2
FED 1	-1	-1	37.0	10.0
FED 2	-1	1	37.0	40.0
FED 3	1	-1	45.0	10.0
FED 4	1	1	45.0	40.0
FED 5	$-\sqrt{2}$	0	35.3	25.0
FED 6	$\sqrt{2}$	0	46.7	25.0
FED 7	0	$-\sqrt{2}$	41.0	3.8
FED 8	0	$\sqrt{2}$	41.0	46.2
FED 9	0	0	41.0	25.0
FED 10	0	0	41.0	25.0

2.7 SURFACE TENSION MEASUREMENT

The surface tension of the culture broth samples and biosurfactant solutions was measured according to the Ring method, as described elsewhere [93]. A KRÜSS K20 Tensiometer (KRÜSS GmbH, Hamburg, Germany) equipped with a 1.9 cm De Noüy platinum ring was used. All the measurements were performed at room temperature (25°C) and at least in triplicate.

2.8 EXTRACTION AND PURIFICATION OF BIOSURFACTANTS

The biosurfactants produced by *B. subtilis* #573 in flask (37°C, atmospheric pressure) and pressurized reactor (41°C, 47 bar) were recovered and purified as described by Pereira and co-workers [94]. At the end of the fermentation, the cells were harvested by centrifugation (7000 rpm, 15 min, 4°C) and the cell-free supernatant was adjusted to pH 2.0 with HCl 6 M; subsequently, it was incubated overnight at 4°C to allow the precipitation of the crude biosurfactant. The crude biosurfactant was collected by centrifugation (5000 rpm, 20 min) and dissolved in a minimal amount of demineralised water by adjusting the pH to 7.0 using NaOH 1 M. A chloroform/methanol mixture (2:1, v/v) was added to the crude biosurfactant solution to achieve a final chloroform/methanol/water ratio of 8:4:3. The mixture was placed in a separation funnel and left overnight to allow the separation of the aqueous and organic phases. Subsequently, the organic phase was collected and evaporated to dryness under N_2 at room temperature (25°C). Thereafter, the purified biosurfactant was dissolved in a minimal amount of demineralised water and freeze-dried. The product obtained was weighed and stored at -20°C for further studies.

2.9 CRITICAL MICELLE CONCENTRATION (CMC)

The CMC is the concentration of an amphiphilic compound in solution at which the formation of micelles is initiated. In order to calculate the CMC of the purified biosurfactants produced by *B. subtilis* #573 grown at different conditions, biosurfactant solutions with concentrations ranging from 1 to 1000 mg/L were prepared in PBS buffer (10 mM KH_2PO_4; 10 mM K_2HPO_4; 150 mM NaCl; pH 7.0). The surface tension of each sample was measured at room temperature (25°C) as previously described. 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 at least in triplicate. As reference, it was also calculated the CMC of commercial surfactin (99% purity, Sigma-Aldrich, USA).

The concentration of biosurfactant in the different culture broth samples was estimated using a calibration curve (surface tension *vs.* logarithm of surfactin concentration; Annex 2). The calibration curve was calculated using different concentrations of commercial surfactin (99% purity, Sigma-Aldrich, USA) below the CMC. In this biosurfactant concentration range it is possible to establish a linear relationship between the biosurfactant concentration and the surface tension. However, in some cases, to estimate the biosurfactant concentration it was necessary to dilute the culture broth samples with demineralised water to adjust the surface tension values to the linear range of the curve (34.6 to 62.4 mN/m).

2.10 CHEMICAL CHARACTERIZATION

The biosurfactants produced by *B. subtilis* #573 in flask (37°C, atmospheric pressure) and pressurized reactor (41°C, 47 bar), and the commercial surfactin (99% purity, Sigma-Aldrich, USA) were characterized by liquid chromatography–mass spectrometry (LC-MS) and Fourier transform infrared spectroscopy (FTIR) in the Department of Inorganic Chemistry, Wroclaw Medical University, Poland, with the collaboration of Dr. Tomasz Janek.

An ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS) system was used for the analysis. The methodology used is based on that proposed by Biniarz and Lukasiewicz [95]. The system consisted of a Primaide (Hitachi, Tokyo, Japan) UPLC System with an Ultimate 3000 Diode Array Detector and a Bruker compact^{TM} QqTOF System (Bruker Daltonics, Bremen, Germany), equipped with an Agilent Eclipse XDB-C18 column (150 mm × 4.6 mm, 3.5 µm particle size) (Agilent Technologies, Santa Clara, USA), which was kept at 40°C. The mobile phase consisted of water with 0.1% (v/v) trifluoracetic acid (TFA) (A) and acetonitrile (ACN) with 0.1% (v/v) TFA (B). For the analysis, the biosurfactants were dissolved in methanol at a concentration of 1.5 mg/mL. 5 µL of each sample were injected onto the column. The flow rate was set to 0.3 mL/min with a 42 min gradient, as follows: injection start (A:B) (50:50), 0.5 min (50:50), 8 min (20:80), 15 min (10:90), 25 min (0:100), 35 min (0:100), 40 min (50:50) and 42 min (50:50). The absorbance between 200 and 400 nm was monitored simultaneously with the total ion count (TIC). MS analysis was conducted in positive mode ESI. The source temperature was set to 150°C, and the desolvation gas temperature was 350°C. Nitrogen was used as the desolvation gas (800 L/h) and the cone gas (20 L/h). The cone voltage was set to 10 V, and the capillary voltage was set to 3 kV. The samples were analyzed in the range of 100-1500 m/z. The retention times, peak areas, and TIC at a given m/z were collected.

The biosurfactants were also characterized by FTIR. The FTIR spectra, with a resolution of 4 cm^{-1}, were collected from 400 to 4000 wavenumbers (cm^{-1}) using a Thermo Nicolet iS50 FT-IR Spectrometer (Thermo Fisher Scientific Co., Waltham, MA, USA) equipped with a single horizontal Golden Gate ATR cell, operating in the attenuated total reflection (ATR) mode.

2.11 ANALYSIS OF THE EXPRESSION OF THE GENE SRFA

The expression of the gene *srfA*, involved in the synthesis of surfactin, was studied through quantitative real-time PCR (qRT-PCR). In order to study the effect of high pressure in the expression of this gene, it was studied in cultures of *B. subtilis* #573 grown in flasks at 37°C and atmospheric pressure, and in pressurized reactor, at 41°C and 47 bar.

The cells from both culture conditions were recovered from the culture medium through centrifugation (10 000 rpm, 10 min, 4°C) and immediately stored at -80°C. Total RNA was extracted using a NZY Total RNA Isolation kit (Nzytech, Lisbon, Portugal), according to the manufacturer's instructions, and stored at -80°C. The amount and the quality of the RNA was assessed by measuring the A260/A280 ratio using a NanoDrop 1000™ (Thermo Fisher Scientific, Waltham, USA), and by analysis on a 0.8% agarose gel. Subsequently, the cDNA was synthesized using a GRS cDNA Synthesis Kit (GRiSP, Porto, Portugal).

The annealing temperature of the primers used in this study was assessed by PCR at a temperature range between 50 and 60°C, using a KAPA Taq PCR Kit (Kapa Biosystems, Basel, Switzerland). A DNA electrophoresis was carried out to check the PCR amplification. The agarose gel 1.3%, was prepared in 1X Tris-acetate-EDTA (TAE) buffer (TAE 50X: 2 M Tris-HCl, 1 M Acetic Acid and 0.05 M EDTA, pH=8.5). The electrophoresis was typically carried out at 90 V for 60 min. The gel was observed using ChemiDoc XRS (Bio-Rad).

The *srfA* gene levels were quantified by qRT-PCR using a RT-PCR CFX96™ System (Bio-Rad, Hercules, USA). The software used was the Bio-Rad CFX Manager 2.0. The qRT-PCR was carried out using the NZY qPCR Green Master Mix (Nzytech, Lisbon, Portugal) according to the manufacturer's instructions. *srfA* transcription levels were normalized to those of the 16S RNA gene. The sequences of the primers used are shown in Table 5.

Table 5. Primers used in this study. According to Jiao and co-workers [4].

Primers	Sequences（5’-3’）
srfA F	TGCTCGCCGCCTATTTGTA
srfA R	GAGATTATACGGATACTTTTGGTGG
16S F	CGCAAGACTGAAACTCAAAGGAA
16S R	ACCCAACATCTCACGACACGA

The qRT-PCR conditions were as follows: 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 20 s. Assessment of the melting curve (65 to 95 °C, Δ=1°C, 5 s) was done to evaluate the specificity of the primers. Neither unspecific product nor primer dimmer formation was observed in the melting curves. The absence of genomic DNA contamination was assessed by including a control (NRT) where the RNA prior to the reverse transcription was used as template. Negative controls were performed replacing the cDNA by ultrapure water. The cycle threshold (Ct) detection of each gene was determined using the standards parameters of the software. The gene expression of srfA was measured and the relative expression ratio was calculated using CFX Manager™ Software Gene Expression Analysis.

2.12 OIL RECOVERY ASSAY USING A SAND-PACK COLUMN

A sand-pack column was used to evaluate the effect of *B. subtilis* #573 in oil recovery at high pressure. The assays were performed using a crude oil sample (CLB) obtained from a Brazilian oil field and provided by PARTEX Oil and Gas. The viscosity of this light-medium oil is 81 ± 5 mPa·s at 40°C.

The vertically oriented stainless-steel column, with a volume of 623 mL, was homogeneously packed with dry sand. The sand was previously sifted with a 0.45 mm sieve and sterilized. After packing the sand tightly, a top sieve and cap were fixed. The caps at both ends of the column were provided with holes for the insertion of stainless steel inlet and outlet tubes. All the fluids were injected into the column upwards using a peristaltic pump, at a constant flow rate of 2 mL/min. The experiment was carried out

in the conditions corresponding to the optimum point obtained from the factorial experimental design (37°C and 46 bar).

The column was first flooded with sterile demineralised water. The pore volume (PV, mL), defined as the empty volume of the column, was calculated by measuring the volume of water required to saturate the column. The porosity (%) of the column was calculated as follows:

$$Porosity = \frac{PV}{Total\ Column\ Volume} \quad (2)$$

In the second step, 200 mL of crude oil (previously sterilized) were injected into the column, and the original oil in place (OOIP, mL) was calculated as the volume of crude oil retained in the column. The initial oil saturation (S_{oi}, %) and the initial water saturation (S_{wi}, %) were calculated as follows:

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

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

Subsequently, the sand-pack column was incubated at 37°C and atmospheric pressure for 48 h. Afterwards it was flooded again with sterile demineralised water to remove the excess of crude oil, until no more oil was observed in the effluent. The amount of crude oil recovered (oil recovered after water flooding (S_{orwf}, mL)) was determined volumetrically. The residual oil saturation (S_{or}, %) was calculated as follows:

$$Sor = \left(\frac{OOIP\ - Sorwf}{OOIP}\right) \times 100 \quad (5)$$

At this point, the residual oil was subjected to the microbial recovery process. The column was inoculated with 170 mL (approximately one pore volume) of a suspension of *B. subtilis* #573 in MSS medium with an optical density (600 nm) of 0.2. After that, the

column was sealed, pressurized at 46 bar using N_2, and incubated for 14 days at 37°C and 46 bar. After the incubation time, the column was flooded again with sterile demineralised water and the volume of crude oil recovered (oil recovered after microbial flooding (S_{ormf}, mL)) was measured volumetrically. The additional oil recovery (AOR, %) was calculated as follows:

$$AOR = \left(\frac{S_{ormf}}{OOIP - S_{ormf}}\right) \times 100 \quad (6)$$

2.13 STATISTICAL ANALYSIS

A two-way ANOVA in conjunction with an Bonferroni multiple comparisons test was used for the statistical evaluation of significant differences; among the different proportions of surfactin isomers produced under the two conditions under study and the standard surfactin; and among the relative expression of *srfA* normalized relative to the 16S reference gene under high pressure and at atmospheric pressure. Statistical analyses were performed in Software GraphPad Prism version 6 (GraphPad Software, San Diego, USA).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 BIOSURFACTANT PRODUCTION UNDER OXYGEN-LIMITING CONDITIONS

The microorganism herein used was *B. subtilis* #573 isolated in a previous work by Gudiña and collaborators [2] from a Brazilian oil field which had a temperature of 40°C and a pressure of 32.4 bar. This microorganism has already been studied as a biosurfactant producer in the context of MEOR, namely its ability to produce surfactin in MSS medium [2] and in MSS medium supplemented with corn step liquor [96]; under aerobic and anaerobic conditions [2][66]; and the additional oil recovery obtained from its use in sand-pack column assays [66]. Furthermore, a crude biosurfactant mixture from this organism was characterized by FTIR, proton nuclear magnetic resonance (1H NMR) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) and it was found to be mainly constituted by different variants of surfactin; C_{13}, C_{14} and C_{15} surfactin [94]. The effect of surfactin produced by this isolate on the viability and proliferation of human breast cancer cells was also evaluated demonstrating a negative influence on cell proliferation [97]. Besides, the ability of this microorganism to degrade hydrocarbons, namely n-alkanes was also demonstrated [2][66].

Initially, a control experiment on serum bottles (Figure 2) was conducted to evaluate microbial growth and biosurfactant production along time under oxygen-limiting conditions at 37°C and atmospheric pressure (section 2.2).

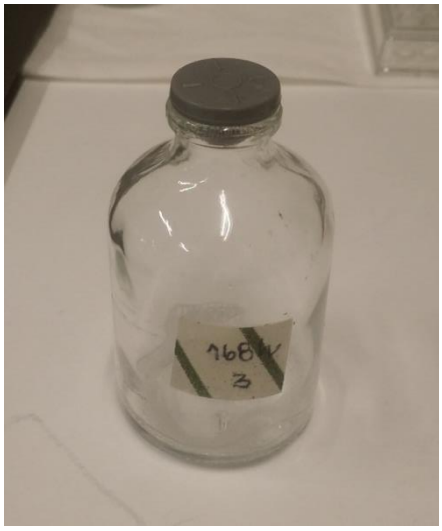


Figure 2. 50 mL serum bottle used in this study.

The microorganism grew under oxygen-limiting conditions up to a maximum of 0.157 ± 0.094 g/L until 24 h of fermentation (data shown in Annex 3) and as can be seen from Figure 3 the surface tension (which is an indicative of biosurfactant production), after 168h of fermentation the values remained constant, drop the surface tension of water from 72mN/m to a value around 26 mN/m at 24h. However, it is important to notice that the surface tension measurements of the cell-free supernatant 10 times diluted decreased along time, however surface tension measurements of the cell-free supernatant 100 times diluted remain constant over time. These diluted surface tension measurements allow to better follow and understand the production of biosurfactants, since at these dilutions a greater variation in surface tension is achieved. Furthermore, it is these diluted surface tension values that allowed to extrapolate the surfactin concentration values in the linear range of the calibration curve (section 2.9). A maximum biosurfactant production was reached at 144h (6 day), considering the lowest value of $ST^{1/10}$, corresponding to a biosurfactant concentration in the fermentative medium of 27.4 mg/L.

In a previous work by Gudiña and coworkers [2], a growth of 0.178 ± 0.041 g/L and a surface tension value of 36.0 ± 2.4 mN/m were obtained for this isolate under anaerobic conditions at 40°C, 120 h and using LB medium. Moreover, using MSS medium, a growth of 0.284 ± 0.057 g/L and a surface tension value of 31.5 ± 0.4 mN/m were reported, suggesting that this microorganism is a good candidate for applications in MEOR. In the present study, the values of surface tension reached were lower than the value mentioned in the previous work.

Indeed, most of the microorganisms that have been reported as potentially useful for MEOR applications belong to the genus *Bacillus*. However, in order to survive the harsh conditions of the wells these organisms must be able to grow and produce biosurfactants under oxygen-limiting conditions and extreme temperatures.

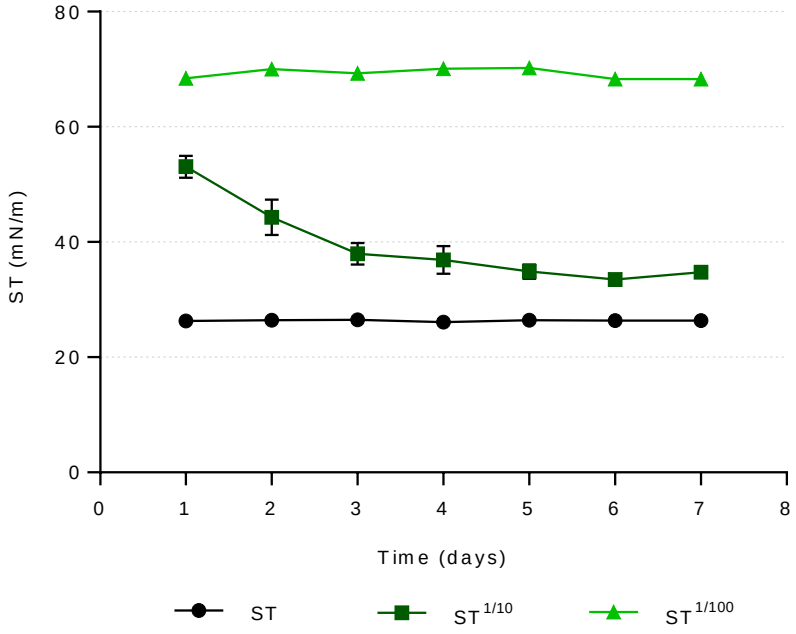


Figure 3. Surface tension values (ST, $ST^{1/10}$ and $ST^{1/100}$, mN/m) obtained throughout the 7-days experiment at 37°C under oxygen-limiting conditions. ST: Surface tension of the cell-free supernatant; $ST^{1/10}$: Surface tension of the cell-free supernatant diluted 10 times with demineralized water; $ST^{1/100}$: Surface tension of the cell-free supernatant diluted 100 times with demineralized water. The results represent the average of 5 measurements ± standard deviation.

Jha and co-workers [39] isolated a microorganism, *B. subtilis* R1, from an oil contaminated desert site in India. This isolate was able to produce biosurfactants at temperatures of 30-45°C, reducing the surface tension to values around 29 mN/m. Sharma and collaborators [36] isolated biosurfactant-producing microorganisms from different oil contaminated sites like automobile shops, petrol pumps and oil refinery, and demonstrated that *B. subtilis* BR-15 is able to reduce surface tension to values below 30 mN/m at 37°C. At a similar temperature, 40°C, Al-Bahry and coworkers [59] studied *B. subtilis* B20 isolated from petroleum contaminated site at the level of the production of biosurfactant and its potential for use in enhancing oil recovery. Reporting that this microorganism was able to lower the surface tension to values ranging from 27 mN/m at 24h.

The studies presented focus on the use of *B. subtilis* strains to produce biosurfactants and the evaluation of their applicability in MEOR. These strains can grow and produce biosurfactants under aerobic conditions.

However, it is important to understand the behaviour of the microorganism under anaerobic conditions or oxygen-limiting conditions such as those occurring in the oil wells. Zhao and coworkers [69] reported that, bacterial strains (*B. amyloliquefaciens* 702 and *P. aeruginosa* 709) isolated from an oil field, under aerobic conditions drop the surface tension of the medium to values around 26 mN/m. However, *B. amylofiquefaciens* 702 under anaerobic conditions at 39°C can drop to 49.5 mN/m the surface tension value, whereas *P. aeruginosa* 709 can drop the surface tension to values around 30 mN/m under these conditions, so it is more suitable and advantageous for *in situ* MEOR applications.

Discussing in situ MEOR application, it is crucial that the microorganism has the ability to grow and produce under oxygen-limiting conditions, such as *B. subtilis* #573 and *P. aeruginosa* 709 [69]. When the aim is an *ex situ* approach, where biosurfactant is produced (in bioreactors) and only then introduced inside the reservoir, oxygen-limiting conditions are not a limiting step in the choice of microorganism. However, several authors focus their studies on an *ex situ* approach, where biosurfactants are produced before under aerobic conditions [36][38][39][41][58].

3.2 STUDY OF BIOSURFACTANT PRODUCTION UNDER AEROBIC CONDITIONS

Experiments were performed under aerobic conditions using shaken flasks containing 200 mL of MSS medium (section 2.3). These experiments were carried out to evaluate the biosurfactant production under aerobic conditions at atmospheric pressure (as a control to assess the effect of pressure on the microbial growth and biosurfactant production), as well as to recover the biosurfactant produced (section 2.8) for further chemical characterization analysis (section 2.10) and to collect cells for the extraction of total RNA (section 2.11). To evaluate cell growth and biosurfactant production two independent experiments were performed and the results are represented in Figure 4 and summarized in Annex 4. The maximum fermentation time, i.e. the time at which the

maximum biosurfactant production is obtained, was found to be 24h. The microorganism grew under aerobic conditions up to a maximum of 0.805 ± 0.030 g/L until 48 h of fermentation. After reaching the minimum surface tension of 26.1 ± 0.1 mN/m at 24h, the values remained constant until the end of the fermentation. The same trend was observed for the cell-free supernatant diluted 10 times with demineralized water and a minimum of 33.3 ± 0.5 mN/m was obtained at 24h, corresponding to a biosurfactant concentration value of 27.9 mg/L. According to these results, the time at which the maximum biosurfactant production is reached is at 24h, and therefore it is not worth to extend the fermentations beyond 48 h under these experimental conditions.

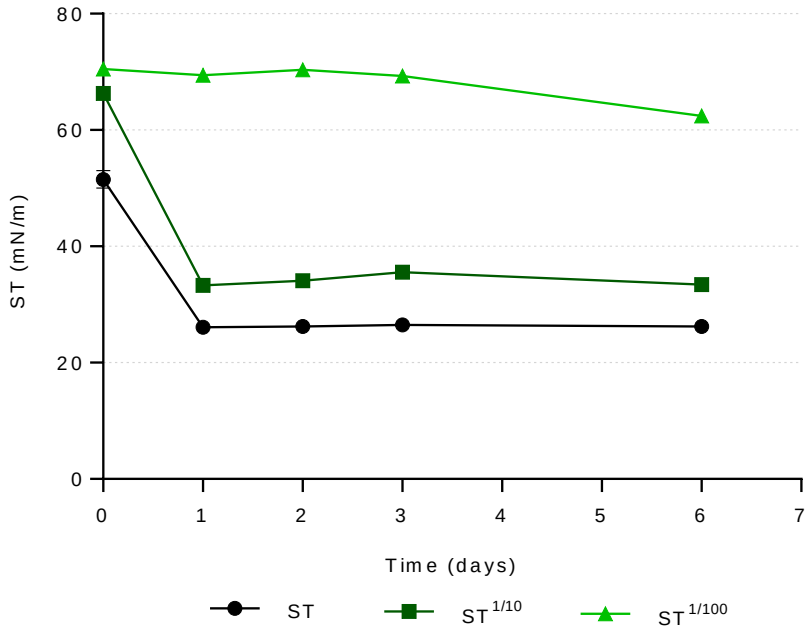


Figure 4. Surface tension values (ST, $ST^{1/10}$ and $ST^{1/100}$, mN/m) obtained throughout the 6-days experiment at 37°C under aerobic conditions. ST: Surface tension of the cell-free supernatant; $ST^{1/10}$: Surface tension of the cell-free supernatant diluted 10 times with demineralized water; $ST^{1/100}$: Surface tension of the cell-free supernatant diluted 100 times with demineralized water. Results represent the average of two independent experiments ± standard deviation.

Growth on aerobic conditions allows a higher biomass value, 0.805 ± 0.030 g/L, when compared to the oxygen-limiting conditions, 0.157 ± 0.094 g/L. However, the production of biosurfactants presents a different time profile. In oxygen-limiting conditions, even if the ST value drop at 24h for values around 26 mN/m, the $ST^{1/10}$ values gradually decrease until reaching a minimum of 144h, as can be seen in Figure 3 and in Annex 3. Under aerobic conditions, a minimum of ST and $ST^{1/10}$ is reached immediately at 24 hours, as can be seen in Figure 4 and in Annex 4. Even if there were differences in production time, in both conditions the same ST value was reached at 24h, around 26 mN/m, being the biosurfactant concentration in the two experiments similar, around 27 mg/L.

In the case of *B. subtilis* #573, the lowest surface tension value (i.e. the highest biosurfactant production) was achieved after 24h of growth under aerobic conditions. However, in most of the *Bacillus* strains reported in the literature, longer incubation times are required to achieve the maximum biosurfactant production. Shakthipriya and co-workers [98] performed a study with a *B. subtilis* thermophilic strain in which maximum production is achieved aerobically in 12 days. Another study [25] reports that *B. subtilis* PT2 reaches a maximum production in 51h at 37°C. Amani and coworkers [99] report that a strain *B. subtilis* strain was able to reduce surface tension to values around 26 mN/m, however the maximum production was reached at 60h under aerobic conditions. Also, a maximum biosurfactant production was achieved at 60 h aerobically in another study [100], and *B. subtilis* LSFM-05 was able to reduce the surface tension to values below 30 mN/m. This represents an advantage of *B. subtilis* #573 over other reported *Bacillus* strains.

Gudiña and coworkers [2], reported the production of biosurfactant from this isolate under aerobic conditions in LB medium at 40°C. A surface tension value of 30.5 ± 0.5 mN/m and a biomass value of 1.686 ± 0.648 g/L were obtained. However, cell growth was lower in this work with the MSS medium when compared to the previous work with LB medium. The LB medium is the rich medium ideal for the growth of microorganisms, however the MSS medium is a less rich medium but allows a good production of biosurfactants. Similarly to what is mentioned in that report [2] herein no relevant differences in the values of surface activity under aerobic and anaerobic conditions were observed, indeed a minimum ST around 26 mN/m and a biosurfactant concentration

around 27 mg/L were found for both conditions. Again, as previously mentioned, this isolate appears to be a good candidate for MEOR applications, and the data presented so far support this claim, both under aerobic and anaerobic conditions.

Two MEOR strategies, *in situ* and *ex situ*, can be adopted for a tertiary oil recovery from a mature reservoir. The direct injection of microorganisms able to produce the desired products *in situ* is more economically advantageous than the *ex situ* strategy (i.e. producing the desired products as biosurfactants and then injecting them in the wells). However, the major challenge to implement *in situ* strategies is to guarantee that the microorganisms can proliferate and be metabolically active under anaerobic conditions and other extreme conditions in the well. This isolate has proven to be able to grow and produce biosurfactants under anaerobic conditions. Indeed, several studies showed that some microorganisms tolerate anaerobic/oxygen-limiting conditions, such as: *B. subtilis* #573 [3], *B. subtilis* l [25], *B. lincheniformis* 421 [42], *B. stearothermophilus* SUCPM #14 [64] and *B. licheniformis* BNP29 [65].

3.3 BIOSURFACTANT PRODUCTION IN BIOREACTOR AT ATMOSPHERIC PRESSURE

Besides being able to grow and produce biosurfactant, the isolate must be capable of surviving the harsh conditions of the well, namely high pressures and temperatures, among others [12]. For this purpose, bioreactor experiments (section 2.4) were first carried out to evaluate the effect of different temperatures (35, 37, 45, 46, 50, 51, 52 and 55°C) on the isolate growth and biosurfactant production. A non-pressurized control experiment at 37°C, corresponding to the R2 experiment, was performed. This experiment, similar to the control experiments performed on the serum bottles and shaken flasks, allowed understand the production and growth profile. Although in the previous experiments a minimum value of surface tension was obtained before 48 h of aerobic fermentation, in this system it was necessary to assess the microorganism behaviour during a longer fermentation period (Annex 5, a graphical representation of these results is available in Figure 5).

The surface tension value of the cell-free supernatant decreased up to 26.6 ± 0.1 mN/m at 24h and remained constant until the end of the fermentation. Likewise, the surface tension values diluted 10 times decreased reaching value of 32.0 ± 0.2 mN/m at 48h, remaining constant until the end of the experiment, with a minimum $ST^{1/10}$ value of 31.3 ± 0.1 mN/m at 144h. The maximum growth of 0.468 ± 0.002 g/L was reached at 72h.

It can be clearly seen that biosurfactants are being produced (i.e. surface tension decreases), along with the cell growth. Contrarily to many secondary metabolites from *bacilli*, which production is induced only when cells deplete one or more essential nutrients, the production of surfactin is actively induced throughout the growth of cells [101]. Based on the results, a fermentation time of 48 h was established.

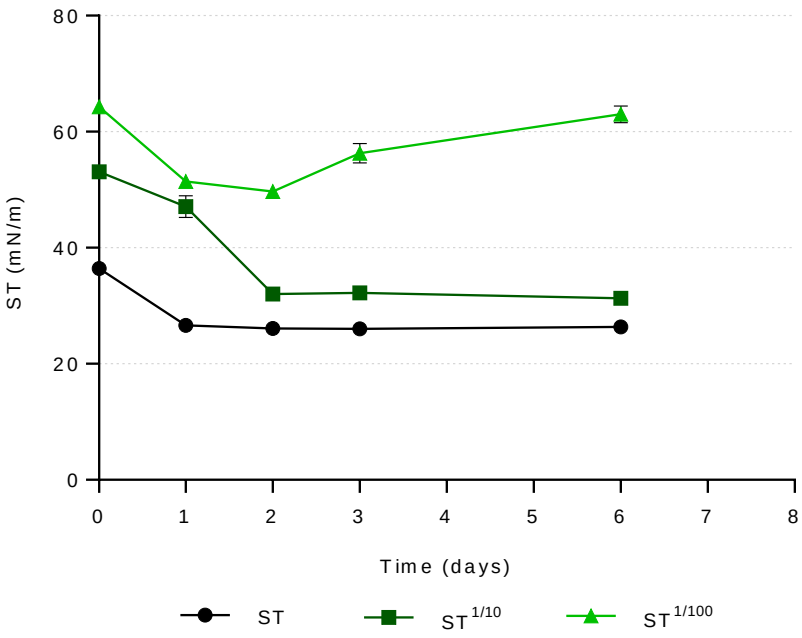


Figure 5. Surface tension values (ST, $ST^{1/10}$ and $ST^{1/100}$, mN/m) obtained throughout the 6-days experiment at 37°C in a non-pressurized reactor. ST: Surface tension of the cell-free supernatant; $ST^{1/10}$: Surface tension of the cell-free supernatant diluted 10 times with demineralized water; $ST^{1/100}$: Surface tension of the cell-free supernatant diluted 100 times with demineralized water. The results represent the average of 5 measurements ± standard deviation.

Afterwards, the experiments at different temperatures were conducted (Table 6). In the temperature range of 35 to 51°C it was possible to observe cell growth. In the experiments R7 and R8, corresponding to the temperatures 52 and 55°C, respectively, no cell growth or production of biosurfactant was observed. At the temperatures of 35, 37, 45 and 46°C not only the microorganism grew but also it was possible to measure surface tension values around 26 mN/m. However, regarding the surface tension 10 times diluted, the $ST^{1/10}$ value reached the lowest value at the temperature of 37°C. Although cell growth was observed at temperatures of 50 and 51°C, the values of surface tension obtained at these temperatures were higher than those verified at lower temperatures, and at 51°C the microorganism could not drop the surface tension of the culture medium. Overall the results suggest that this isolate is able to grow at temperatures up to 51°C but only for temperatures below 50°C a drop of the surface tension could be observed, which is an indication of biosurfactant production. According with the results obtained, it can be concluded that this isolate can grow and produce biosurfactants at temperatures up to 50-51°C.

Most of the studies reported in the literature work with temperatures around 37-40ºC [99][102][69]. Because it is a temperature that is in the oil well under study, or because it is the optimum temperature for the growth and production of biosurfactants. However, the crude oil may be trapped in oil reservoirs with higher temperature conditions. It is therefore important that microorganisms are able to grow and produce biosurfactants over a greater temperature range, so that their applicability is more comprehensive.

A microorganism capable of grow and produce biosurfactants in a wider range of temperature is clearly more advantageous. Like *B. subtilis* #573 can grow and efficiently drop the surface tension to values around 26 mN/m up to 50°C, others *B. subtilis* strains exhibit the same behaviour at these higher temperatures.

Joshi and co-workers [103] isolated a biosurfactant-producing strain, *B. subtilis* 20B. This isolate was able to grow to temperatures of 55°C. Also, Jha and co-workers [39] found that *B. subtilis* R1 has been able to grow up to 50°C, but can only produce biosurfactants up to 45°C. The authors suggest these strains as useful for MEOR applications at higher temperatures.

Table 6. Growth and surface tension values of experiments R1 to R8 performed under atmospheric pressure in a non-pressurized bioreactor at 8 different temperatures. Cell growth is assessed as YES or NO; ST: Surface tension of the cell-free supernatant; $ST^{1/10}$: Surface tension of the cell-free supernatant diluted 10 times with demineralized water. The time at which the maximum biosurfactant production occurs is indicated. The surface tension of water is around 72 mN/m. Results represent the average of 5 measurements ± standard deviation.

Experiment	Temperature (°C)	Growth	Time (h)	ST (mN/m)	$ST^{1/10}$ (mN/m)
R1	35	Yes	48	26.0 ± 0.1	34.4 ± 0.6
R2	37	Yes	48	26.1 ± 0.1	32.0 ± 0.2
R3	45	Yes	48	26.2 ± 0.1	33.8 ± 0.3
R4	46	Yes	24	26.3 ± 0.1	43.5 ± 2.7
R5	50	Yes	24	28.9 ± 1.2	53.5 ± 1.7
R6	51	Yes	-	54.4 ± 2.2	69.6 ± 0.5
R7	52	No	-	53.8 ± 1.2	67.4 ± 0.3
R8	55	No	-	52.8 ± 2.7	69.7 ± 0.1

3.4 BIOSURFACTANT PRODUCTION IN PRESSURIZED REACTOR

One of the main goals of the current work is to evaluate and understand the growth and metabolism of this isolate under high pressures, similar to those that exist in oil reservoirs, aiming at its use in MEOR to improve the additional oil recovery. A pressurized reactor (section 2.4) was used to perform these experiments under pressure. Some authors affirm that under pressure the indigenous microorganisms grow more slowly and that fewer cells are able to adapt to these conditions [92], while others

do not observe negative effect of pressure on indigenous microorganisms [70][88]. Initially, an experiment was performed at 40°C and 40 bar to evaluate if the pressure had an effect in the biosurfactant production profile, as literature suggests that microorganisms can grow more slowly under pressurized conditions.

The results obtained in this control experiment are shown in Annex 6 and a graphical representation of these results is available in Figure 6. The isolate was able to drop the surface tension of the culture medium to an ST value of 26.1 ± 0.2 mN/m and an $ST^{1/10}$ value of 40.7 ± 2.6 mN/m after 24 h of fermentation. At 48 h, an ST value of 26.6 ± 0.2 mN/m and an $ST^{1/10}$ value of 39.7 ± 3.0 mN/m was obtained, remaining constant throughout the 6 days of fermentation. A biomass value estimate of 0.677 ± 0.021 g/L was determined at 24 h, which is in accordance with the biomass values reported in the previous sections. Similar trends for growth and biosurfactant production were found as compared to the previous control experiments.

Besides the microorganisms being able to tolerate adverse conditions of the oil wells as high temperatures and oxygen-limiting conditions, they must proliferate and produce biosurfactants under high pressures. In this control assay at 40°C and at 40 bar, *B. subtilis* #573 was able to grow and produce biosurfactants. Biomass and surface tension values were similarly reached, similar to previous control experiments. The study of the effect of pressure on useful microorganisms for MEOR applications is scarce and a long way is still needed to better understand the effect of this parameter, which can not be neglected when it is discussed *in situ* MEOR applications.

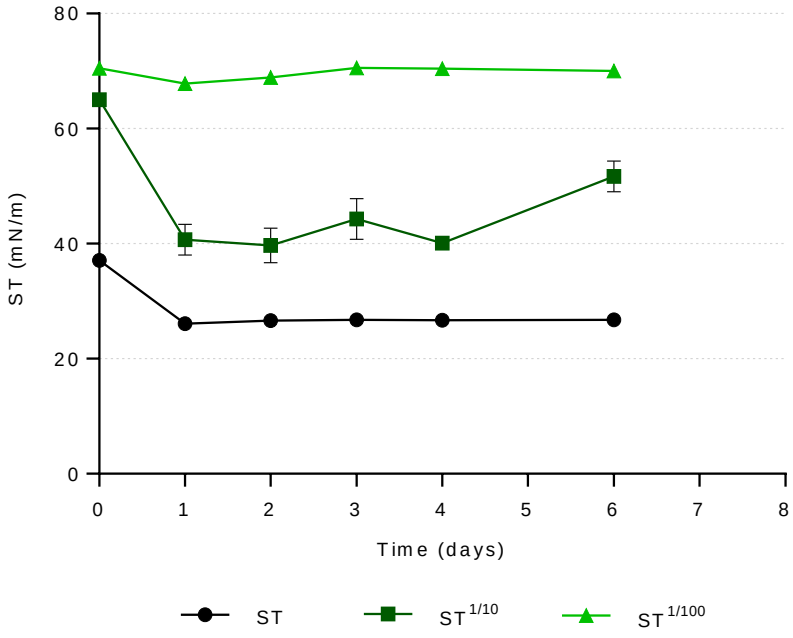


Figure 6. Surface tension values (ST, $ST^{1/10}$ and $ST^{1/100}$, mN/m) obtained throughout the 6-days experiment at 40°C and 40 bar in a pressurized reactor. ST: Surface tension of the cell-free supernatant; $ST^{1/10}$: Surface tension of the cell-free supernatant diluted 10 times with demineralized water; $ST^{1/100}$: Surface tension of the cell-free supernatant diluted 100 times with demineralized water. The results represent the average of 5 measurements ± standard deviation.

Yue and co-workers [92] performed experiments and observed that endogenous mixed bacterium from Shengli Oil Field, grow more slowly under high pressures (namely 100 bar) when compared to atmospheric pressure conditions. However, even under high pressure conditions the displacement of remaining oil is observed due to the concentration of biosurfactant produced by the metabolism of the microorganisms. Another study [81] focused on the study of bioremediation of toxic solvents to enhanced oil recovery processes by stimulating resident bacteria of oil reservoirs (Thauera sp. TK001). An increase in oil recovery was observed in a high pressure bioreactor model (namely 27.2 bar) when compared to low pressure model, due to the action of the microorganism. The authors suggest that increased oil recovery is due to bioplugging or increased oil emulsification, which contributed to MEOR. Formation the oil water microemulsion improves mobility of entrapped oil and hence MEOR [104]. Also Zhao

and collaborators [69] demonstrated that *P. aeruginosa* 709 can grow under anaerobic conditions and high pressures (100 bar) and efficiently produced biosurfactant under these conditions, reducing the surface tension to 33.7 mN/m.

B. licheniformis BNP29 was reported by Yakimov and co-workers [65] as a producer of biosurfactants, polymers and acids under anaerobic conditions at 47°C and 95 bar. All these reported studies examining the effect of pressure, culminated in positive assays for additional oil recovery. These authors suggest that the pressure does not have a negative effect on the metabolism of oil reservoir microorganisms. These studies are in agreement with the behaviour of *B. subtilis* #573 under high pressure conditions (40 bar).

3.5 EXPERIMENTAL DESIGN TOWARDS THE STUDY OF BIOSURFACTANT PRODUCTION UNDER THE SIMULATED OIL RESERVOIR CONDITIONS

A response surface methodology (RSM) with a central composite design (CCD) was used to find an optimum point of temperature and pressure at which the surface tension is minimized by the biosurfactant from *B. subtilis* #573. Temperature (X_1) and pressure (X_2) are the independent factors selected to find the optimum point that maximizes the biosurfactant production. The factors range according to the levels of the factorial design are summarized in Table 7.

Table 7. Central composite design factors and corresponding ranges according to the experimental levels.

			Levels		
Factor	-1.41	-1	0	+1	+1.41
			Temperature (°C) / Pressure (Bar)		
Temperature(X_1)	35.3	37	41	45	46.7
Pressure(X_2)	3.8	10	25	40	46.2

From section 3.3 where the temperature (35 to 55 °C) was evaluated in the growth and biosurfactant production by *B. subtilis* #573, it was observed that the microorganism is able to grow up to 50 °C and reduce the surface tension to values below 30 mN/m. From the previous section it was possible to verify that a pressure of 40 bar not affect the production and the growth of the microorganism. Additionally, this microorganism was isolated from a Brazilian oil field which has a temperature of 40°C and a pressure of 32.4 bar. Based on these data it was possible to delimit the factorial design from 37 to 45°C and from 10 to 40 bar.

The design matrix and the respective observed and predicted results of the RSM experiments to determine the effect of temperature and pressure on the response factor, surface tension ($ST^{1/5}$), are shown in Table 8.

Table 8. Matrix of the central composite design showing the values of the different factors for the experimental design and the results (predicted and observed) of the experimental runs. $ST^{1/5}$: Surface tension of the cell-free supernatant diluted 5 times with demineralized water.

Experiment	Temperature (°C)	Pressure (Bar)	$ST^{1/5}$ (mN/m)	
	X_1	X_2	Predicted	Observed
1	37.0	10.0	32.4	30.9
2	37.0	40.0	31.8	31.7
3	45.0	10.0	34.0	33.5
4	45.0	40.0	34.3	35.1
5	35.3	25.0	32.1	33.1
6	46.7	25.0	35.0	34.6
7	41.0	3.8	32.8	34.1
8	41.0	46.2	32.6	32.0
9	41.0	25.0	39.1	39.6
10	41.0	25.0	39.1	38.6

The regression analysis of this study indicated that the temperature in the linear and quadratic term (X_1), (X_1^2) and pressure (X_2^2) are significant factors ($p < 0.1$) in the production of biosurfactants by this isolate, as can be seen in the Table 9.

Table 9. Coefficient regression values for the model that describes the effects of the temperature and the pressure in the production of biosurfactant which is given by the surface tension ($ST^{1/5}$).

Factor	Terms	Regression coefficients	Standard error	p-value	Significant (p<0.1)
X_1	Linear	14,45640	0.46199	0.09292	Yes
X_1^2	Squared	-0,17422	0.61115	0.01033	Yes
X_2	Linear	0,56692	0.46199	0.88493	No
X_2^2	Squared	-0,14167	0.61115	0.00644	Yes
$X_1 X_2$	Interactive	0,33333	0.65335	0.77479	No

The interaction between the two factors and pressure in the linear term were found to be non-significant. The results were then fitted to a second order polynomial equation that describes the surface tension (response factor) based on the effects of the significant factors:

$$Y = -269{,}48594 + 14.45640X_1 - 0.17422X_1^2 - 0.14167X_2^2 \quad (7)$$

Where Y is the surface tension ($ST^{1/5}$), X_1 is the temperature in the linear term, X_1^2 is the temperature in the squared term and X_2^2 is the pressure in the squared term. The regression equation obtained from ANOVA showed that R^2 was 0.9065, indicating

that 90.7% of the variability in the responses could be explained by the second order polynomial equation given above (Equation 7). In order to find the optimum point, a three-dimensional response surface graph (Figure 7) was obtained by plotting the response (surface tension, $ST^{1/5}$) as a function of the two independent factors, and the model equation was minimized to find the lowest value of surface tension (i.e. the higher production of biosurfactant).

The optimum point obtained corresponds to a temperature of 37°C and a pressure of 46 bar with an estimated surface tension ($ST^{1/5}$) of 29.7 mN/m. To validate the optimal point, three extra experiments were run at these conditions and a value of 32.3 ± 2.4 mN/m was obtained, which falls in the range of values close to that predicted by the model. In Figure 8 a picture of one of the three validation experiments shows the amount of foam formed during fermentation under high pressures when the pressurized reactor was opened. This formed foam is due to the presence of biosurfactants produced by microorganism *B. subtilis* # 573.

In addition to the mentioned optimum point, several other optimum points are found, as can be seen in Figure 2. The optimum points found vary along the RSM completing a circle in the yellow region. The inflection point of the curvature corresponds to the point at which the lowest concentration of biosurfactant can be found (i.e. the highest value of $ST^{1/5}$). And these optimum points (below the yellow region of the RSM) vary according to the temperature at the pressure, that is, this microorganism presents a great versatility with respect to these two factors. The optimal point chosen, presents the highest pressure of the optimum points found by the model, however, other combinations can be used, varying the temperature and the pressure, and this versatility of the microorganism is a great advantage found because it allows a future applicability in reservoirs with conditions of temperature and pressure that the same effect can be observed.

All these experiments were conducted under pressurized conditions and it is important to mention that, in all of them it was possible to observe cell growth and reduction of the surface tension values (up to 26 mN/m, Table 10). To follow the production of biosurfactants, dilutions of the supernatants were carried out 5 times and, in this dilution range it is possible to evaluate the surface tension ($ST^{-1/5}$) differences (Table 8). According to the data obtained from the Doe analysis, the pressure (quadratic

term) and temperature (linear and quadratic term) are significant factors ($p < 0.1$) in the biosurfactant production by this microorganism. Comparing the ST results from the factorial design assays with the ones obtained in the other reactor models, it was found that even under pressurized conditions, the microorganism is able to drop the surface tension of the medium to values around 26 mN/m (Table 10).

Table 10. Results obtained in the 10 experimental runs: ST: Surface tension of the cell-free supernatant; Time: The time at which the maximum production of surfactin is reached; [Surfactin]: Surfactin concentration considering the lowest value of $ST^{1/5}$; [Biomass]: Maximum concentration of biomass attained in each experiment.

Experiment	ST (mN/m)	Time (h)	[Surfactin] mg/L	[Biomass] g/L
1	26.1 ± 0.18	24	34.45	0.443
2	26.3 ± 0.17	24	32.12	0.592
3	26.3 ± 0.27	24	27.43	0.666
4	26.1 ± 0.07	24	23.84	0.545
5	25.4 ± 0.05	48	28.41	0.289
6	25.3 ± 0.17	24	24.90	0.328
7	25.9 ± 0.15	24	26.02	0.405
8	25.6 ± 0.12	24	31.28	0.585
9	26.3 ± 0.19	24	16.06	0.233
10	25.8 ± 0.07	24	20.71	0.441

When comparing the maximum biomass values reached in the pressure experiments, a great variability of results was observed, never exceeding a maximum of 0.666 g/L. In the shaken flask experiments biomass values of 0.805 g/L and values of 1.236 g/L and 1.084 g/L (date not shown) are reached. Even if the microorganism grows less under high pressure conditions, it reaches similar values of ST.

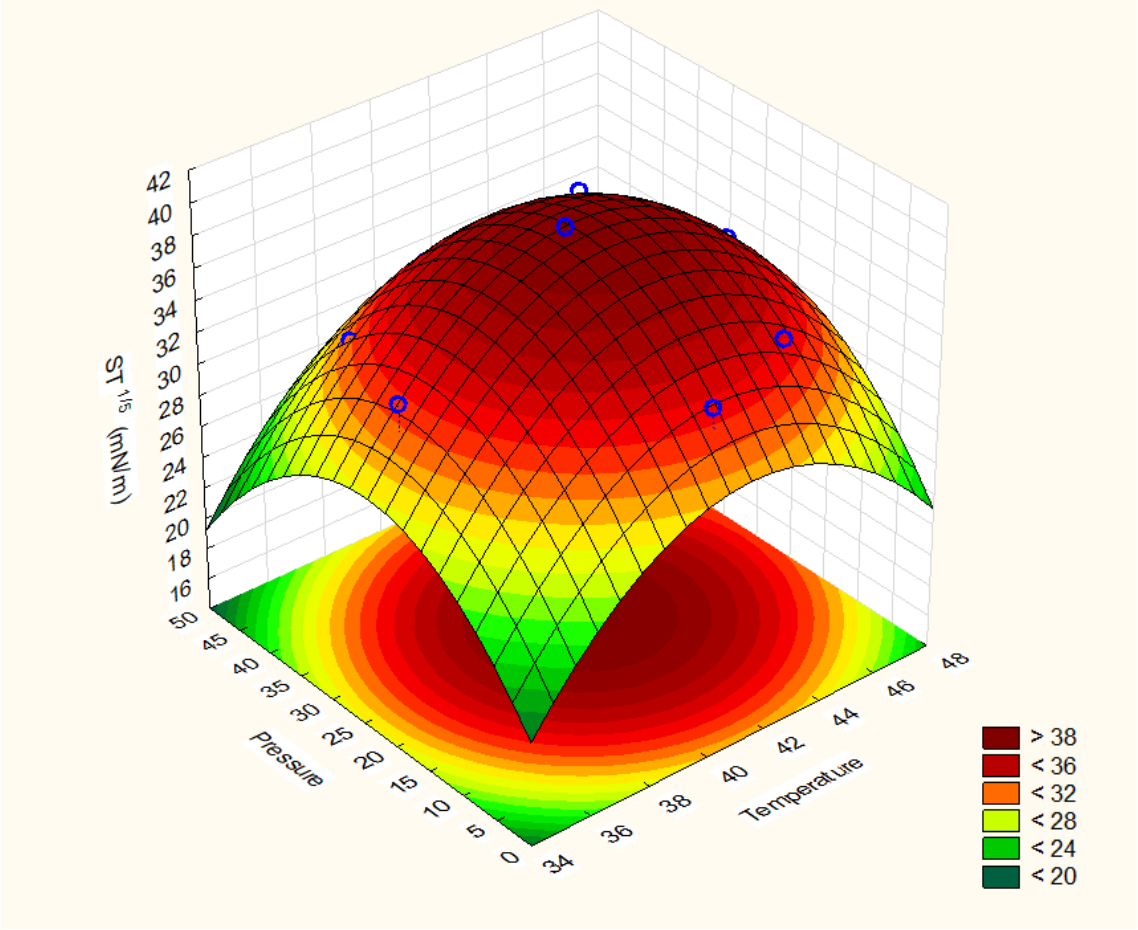


Figure 7. Response surface representing the effect of Pressure (bar) and Temperature (°C) on the surface tension (mN/m).

B. subtilis #573 under pressurized conditions exhibits lower cell growth than under oxygen-limiting conditions at atmospheric pressure. These data are in agreement with is reported by Yue and co-workers [52][92] in which it observed that the endogenous mixed bacterium, grows slowly under high pressures when compared to atmospheric conditions, however observe that the concentration of biosurfactant reached is able to displace trapped oil. Also Zhao and collaborators [69] demonstrated that *P. aeruginosa* 709 can grow under anaerobic conditions and high pressures (100

bar) and efficiently produced biosurfactant under these conditions, reducing the surface tension to 33.7 mN/m. With these results, it is expected that, when *B. subtilis* #573 acts in *in situ* MEOR strategy, there is an increase in the oil recovery, as verified by some authors with microorganisms isolated from oil reservoirs [92][65][69].

Figure 8. Picture of one of the three validation experiments, where the amount of foam formed is visible due to the presence of biosurfactants produced by *B. subtilis* # 573.

3.5 BIOSURFACTANT CRITICAL MICELLAR CONCENTRATION

The biosurfactants produced by *B. subtilis* #573 in flask (37°C, atmospheric pressure) and pressurized reactor (41°C, 47 bar, these conditions were used, not the optimum point, because the optimum point was not yet known) were extracted, purified and freeze-dried (section 2.8). The product obtained from each experiment was then used to prepare biosurfactant solutions with concentrations ranging from 1 to 1000 mg/L and the CMC was determined by plotting the surface tension as a function of the logarithm of biosurfactant concentration, as can be seen in Figure 9. Two independent experiments were performed for each condition. Surfactin concentration obtained in the experiment, estimated using a calibration curve (surface tension *vs.* logarithm of surfactin concentration), as well as the CMC values are gathered in Table 11.

The standard surfactin from Sigma-Aldrich showed the most efficient performance, with a CMC value of 9.31 mg/L. The CMC values obtained in the different experiments, although higher than the one for the commercial surfactin (so, not as pure as the commercial surfactin), are low and suggest that the biosurfactants extracted have high purity levels. The extraction and purification method (section 2.8) herein used is a simple, effective and efficient method to recover surfactin produced by *B. subtilis #573*.

However, these high levels of purity differ from experiment to experiment. Different values of CMC and amount of crude extracted biosurfactants were found for the four experiments. Considering the experiments performed in shaken flask it is possible to verify that the amount of biosurfactant crude extracted is different in both cases (Table 11); However, the assay volume was the same.

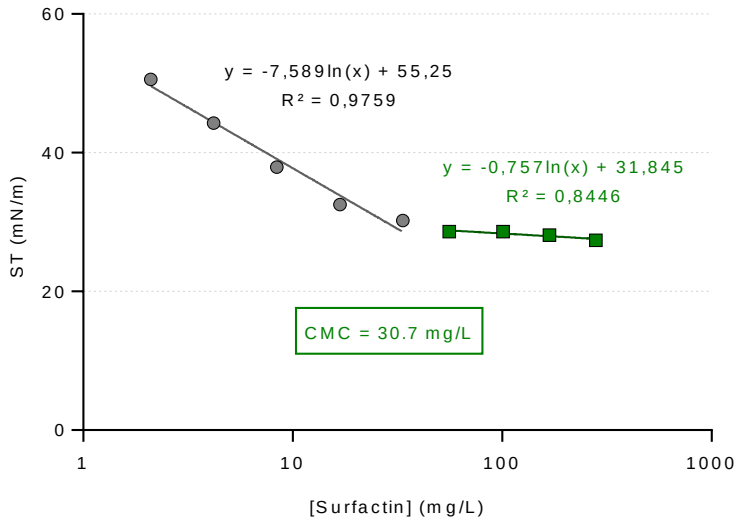
A higher amount of crude biosurfactants extracted from 40.6 mg in the Flask1 experiment resulted in a higher CMC value (30.7 mg/L); and a lower amount of biosurfactant crude extracted from 16.5 mg in the Flask 2 experiment led to a lower CMC value (16.5 mg/L). And the same trend is observed in the other experiments, the higher the amount of crude biosurfactants extracted the higher the CMC value was found. This method of solvent extraction is not 100% specific and efficient to recover surfactin, however it is presented as a simple, inexpensive and efficient method for the extraction and recovery of biosurfactants. When extracting the biosurfactants other impurities are also extracted, thus a greater amount of crude biosurfactants extracted

does not imply that it is a greater amount of surfactin with a high level of purity. For the obtained data a smaller quantity of crude biosurfactants extracted (i.e. an amount with less percentage of impurities and a greater percentage of surfactin) infers lower values of CMC, that is, the extraction process was more efficient in those cases in which smaller amount of crude biosurfactants and a lower value of CMC was obtained.

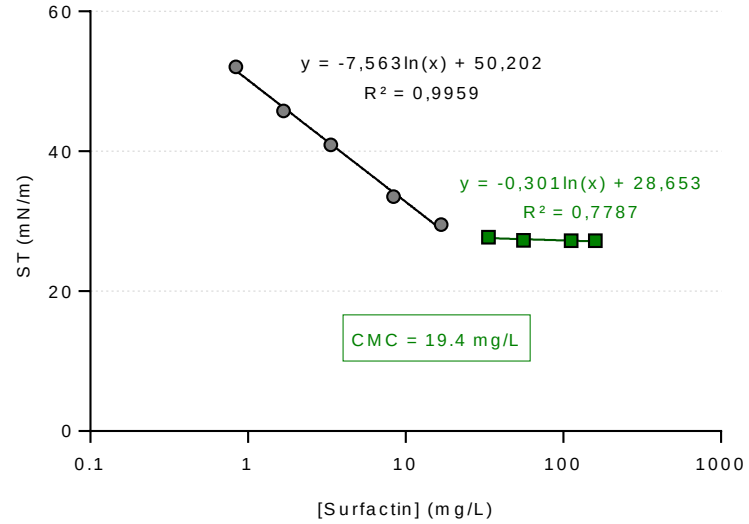
Table 11. Surfactin concentration (mg/L) determined, amount of crude biosurfactant extracted (mg) and critical micellar concentration (CMC) (mg/L) of the biosurfactants produced by *B. subtilis* # 573 in flask (37°C, atmospheric pressure) and pressurized reactor (41°C, 47 bar). The CMC of the standard commercial surfactin was also determined. The results represent the mean of 5 measurements ± standard deviation for the values of surfactin concentration.

Experiment	Reactor	[Surfactin] (mg/L)	Biosurfactant crude (mg)	CMC (mg/L)
Flask1	Shake Flask	29.7 ± 1.5	40.6	30.7
Flask2	Shake Flask	34.2 ± 0.9	16.5	19.4
RP1	Pressurized Reactor	24.9 ± 2.8	28.6	23.0
RP2	Pressurized Reactor	34.8 ± 1.1	8.4	15.0
Standard surfactant (Sigma-Aldrich)		CMC = 9.31 mg/L		

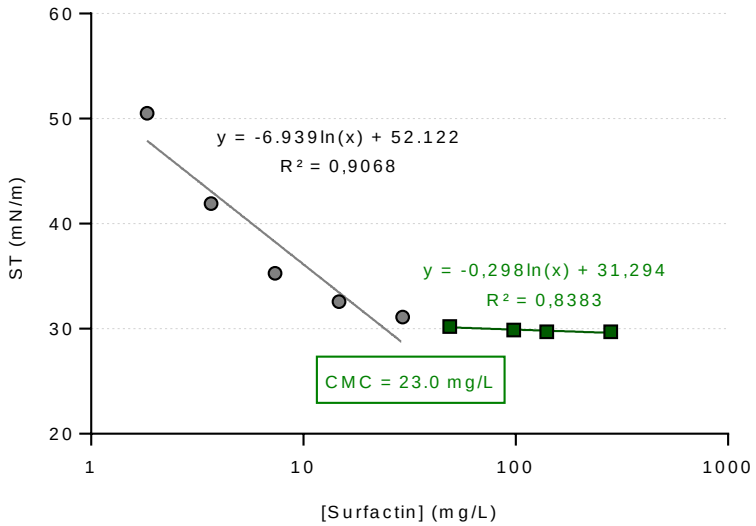
a)


y = -7,589ln(x) + 55,25
R² = 0,9759
y = -0,757ln(x) + 31,845
R² = 0,8446
CMC = 30.7 mg/L
ST (mN/m)
[Surfactin] (mg/L)
60
40
20
0
1
10
100
1000

b)


y = -7,563ln(x) + 50,202
R² = 0,9959
y = -0,301ln(x) + 28,653
R² = 0,7787
CMC = 19.4 mg/L
ST (mN/m)
[Surfactin] (mg/L)
60
40
20
0
0.1
1
10
100
1000

c)


y = -6.939ln(x) + 52.122
R² = 0,9068
y = -0,298ln(x) + 31,294
R² = 0,8383
CMC = 23.0 mg/L
ST (mN/m)
[Surfactin] (mg/L)
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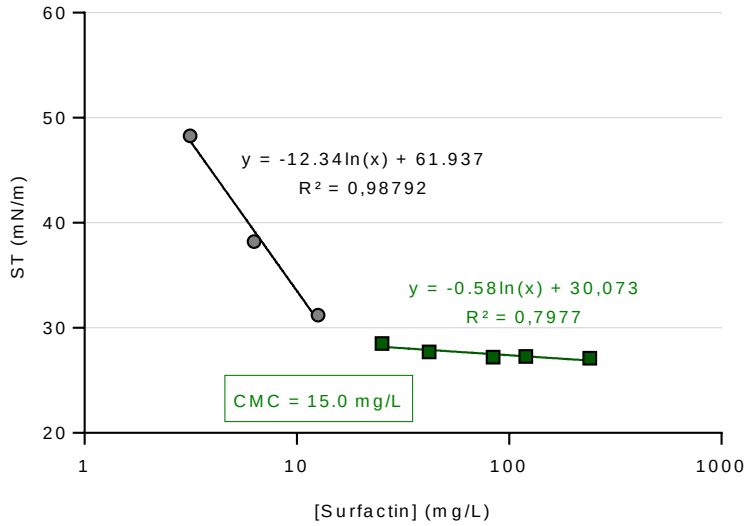
50
40
30
20
1
10
100
1000

d)



e)

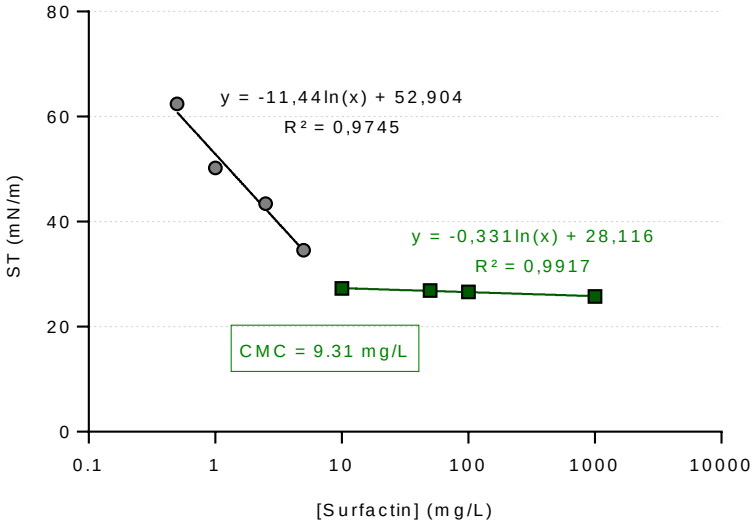


Figure 9. CMC values determined by plotting the surface tension as a function of the logarithm of biosurfactant concentration, for each experiment and the standard surfactin. **a)** experiment Flask1 (flask, 37°C, atmospheric pressure); **b)** experiment Flask2 (flask, 37°C, atmospheric pressure); **c)** experiment RP1 (pressurized reactor, 41°C, 47 bar); **d)** experiment RP2 (pressurized reactor, 41°C, 47 bar) and **e)** standard surfactin.

When comparing crude biosurfactant amount obtained, the values differ because in some experiments the extraction and recovery was more efficient than in others experiments, thus the CMC value also varies. Even a CMC value of 15 mg/L was obtained in the RP2 experiment and 30.7 mg/L in the Flask1 experiment, a high pressure condition and a condition at atmospheric pressure, respectively, these values are influenced by the purity level of the biosurfactants extract extracted and purified.

Gudiña and coworkers[2] obtained biosurfactants using the same method of extraction and purification of the same isolate and a CMC value of 30 mg/L was found. Several authors obtained CMC values between 10 and 63 mg/L for biosurfactants produced by different *B. subtilis* strains [105],[106],[107].

The values of CMC reported for the different assays were higher or very close to the CMC value herein obtained, and low biosurfactant concentrations (i.e. close to the CMC value) have been reported to be sufficient to mobilize entrapped oil [25], [108]. At 47 bar and at atmospheric pressure, no difference in the reduction of the value of surface tension reached (i.e., similar biosurfactant concentrations), also it was not observed through the recovery and purification of biosurfactants differences between the two conditions under study.

3.7 BIOSURFACTANT CHEMICAL CHARACTERIZATION

The biosurfactants produced by *B. subtilis* #573 in flask (37°C, atmospheric pressure) and pressurized reactor (41°C, 47 bar), and the commercial surfactin (99% purity, Sigma-Aldrich, USA), were characterized by liquid chromatography–mass spectrometry (LC-MS) and Fourier transform infrared spectroscopy (FTIR). A fast and direct characterization of the biosurfactants mentioned above was performed using a FTIR analysis (Figure 10). The spectra obtained for the two conditions under study, namely the experiment Flask1 (shaken flask, 37°C, atmospheric pressure), experiment RP1 (pressurized reactor, 41°C, 47 bar) and standard commercial surfactin showed a great similarity among them.

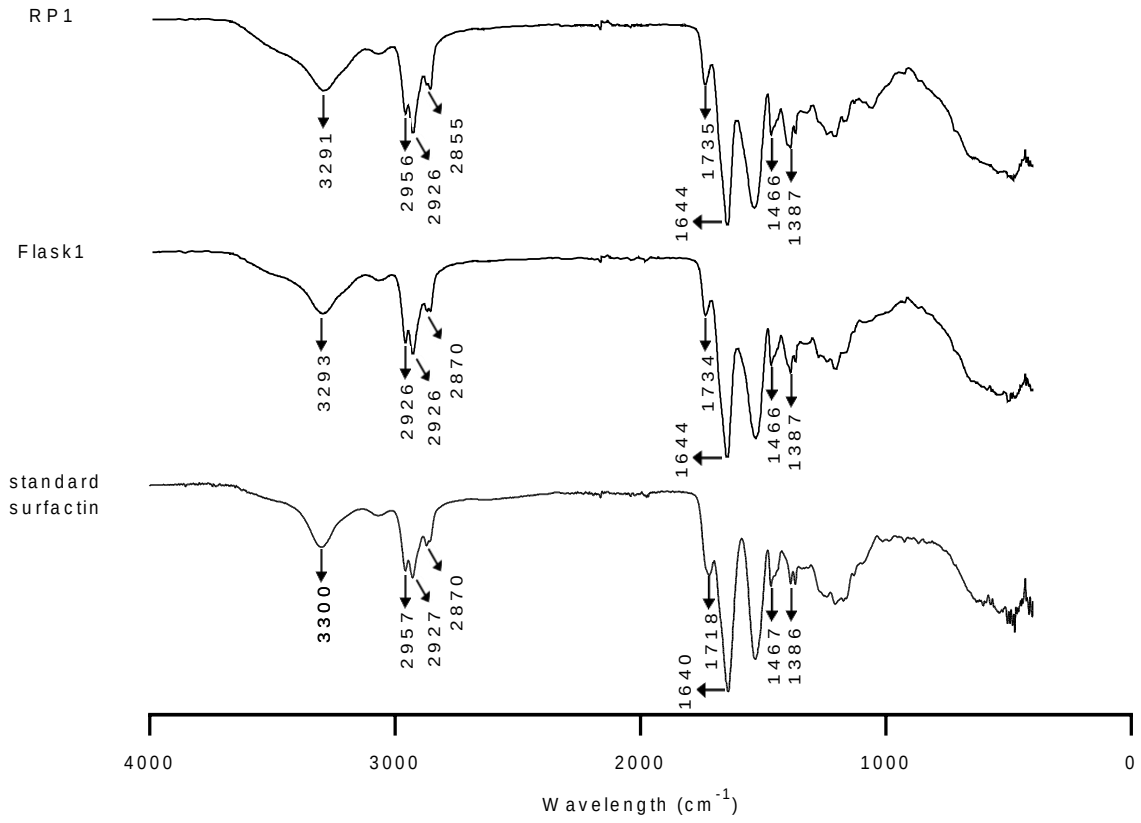


Figure 10. FTIR spectra of the biosurfactants produced by *B. subtilis* #573 in shaken flask (37°C, atmospheric pressure), experiment Flask1, and pressurized reactor (41°C, 47 bar), experiment RP1, and commercial surfactin (standard).

This analysis reveals that the biosurfactants produced under both conditions present the main characteristic groups of lipopeptide biosurfactants, indicating the presence of aliphatic hydrocarbon, as well as a peptide fraction. The most important absorption bands were assigned by comparison with spectra obtained from the literature [37][66][100][109]. In the spectrum shown in Figure 10, eight main bands can be observed.

The absorbance of N-H stretching bond at 3300 cm^{-1} indicates the presence of a peptide residue. Bands between 2957 cm^{-1} - 2855 cm^{-1} and 1467 cm^{-1} - 1386 cm^{-1}, corresponding to the C–H (CH_3) and (CH_2) stretch, can be associated with the lipopeptide

portion of the molecule. Between 1735 cm^{-1} - 1718 cm^{-1}, a band is observed that can be related to the absorption of C=O groups. Bands between 1644 cm^{-1} - 1640 cm^{-1} are related to CO–N stretch points to the amide group. These results suggest that the biosurfactant produced by *B. subtilis* #573 under the two conditions studied have a purity similar to that of commercial surfactin, since all the spectra have the same functional groups and the same profile, that is, there are no other impurities in the samples.

Each peak obtained in the chromatograms was then analyzed by MS, and the peaks obtained in the mass spectra (data not shown) were analyzed and corresponded to pseudomolecular ions ([M + H] +) formed from the biosurfactant molecules. The spectra obtained show similarity between themselves and were similar to that reported with surfactins produced by *B. subtilis* strains [94][36][106] .The peaks at m/z values 994, 1008, 1022, 1036 and 1050 differ by 14 units (equivalent to a CH_2 group) indicating the presence of homologues series. Hence, biosurfactants produced by *B. subtilis* #573 were found to be surfactin isoforms, namely isomers of surfactin with C_{12}, C_{13}, C_{14}, C_{15} and C_{16} acyl chains.

By integrating the peaks obtained in the LC-MS chromatograms (Figure 12) it is possible to obtain the peak area corresponding to each surfactin isoform and thus to calculate the relative percentage of each isoform in the two conditions under study, as can be seen in figure 11. When a Bonferroni multiple comparisons test (section 2.13) was applied to the results, no significant differences were found, with a confidence level of 95%. Statistically significant differences in the relative percentage of C_{12} are found when the confidence level is lowered from 95% to 90%.

Youssef *et al.* [110] showed that C_{14} surfactin exhibits the optimum hydrophilic–lipophilic balance required for optimum surface activity. Also, Bacon *et al.* [111] reported that surfactins with C_{15} acyl chains possess a higher surface activity. Liu and coworkers[41] have shown that in their studies with a *B. subtilis* strain, the more C_{15} surfactin content in the products, oil wash efficiency and oil displacement efficiency were better. In this study the percentage of C_{13} C_{14} and C_{15} are higher than the relative percentages of C_{12} and c_{16} found in both conditions. A higher percentage of C_{15} and even C_{14} in the crude constitution of biosurfactants allows better values of surface activity.

This higher percentage of isoforms with better surface activity helps to explain the low values of surface tension and the values of CMC found in both, flasks and in pressurized reactor.

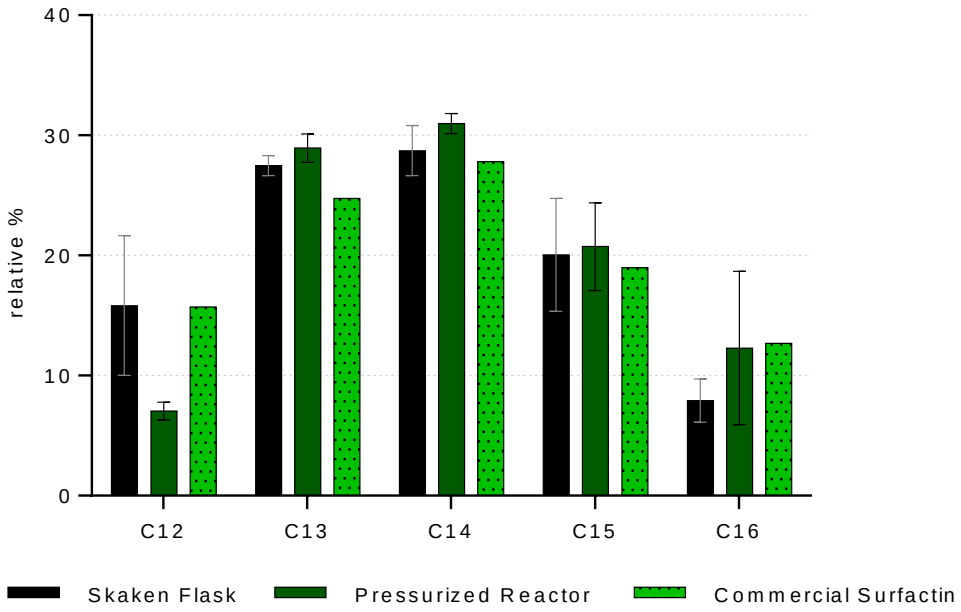
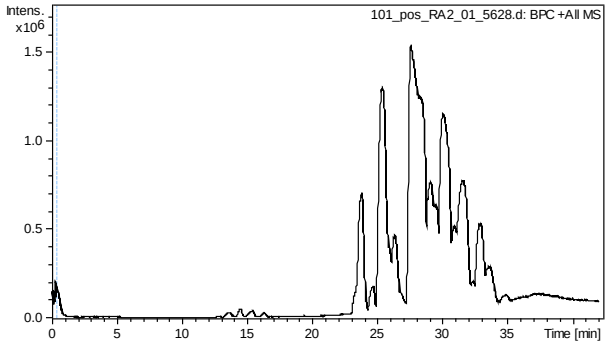
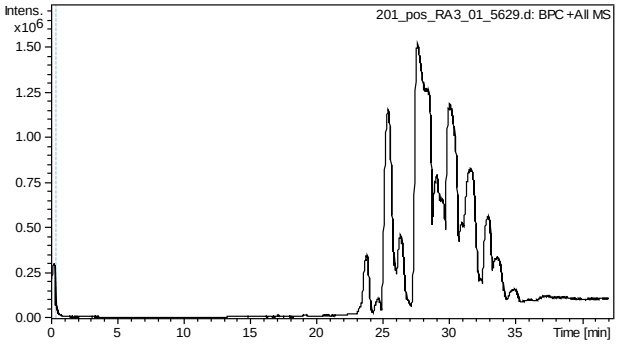


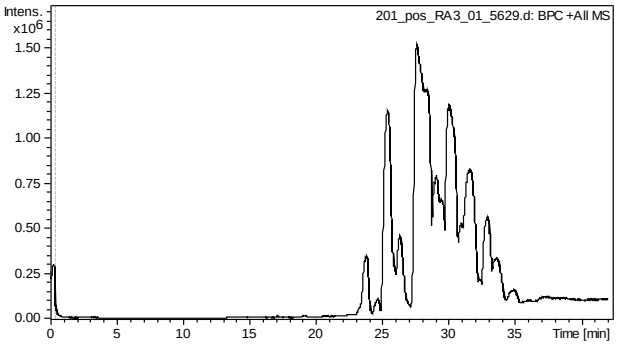
Figure 11. Relative percentage of different surfactin isoforms found in the two different conditions under study: shaken flask (37°C, atmospheric pressure) and pressurized reactor (41°C, 47 bar); and commercial surfactin (standard). The results represent the mean of 2 independent experiments ± standard deviation, for extracted biosurfactants.



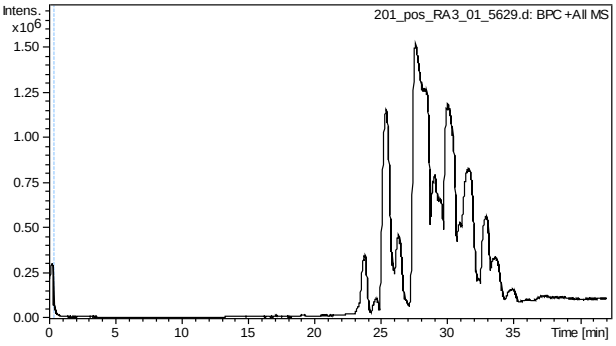
UPLC (TIC) - **Flask1**



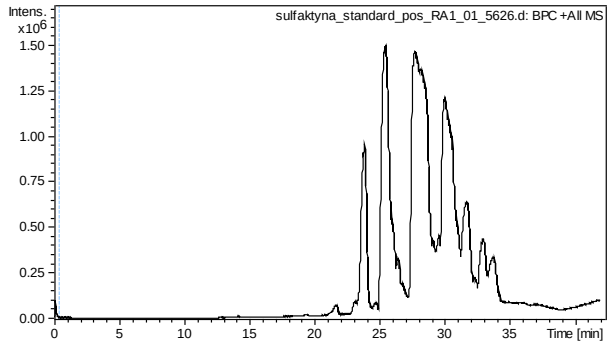
UPLC (TIC) - **Flask2**



UPLC (TIC) - **RP1**



UPLC (TIC) - **RP2**



UPLC (TIC) – **Commercial Surfactin**

Surfactin Isoform	m/z value [M+H] +
C_{12}	994
C_{13}	1008
C_{14}	1022
C_{15}	1036
C_{16}	1050

Figure 12. LC-MS chromatograms (TIC) obtained from the analysis of biosurfactants produced by *B. subtilis* #573 in flask (37°C, atmospheric pressure) (Experiment Flask1 and Flask2) and pressurized reactor (41°C, 47 bar) (Experiment RP1 and RP2, and the commercial surfactin (99% purity, Sigma-Aldrich, USA) and assignments of surfactin isoforms for pseudomolecular ions detected by LC-MS.

3.6 ANALYSIS OF THE EXPRESSION OF THE GENE *SRFA*

Surfactin is synthesized by surfactin synthase (SrfA), a seven module non-ribosomal peptide synthase, and then transported outside the cells during fermentation[112] . The expression of the gene *srfA*, involved in the synthesis of surfactin, was studied through qRT-PCR. To study the effect of high pressure in the expression of this gene, it was studied in cultures of *B. subtilis* #573 grown in flasks at 37°C and atmospheric pressure, and in pressurized reactor, at 41°C and 47 bar (section 2.11). First, the annealing temperature of the primers used for the genes *srfA* and 16S was assessed by PCR at a temperature range between 50 and 60°C. In Figure 13 it can be observed that the primers used were able to amplify the regions of interest of the *B. subtilis* #573 genome at these temperatures. It was possible to observe in the PCR reactions an amplified fragment of 226 bp for the gene *srfA* and an amplified fragment of 193 bp for the gene 16S.

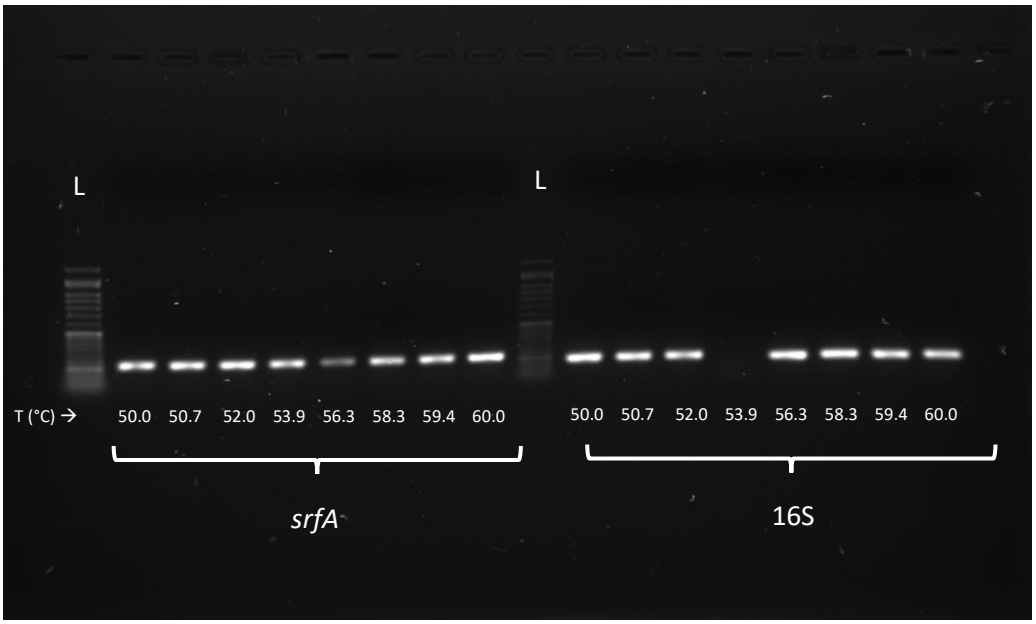


Figure 13. Agarose gel showing the products obtained from the PCR reaction (the cDNA obtained from the assays performed in flasks was used as template) at different temperatures between 50 and 60°C. L: 1kb DNA Ladder; srfA: wells relative to the PCR reactions with srfA primers at different temperatures; 16S: wells relative to the PCR reactions with 16S primers at different temperatures.

The *srfA* gene expression profile obtained from the cultures performed in the pressurized reactor was compared with that of bacteria grown in flasks. For an efficiency of the *srfA* and 16S primers estimated at 102.3% and 105.7%, respectively (Annex 7), our qRT-PCR experiments revealed that the normalized expression values of the *srfA* gene were higher those obtained in the flask analysis, 19.84 ± 2.33 for normalized expression value in flasks and 23.95 ± 2.42 for the normalized expression value in pressurized reactor. Obtaining a relative normalized expression higher in pressurized reactor than in flask, as can be seen in figure 14. However, when a Bonferroni multiple comparisons test (section 2.13) was applied to the results, no significant differences were found, with a confidence level of 95%.

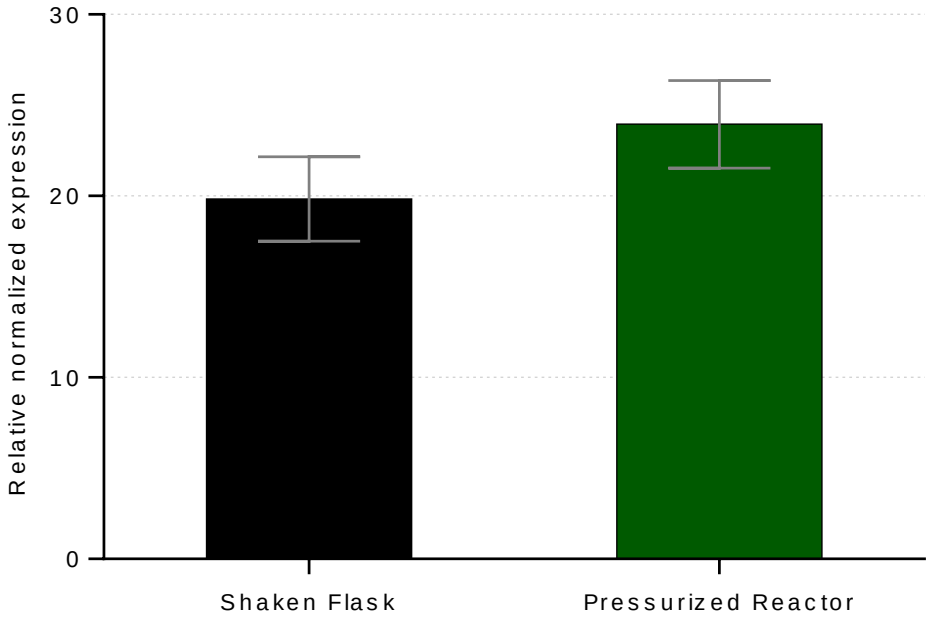


Figure 14. Relative expression of *srfA* normalized relative to the 16S reference gene.

To ensure the absence of genomic DNA contamination, a negative control (NRT) was included in the reverse transcriptase reaction. Also, other negative controls (NTC) were performed where the cDNA was replaced by ultrapure water. In none of the controls

should a CT value appear in the qRT-PCR run. Controls in qRT-PCR reactions prove that signal obtained from experimental samples represent the amplicon of interest, thereby validating specificity. The appearance of signal in these controls does not allow an accurate evaluation of gene expression. Neither unspecific product was observed in the melting curves, however, with the controls performed in this work, a CT value was obtained that did not allow to verify if these results were conclusive.

The primers used in this study were obtained from the bibliography [4],however, there is always the possibility that even well-designed primers may form primer dimer or amplify a nonspecific product. Another problem that may have occurred is a contamination with genomic DNA or DNA contaminated work spaces, gDNA can compromise the efficiency of the reaction due to competition for reaction components such as dNTPs and primers. Thus, as future perspective new extractions of RNA must be made and the use of different primers, so that, concrete data can be reached on the influence of pressure on the expression of the gene involved in surfactin synthesis.

Even these data are not conclusive, a lower growth observed in a pressurized reactor (up to a maximum of 0.666 g/L) when compared to the shaken flask (up to a maximum of 1,236 g/L) and a similar surfactin production in both cases, perhaps these results make sense. If the microorganism grows less but has an equal production. There are fewer cells but a similar production of surfactin, so it is presumable that, the expression of genes related to surfactin synthesis will be greater.

3.7 OIL RECOVERY ASSAY USING A SAND-PACK COLUMN

All data up to this point indicate that this microorganism is a useful candidate for *in situ* MEOR applications, under extreme conditions such as those occurring in oil wells, namely the focus of this thesis, the high pressures. The laboratory scale sand-pack column assay under high pressure simulated the natural oil reservoir with entrapped crude oil. Several oil recovery parameters considered in this assay are listed in Table 12. This experiment was performed during 14 days in the optimum conditions obtained from the factorial design, of 37°C and 46 bar. And after incubation with *B. subtilis* #573 was obtained an additional oil recovery of 14.5%.

It is important to note that a value of 14.5% of additional oil recovery is due to the action of the microorganism by reducing the capillary forces that trap the oil, and perhaps due to the degradation of the hydrocarbons present in the column. *B. subtilis #573* is described by Gudiña and co-workers [2] as an isolate that degraded n-alkanes between C18 and C20 into lighter ones, under aerobic and anaerobic conditions. And in sand-pack column assays [94], the isolate showed the ability to degrade long-chain n-alkanes (C> 25).

Considering the effect of the pressure in a sand pack column experiment, this system recreates as close as possible the conditions inside the oil reservoir. These experiments allow to establish the high applicability of potentially useful microorganisms in MEOR in recovering residual oil.

Table 12. Summary of the results obtained in the MEOR sand-pack experiment performed with *Bacillus subtilis* #573.

OIL RECOVERY PARAMETERS	
Porosity (%)	54.5
OOIP (mL)	210
S_{oi} (%)	61.8
S_{orwf} (mL)	137.5
OOIP - S_{orwf} (mL)	72.5
S_{or} (%)	34.5
S_{ormf} (mL)	10.5
AOR (%)	**14.5**

This microorganism was already studied in sand pack column at atmospheric pressure with different hydrocarbons mixture at 40°C by Gudiña and coworkers [66], and after 14 days an additional oil recovery between 17.7% and 22.4% was reported. With this assay the recovery was lower than in the previously reported trials, this

variation may be due to the use of a different crude oil (CLB). A larger study with controls and more replicates should be done so that successful conclusions can be made.

Some authors [65][69][81] after investigating the growth and production of metabolites by isolated microorganisms from oil reservoirs and conclude that the microorganisms used were good candidates for the in situ MEOR applications, since they proliferated under oxygen limiting conditions, they had excellent profiles of metabolite production and/or presented desirable characteristics even at high pressures, reported assays in models that recreate the conditions of oil wells at high pressures.

These authors also obtained positive results in oil recovery assays with microorganisms at high pressures, as was verified in this work with *B. subtilis* #573. Yakimov and co-workers [65] studied the potential of several strains of *Bacillus licheniformis* in enhanced oil recovery and reported that strain BNP29 produces a significant amount of a surfactant similar to surfactin at elevated temperatures of 55°C. In experiments with the presence of oil verified that the oil reduces the growth rate and production of polymer and surfactant. However, in core flooding experiments at 95 bar and at 47°C or 30°C, oil recovery efficiencies in situ varies from 9.3 to 22.1%, anaerobically. The BNP29 strain grown under stimulated reservoir conditions produced besides biomass, significant amounts of extracellular polymer and fermentation products, such as acetate, lactate and CO2, which may be useful for MEOR.

At a similar pressure range (100 bar) Zhao and collaborators [69] studied the growth and production of biosurfactants by *P. aeruginosa* 709 isolated from an oil field in china under aerobic conditions, anaerobic conditions and under high pressure conditions. This strain efficiently produced biosurfactants under these conditions. Results of core flooding experiments showed that 7.04% of crude oil was displaced through *in situ* production of biosurfactant by *P. aeruginosa* 709 at 39°C and at atmospheric pressure. The authors suggest that this strain is beneficial to mobilize entrapped oil in core through *in situ* biosurfactant production, since it efficiently produces biosurfactants under anaerobic conditions and high pressure conditions.

Also, Fida and co-workers investigated the effect of growth and metabolite production by *Thauera sp.* TK001 in additional oil recovery in sand-pack columns,

containing heavy oil at atmospheric or high pressure (27.2 bar). After incubation in sand-pack columns for 15 days at 27.2 bar, an additional oil recovery of 18.3% was observed. The increased production of oil was suggested by the authors due to the bioplugging or increased oil emulsification (possibly due to the production of biosurfactants).

The results obtained from the oil recovery assay suggest that the produced biosurfactants by *B. subtilis* #573 can efficiently mobilize the trapped oil under extreme environmental conditions, such as the high pressures of oil reservoirs.

CHAPTER 4

CONCLUSIONS AND FUTURE PERPECTIVES

In the present study, growth and biosurfactant production by *Bacillus subtilis* #573 isolated from a Brazilian oil field were studied under diverse conditions, namely different temperatures, pressures, under aerobic conditions and oxygen-limiting conditions and using five different culture scales (serum bottles, shaken flask, non-pressurized reactor, pressurized reactor and sand-pack column).

The main conclusion of this thesis is that this isolate exhibit the desirable proprieties towards its applications in MEOR. The microorganism was able to grow and produce biosurfactants under aerobic and oxygen-limiting conditions up to 50-51°C. In the range of pressure values studied (1-47 bar), it was not observed an inhibition of growth or biosurfactant production by *B. subtilis* #573, and the optimum biosurfactant production point was reached at 46bar and 37°C.

However, it is important to mention that other points with more extreme conditions (45°C and 40bar; 46.7°C and 25bar; 41°C and 47bar) also vallowed a good biosurfactant production. In addition to the validated optimum point, several other points were found where the same production can be achieved, and this isolate presents a good versatility in the studied temperature and pressure values. Additionally, it has the ability to reduce the surface tension of culture medium to values around 26 mN/m under aerobic, oxygen-limiting conditions and high pressure conditions, thus demonstrating its great biosurfactant potential.

Even though the data from the gene expression analysis are not conclusive, these data suggest a higher relative expression of the *gene* srfA under high pressure conditions. However, the surfactin production was similar in both cases, and no significant differences could be observed. The extracted biosurfactants were found to be constituted by surfactin isoforms between C_{12} and C_{16}. These data suggest that the pressure does not have a negative effect on the production of biosurfactants by *B. subtilis* #573.

In addition, a sand-pack column assay was performed and an additional oil recovery of 14.5% was achieved which represents a great improvement and reinforces the potential of this microorganism for MEOR.

As future perspectives and to further support the claim that it is a good candidate for MEOR applications, additional studies on the degradation of hydrocarbons under pressure as well as experiments at even higher pressure ranges are recommended. In addition, it would also be interesting from the standpoint of molecular biology to conduct studies on the impact of extreme conditions on the expression of the genes related with surfactin synthesis and the degradation of hydrocarbons. Finally, and as the ultimate goal is the application in field studies, it will be necessary to understand if this microorganism can be adversely affected when injected into the reservoirs by the presence of other microorganisms.

CHAPTER 5

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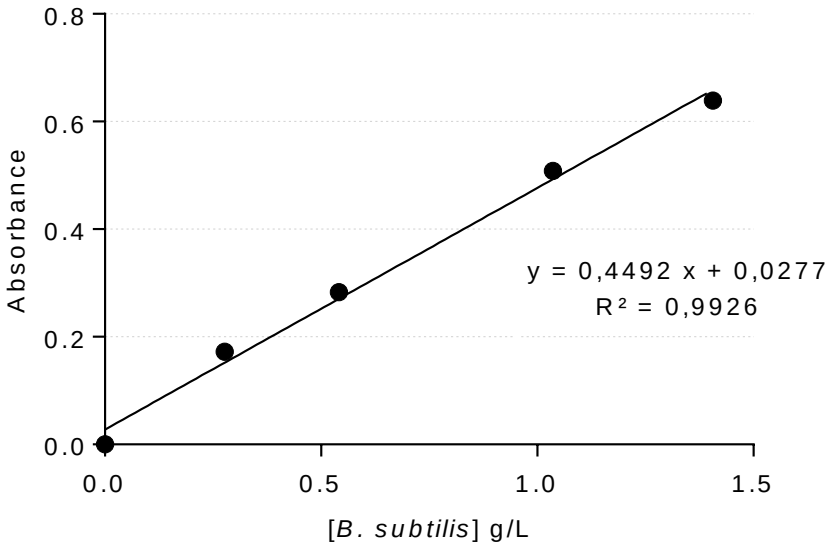
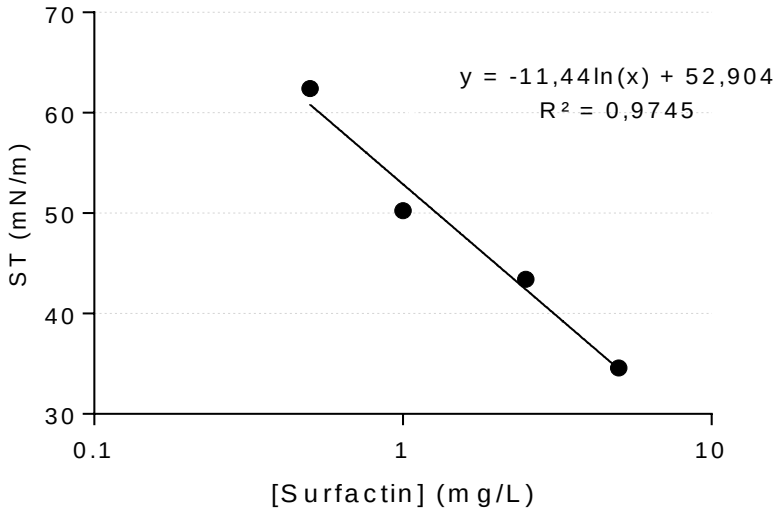
CHAPTER 6

APPENDIXES

Annex 1- Linear regression calibration curve, absorvance (by reading the optical density of the samples at 600 nm) as a function of the *B. subtilis* concentration (g/L). Linear range of absorbance of the calibration curve between 0 to 0.7 absorbance units.



Annex 2- Linear regression calibration curve, surface tension (mN/m) as a function of the logarithm of the biosurfactant concentration (mg/L). Linear range of ST of the calibration curve between 34.6 to 62.4 mN/m.



Annex 3. Surface tension values (ST and ST^{-1}, mN/m) obtained throughout the 7-days experiment at 37°C under oxygen-limiting conditions. Surface tension values were measured five times at room temperature (25°C). ST: Surface tension of the cell-free supernatant; ST^{-1}: Surface tension of the cell-free supernatant diluted 10 times with demineralized water. Results represent the average of five independent experiments ± standard deviation.

Time (h)	ST (mN/m)	ST^{-1} (mN/m)
24	26.3 ± 0.2	53.1 ± 1.9
48	26.4 ± 0.2	44.3 ± 3.0
72	26.5 ± 0.3	38.0 ± 1.8
96	26.1 ± 0.9	36.9 ± 2.4
120	26.4 ± 0.2	34.9 ± 1.2
144	26.4 ± 0.2	33.5 ± 0.5
168	26.4 ± 0.1	34.8 ± 0.6

Annex 4. Surface tension values (ST and $ST^{1/10}$, mN/m) obtained throughout the 6-days experiment at 37°C and 200 rpm under aerobic conditions. Surface tension values were measured five times at room temperature (25°C). ST: Surface tension of the cell-free supernatant; $ST^{1/10}$: Surface tension of the cell-free supernatant diluted 10 times with demineralized water. Results represent the average of two independent experiments ± standard deviation.

Time (h)	ST (mN/m)	$ST^{1/10}$ (mN/m)
0	51.5 ± 1.5	66.3 ± 0.4
24	26.1 ± 0.1	33.3 ± 0.5
48	26.2 ± 0.1	34.1 ± 0.5
72	26.5 ± 0.1	35.6 ± 1.0
144	26.2 ± 0.2	33.4 ± 0.1

Annex 5. Surface tension values (ST and $ST^{1/10}$, mN/m) obtained throughout the 6-days experiment at 37°C in a non-pressurized bioreactor. ST: Surface tension of the cell-free supernatant; $ST^{1/10}$: Surface tension of the cell-free supernatant diluted 10 times with demineralized water. The results represent the mean of five measurements ± standard deviation.

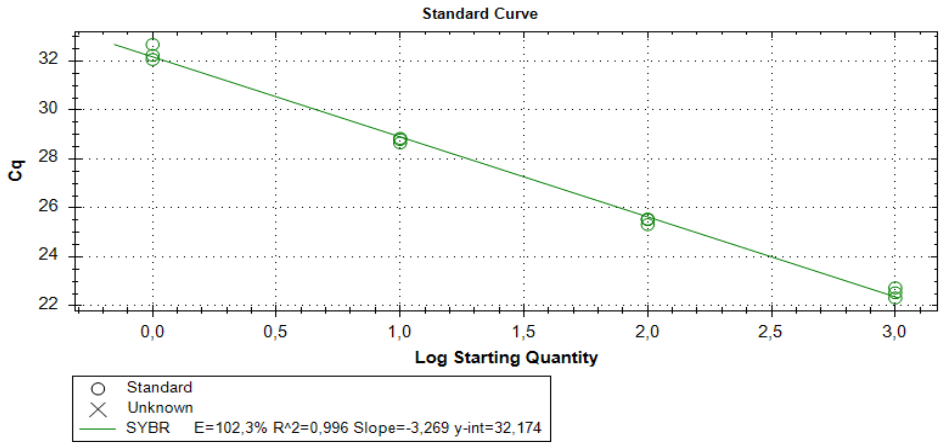
Time (h)	ST (mN/m)	$ST^{1/10}$ (mN/m)
0	36.4 ± 1.1	53.1 ± 0.2
24	26.6 ± 0.1	47.1 ± 1.9
48	26.1 ± 0.1	32.0 ± 0.2
72	26.0 ± 0.1	32.2 ± 0.4
144	26.4 ± 0.2	31.3 ± 0.1

Annex 6. Surface tension values (ST and $ST^{1/10}$, mN/m) obtained throughout the 6-days experiment at 40°C and 40 bar in a pressurized reactor. ST: Surface tension of the cell-free supernatant; $ST^{1/10}$: Surface tension of the cell-free supernatant diluted 10 times with demineralized water; The results represent the mean of 5 measurements ± standard deviation.

Time (h)	ST (mN/m)	$ST^{1/10}$ (mN/m)
0	37.1 ± 0.9	65.0 ± 0.6
24	26.1 ± 0.2	40.7 ± 2.6
48	26.6 ± 0.2	39.7 ± 3.0
72	26.8 ± 0.1	44.3 ± 3.5
96	26.7 ± 0.1	40.1 ± 1.1
144	26.8 ± 0.2	51.7 ± 2.7

Annex 7. a) Standard curve with efficiency of the *srfA* primers. **b)** Standard curve with efficiency of the 16S primers.

a)



b)