Universidade do Minho
Escola de Engenharia

Helena de Fátima Ribeiro

Construction of engineered microorganisms for application in Microbial Enhanced Oil Recovery

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Dissertação de Mestrado
Mestrado em Biotecnologia

Trabalho efetuado sob a orientação da
Professora Doutora Lígia Raquel Marona Rodrigues
e do
Doutor Eduardo José Gudiña Pérez

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

Construção de microrganismos para aplicação na Recuperação Avançada de Petróleo

A recuperação avançada de petróleo com microrganismos (*MEOR*) é uma técnica de recuperação terciária que usa microrganismos e os seus metabolitos para recuperar o óleo que ficou preso nos reservatórios. Uma das principais desvantagens na aplicação de processos de *MEOR in situ* é a escassez de microrganismos capazes de crescer e produzir os bioprodutos desejados nas condições adversas do reservatório petrolífero. Assim, o objetivo desta tese foi a construção de microrganismos capazes de produzir biopolímeros quando expostos a altas temperaturas e em condições limitantes de oxigénio através de fusão de protoplastos. Nesse sentido, uma estirpe produtora de biopolímero (*Rhizobium viscosum* CECT 908) e a estirpe *Bacillus subtilis*, isolada a partir de um reservatório petrolífero, foram usadas como estirpes parentais. Diversos parâmetros foram otimizados de modo a aumentar as frequências de formação e regeneração de protoplastos para cada estirpe parental. As condições mais favoráveis para a formação de protoplastos foram uma incubação com EDTA (2.9 g/L) durante 60 min a 30°C seguida de um tratamento com 2 g/L de lisozima durante 1 h a 37°C para *R. viscosum* CECT 908, e a incubação com 3 g/L de lisozima durante 1 h a 37°C sem tratamento com EDTA para *B. subtilis* PX573. Outros parâmetros foram também avaliados para melhorar a regeneração e fusão de protoplastos, incluindo o uso de diferentes estabilizadores osmóticos e meios de regeneração, e o tempo de incubação com o agente fusogénico. Contudo, após a fusão de protoplastos, não foi possível obter estirpes recombinantes contendo as propriedades desejadas das estirpes parentais. Adicionalmente, quatro estirpes de *Paenibacillus* sp. foram identificadas como novas e promissoras produtoras de biopolímeros. Estes isolados produziram até 30 g/L de biopolímero no meio de cultura otimizado, atingindo valores de viscosidade aparente de 54000 mPa s (80 vezes maiores quando comparado com o biopolímero de *R. viscosum* CECT 908). Nos ensaios de recuperação de óleo em colunas de areia usando um óleo de elevada viscosidade ($\eta_{40°C}$≈545 mPa s), estes biopolímeros permitiram a recuperação de 41.9 ± 0.7% do óleo retido, sendo candidatos promissores para a aplicação em *MEOR*. O efeito da combinação de biosurfactante e biopolímero na recuperação de óleo foi também estudado usando o mesmo óleo. Os resultados obtidos demonstraram que a combinação de biopolímero (*R. viscosum*) e surfactina (*B. subtilis*) levou a recuperações de óleo semelhantes (47.2 ± 0.7%) quando comparada com o biopolímero isolado (46.2 ± 3.8%), enquanto que a surfactina isolada foi incapaz de recuperar este tipo de óleo.

Palavras chaves: Biopolímeros; Biosurfactantes; Fusão de protoplastos; Microrganismos geneticamente modificados; Recuperação avançada de petróleo com microrganismos.

ABSTRACT

Construction of engineered microorganisms for application in Microbial Enhanced Oil Recovery

Microbial enhanced oil recovery (MEOR) is a tertiary oil recovery technique that uses microorganisms and their metabolites to recover the oil entrapped in the reservoirs. One of the main drawbacks related with the application of *in situ* MEOR is the shortage of microorganisms capable of growing and producing the desired bioproducts at the harsh conditions of the oil reservoir. Thus, the aim of this Thesis was the design and construction of microorganisms with the ability of producing biopolymers at high temperatures and oxygen-limiting conditions through protoplasts fusion. For this purpose, a biopolymer-producing strain (*Rhizobium viscosum* CECT 908) and a *Bacillus subtilis* strain isolated from an oil reservoir were used as parental strains. Several parameters were optimized to increase the protoplasts formation and regeneration frequencies for each parental strain. The most favourable conditions for protoplasts formation were an incubation with EDTA (2.9 g/L) for 60 min at 30°C followed by a treatment with 2 g/L of lysozyme for 1 h at 37°C for *R. viscosum* CECT 908, and incubation with 3 g/L of lysozyme for 1 h at 37°C without EDTA treatment for *B. subtilis* PX573. Other parameters were also evaluated to improve the protoplasts regeneration and fusion, including the use of different osmotic stabilizers and regeneration media, and the incubation time with the fusogenic agent. However, after protoplasts fusion, it was not possible to obtain recombinant strains combining the desired properties of the parental strains. In addition, four *Paenibacillus* sp. strains were identified as new and promising biopolymer producers. These isolates produced up to 30 g/L of biopolymer in the optimized culture medium, achieving apparent viscosity values as high as 54000 mPa s (80 times higher when compared with *R. viscosum* CECT 908). In oil recovery assays performed in sand-pack columns using a heavy crude oil ($\eta_{40\,^{\circ}C}$≈545 mPa s), these biopolymers allowed a recovery of 41.9 ± 0.7% of the entrapped oil, thus being promising candidates for application in MEOR. The effect of the combination of biosurfactants and biopolymers in oil recovery was also studied using the same crude oil. The results obtained demonstrated that the combination of biopolymer (*R. viscosum*) and surfactin (*B. subtilis*) resulted in similar oil recoveries (47.2 ± 0.7%) when compared with the biopolymer alone (46.2 ± 3.8%), whereas surfactin alone was unable of recovering this type of oils.

Keywords: Biopolymers; Biosurfactants; Engineered microorganisms; Microbial Enhanced Oil Recovery; Protoplasts fusion.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AOR	Additional Oil Recovery
BP #540	Biopolymer produced by *Paenibacillus* sp. #540
BP 908	Biopolymer produced by *Rhizobium viscosum* CECT 908
BS	Biosurfactant produced by *Bacillus subtilis* PX573
cmc	Critical micelle concentration
CSL	Corn Steep Liquor
CTAB	Hexadecyltrimethylammonium bromide
DMSO	Dimethyl sulfoxide
E_{24}	Emulsifying index measured after 24 hours
EDTA	Ethylenediaminetetraacetic Acid
EOR	Enhanced Oil Recovery
LB	Luria-Bertani medium
MEOR	Microbial Enhanced Oil Recovery
MSS	Mineral Salt Solution
MSS-S	MSS medium supplemented with sucrose (200 g/L)
$OD_{600\ nm}$	Optical Density at 600 nm
OOIP	Original Oil In Place
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PV	Pore Volume
rpm	Rotations per Minute
S_{oi}	Initial Oil Saturation
S_{or}	Residual Oil Saturation
S_{orbf}	Oil Recovered after Bioproduct Flooding
S_{orwf}	Oil Recovered after Water Flooding
UV	Ultraviolet
$\eta_{40°C}$	Apparent viscosity measured at 40 °C

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1. INTRODUCTION

1.1. Natural resources

Energy is an essential asset to the society and its demand has increased to guarantee the quality of life and the proper functioning of the economy. Consequently, the utilization of the energy resources is considered one of the most important and actual questions in the modern times (Hussain et al., 2017).

Since the beginning, the humans used fossil fuels, which demonstrated to be essential for the civilizations development and contributed to this development in large scale (Silva et al., 2014). The excessive use of fossil fuels conducted to the development of new strategies to use the renewable energy sources in order to reduce the negative environmental impact caused by the fossil fuels and to supply the energetic demand caused by the fast increase of World's population (Geetha et al., 2018; Lopez-Hidalgo et al., 2017). Despite the efforts to increase the use of renewable energy sources, fossil fuels will continue to represent the largest fraction of energy consumed in the next decades (Ismail et al., 2017).

The energy sources can be divided into three main categories: fossil fuels, nuclear resources and renewable energy sources (Demirbas, 2000). Within the fossil fuels, petroleum is one of the most important (Silva et al., 2014). Between 2000 and 2030, the number of barrels of oil produced per year is expected to increase by 1.7%. However, if the current levels of petroleum consumption are maintained, the oil reservoirs will only meet the World's demand for 40 years (Silva et al., 2014). Additionally, petroleum recovery, transportation and refining are associated to serious environmental pollution issues (Zhang et al., 2011). Besides that, these processes are costly, use large amounts of energy and, in some cases, they are not efficient (Ismail et al., 2017).

The growing demand for petroleum may not be satisfied by finding new oil fields (Siegert et al., 2014). Consequently, the petroleum industry will be forced to produce and refine large amounts of fossil fuels due to the increasing demand. At the same time, the increase of renewable energy sources demand and the exhaustion of crude oil reservoirs will result in the need of developing and applying alternative technologies to make the production process more sustainable and cost-effective (Bachmann et al., 2014; Ismail et al., 2017; Silva et al., 2014).

1.2. Characterization of oil reservoirs

Petroleum can be defined as a complex mixture of hydrocarbons, minor quantities of compounds containing nitrogen, sulphur and oxygen, and trace amounts of metals such as vanadium and nickel (Speight & El-Gendy, 2018b; Van Hamme et al., 2003). Petroleum is derived from organic matter of microorganisms and algae that died hundreds of millions of years ago. This organic matter was deposited over the years, accumulating in the bottom of the oceans and being covered by sediments. The decomposition of organic matter gave rise to petroleum, which migrated from the original deposits to more porous deposits with more permeable rocks, where it was retained forming reservoirs. The oil formation was the result of the interactions between organic matter, sediments and appropriated thermochemical conditions (De Almeida et al., 2016; Speight & El-Gendy, 2014, 2018b).

The possibility of microorganisms to survive or thrive in oil reservoirs depends on the physical characteristics and the chemical composition of these environments, since these ecosystems constitute an extreme environment with high temperatures, pressures, and strict anoxic conditions (De Almeida et al., 2016; Li et al., 2010; Magot et al., 2000).

The temperature is one of the most important limiting factors to the microorganism's growth. The temperature of most reservoirs ranges between 40 and 80°C (Bachmann et al., 2014). The temperature increases with the increase of depth at an average rate of 3°C/ 100 m, but the regional geothermic gradients may vary. Temperatures higher than 80°C may prevent the anaerobic degradation of oil (Magot et al., 2000). Some crude oil reservoirs can reach temperatures between 130 and 150°C when the depth reaches between 4030 and 4700 m (Magot et al., 2000; Varjani, 2017); these reservoirs do not allow the growth of microorganisms, as this temperature range is considered above the theoretical limit to the microbial growth, due to the thermal instability of biological molecules (Magot et al., 2000; Tatar, 2018; Varjani, 2017).

Pressure is also an important parameter affecting the microbial growth. The pressure inside the reservoirs is not considered an impediment for microbial growth (Magot et al., 2000). However, it can influence the physiologic properties of the microorganisms, because the increase of pressure influences the redox potential and increases the gases solubility (Magot et al., 2000; Saikia et al., 2013). At ambient temperature, the increase of pressure results in a decrease in the rate of hydrocarbons metabolism when compared to the atmospheric pressure (Schwarz et al., 1975).

An extensive range of salinity and pH can be observed in reservoirs. The variations in temperature and pressure of crude oil reservoirs influence their salinity (Nmegbu, 2014). The reservoirs salinity ranges between 100 mg/L and 300 g/L according to their depth. The reservoir water can contain high concentrations of dissolved salts. Sodium chloride corresponds to 90% of the total dissolved solids found in the oil reservoirs (Jimoh, 2012). The presence of microorganisms in the oil reservoir will be affected by their tolerance to the different salinities (Nmegbu, 2014; Safdel et al., 2017). The pH can influence the microbial growth, since it affects the surface charge and the enzymatic activities (Safdel et al., 2017). In general, the pH of oil reservoirs varies between 5 and 8 (H. Li et al., 2013). However, the pH measured does not always corresponds to the *in situ* pH, since it is influenced by the dissolution of gases at high pressures. Usually the *in situ* pH varies between 3 and 7 (Varjani & Gnansounou, 2017).

In addition to the environmental conditions mentioned above, the reservoirs may also have different geologic structures and sizes (Speight & El-Gendy, 2018b). The reservoirs are generally made up of sandstones and fractured limestones. Two of the main physical characteristics of a reservoir are porosity and permeability. The porosity allows to estimate the volume of crude oil present in the reservoir, while the permeability allows to estimate the ability of the crude oil to flow from the reservoir (Speight & El-Gendy, 2018b).

1.3. Crude oil recovery

The process of crude oil recovery is affected by the different characteristics of the reservoirs, and it can be carried out through various recovery methods (Speight, 2016). The crude oil recovery is performed in three phases: primary, secondary and tertiary recovery. The primary recovery depends on the reservoir energy leading the oil through the complex network of well pores. When the pressure in the reservoir is high, the oil flows to the production wells and then to the surface (Gao & Zekri, 2011; Speight & El-Gendy, 2018a). Although this recovery method is the less expensive, it allows only the recovery of 10-20% of the original oil in place (OOIP) (Bachmann et al., 2014; De Almeida et al., 2016).

The secondary recovery is used when the natural pressure of the reservoir decreases. In this phase, the techniques used to aid the crude oil recovery include the use of pumps (on the surface or submerged) to bring the oil to the surface. In addition, techniques such as water or gas injection can be applied to increase the pressure of the reservoir. These recovery methods are more expensive when

compared with the primary recovery, but they can contribute to recover up to 45-50% of the OOIP (Bachmann et al., 2014; Speight & El-Gendy, 2018a). However, half of the original oil present in the reservoir is trapped in the small pores of the rocks due to the high capillarity forces and the interfacial tension between the aqueous phase and the hydrocarbons (De Almeida et al., 2016; Santos et al., 2016). This oil can be recovered by tertiary techniques (Al-Sulaimani et al., 2011).

The tertiary recovery methods or enhanced oil recovery (EOR) techniques are used to recover the residual oil, and their application depends on the characteristics of the crude oil and the reservoir (Bachmann et al., 2014; Gao & Zekri, 2011). These methods include technologies such as gas injection, thermal and chemical treatments (Al-Sulaimani et al., 2011; De Almeida et al., 2016; Sen, 2008). The thermal processes are used to increase the temperature of the oil in the reservoir, making it easier to flow to the production wells. This technique is the most common and it can be applied by *in situ* combustion, steam injection and hot water injection (De Almeida et al., 2016; Geetha et al., 2018). However, the application of thermal processes can produce polar compounds, which are incompatible with the asphaltenes present in the oil and can therefore result in the blockage of the pores and channels through which the oil should move (Bachmann et al., 2014). The chemical processes involve the injection of chemical compounds which can change the properties of the fluids, improving the recovery mechanisms (Al-Sulaimani et al., 2011). These processes comprise the injection of solvents, surfactants, gases or combinations of them (Brown, 2010; De Almeida et al., 2016). In the gas injection various types of miscible or immiscible gases are used, such as carbon dioxide (CO_2), nitrogen (N_2) and combustion gas, to move the oil to the extraction point (Bachmann et al., 2014; Sen, 2008).

However, as these technologies are expensive and environmentally hazardous, it was necessary to look for more environmentally friendly and economic alternatives. Thus, the petroleum biotechnology is becoming an emergent technology (De Almeida et al., 2016; Ismail et al., 2017). Among the various applications of this technology, the microbial enhanced oil recovery (MEOR) is one of the most important ones (Ismail et al., 2017).

1.4. MEOR

MEOR is a tertiary oil recovery technique that uses microorganisms and their metabolic products to recover the oil entrapped in the reservoirs (Safdel et al., 2017).

3. MATERIALS AND METHODS

Microbial life in the oil fields was reported for the first time by Bastin (Bastin, 1926). In 1926, Beckman suggested the utilization of microorganisms in the oil recovery processes (Beckman, 1926). In the 1940s, ZoBell and co-workers started a series of laboratory investigations which marked the beginning of a new era for investigation in petroleum microbiology for application in oil recovery (ZoBell, 1947).

MEOR is a favourable technology since it is economically efficient (the injection of microorganisms and nutrients into the oil wells has low cost and low energy consumption); furthermore, it is more environmentally friendly comparing with the use of chemical methods. Besides that, this technology is considerably efficient in sandstone and carbonate reservoirs; and it can be applied to both light and heavy crude oils (Safdel et al., 2017).

On the other hand, the drawbacks of this technology are the corrosion of equipment as a result of the metabolic activities of aerobic bacteria; the logistic problems associated to the injection of high concentrations of nutrients; the complexity of developing a comprehensive model to understand all the aspects of the MEOR process; the toxicity of specific heavy metal ions; and the limited tolerance of microorganisms to the reservoir conditions (De Almeida et al., 2016; Safdel et al., 2017; Speight & El-Gendy, 2018a).

The MEOR processes can be classified as *in situ* or *ex situ*, according to the place where the bioproducts are synthesized (Al-Sulaimani et al., 2011; Geetha et al., 2018). In *ex situ* MEOR processes, the bioproducts are synthesized out of the reservoir and then they are injected into the oil wells (Geetha et al., 2018). This process is relatively expensive due to the costs associated with the operation of bioreactors, bioproducts purification and their introduction into the oil reservoirs (Gudiña et al., 2012). On the contrary, in the *in situ* MEOR, the microorganisms and/or the nutrients required for their growth are injected into the oil reservoirs. The exogenous or indigenous microorganisms will grow inside the oil well where they will produce specific metabolic compounds that are expected to aid in the oil recovery (Al-Sulaimani et al., 2011; Geetha et al., 2018). This strategy is economically efficient but requires the use of microorganisms that can produce enough amounts of bioproducts inside the oil reservoir to mobilize the entrapped oil (Gudiña et al., 2012). In addition, when exogenous microorganisms are injected into the oil wells, it is important to perform compatibility studies to understand the interaction between the exogenous and the indigenous microorganisms, the nutrients, the crude oil and the rocks (Al-Sulaimani et al., 2011).

MEOR processes usually occur through a combination of different mechanisms. These mechanisms include hydrocarbon metabolism, reduction of the viscosity of the entrapped oil, increase of the water viscosity, selective plugg of high permeability channels to modify the permeability profile, dissolution of some parts of the reservoir rocks, wettability alteration, crude oil emulsification, surface and interfacial tension reduction, re-pressurization of the oil reservoir, oil swelling and well stimulation by removing the wellbore damages (Sen, 2008; Tatar, 2018).

However, until now, MEOR has still not become a popular technology mainly due to the inconsistency between the laboratory results and the field trials, as well as the lack of understanding about all the details involved in the different *in situ* processes (Maudgalya et al., 2007).

1.4.1. Microbial ecology of oil reservoirs

Numerous groups of microorganisms have been isolated from oil reservoirs (Youssef et al., 2009). Bacteria are considered promising microorganisms for application in MEOR due to their small size, their ability to tolerate harsh environmental conditions and their capacity to produce metabolic compounds that promote oil recovery (Al-Sulaimani et al., 2011). The average size of bacterial cells varies between 0.5 μm and 5.0 μm, which facilitates their penetration through the porous media of the reservoir (Al-Sulaimani et al., 2011). In MEOR processes that involve the injection of bacteria into the reservoir, it has been calculated that the microorganisms need to be spherical and their size less than 20% of the size of the pore throat in the formation (Jack at al., 1991).

The microbial growth and the effect of their bioproducts depend on chemical (*e.g.,* nutrients composition, electrolyte composition, redox potential) and physical factors (*e.g.,* porosity, permeability, pore size, pore geometry, pressure, temperature, dissolved solids, pH, salinity) (Bachmann et al., 2014; Tatar, 2018).

The microorganisms used for MEOR can be classified based on their origin, mechanism of action and metabolic processes (Safdel et al., 2017; Tatar, 2018). According to their origin, microorganisms can be classified in indigenous or exogenous. Indigenous microorganisms are those that are originally present in the reservoir, while exogenous microorganisms are those that were transferred or injected in the reservoir (Tatar, 2018).

Considering their metabolism, the microorganisms can be classified in methanogens, fermentative microorganisms, sulphate-reducing bacteria, nitrate-reducing bacteria and iron-reducing bacteria. Methanogens are microorganisms that generate methane trough a metabolic process called methanogenesis. These microorganisms metabolize substrates such as carbon dioxide and hydrogen, dimethylsulphides, acetate and methylamines (Tatar, 2018; Youssef et al., 2009). Fermentative microorganisms are considered an important community in oil reservoirs. These microorganisms obtain energy through substrate phosphorylation. They can use organic acids and organic compounds, such as amino acids, peptides and sugars as substrates. Some of the fermentative microorganisms can use electron acceptors such as ferric iron, nitrate and inorganic sulphur compounds to oxidize the different substrates. Furthermore, these microorganisms can grow in a wide range of salinities (Ollivier & Cayol, 2005). Sulphate-reducing bacteria are microorganisms that use sulphate as the terminal electron acceptor (Tatar, 2018). These bacteria can use low molecular weight organic compounds, such as ethanol, succinate, lactate, acetate, propionate, pyruvate and sugars to grow while reducing sulphate to hydrogen sulphide (Song et al., 2013). These microorganisms have been extensively studied due to their negative effects in the reservoir (corrosion and *in situ* reservoir souring) (Varjani & Gnansounou, 2017). Several nitrate-reducing bacteria have been isolated from oil fields. These bacteria can be classified as microaerophilic, aerobic, facultative anaerobic or strict anaerobic. The interest in these microorganisms increased due to their capacity to decrease the sulphate concentration, reducing the toxicity and the corrosive effect of this compound in the oil reservoirs (Ollivier & Cayol, 2005; Tatar, 2018). Iron-reducing bacteria have been detected in some oil reservoirs; however, the ecology of these microorganisms has been poorly studied (Magot et al., 2000; Ollivier & Cayol, 2005).

The choice of the right microorganism(s) that produces the target bioproduct and can survive to the physicochemical conditions of the reservoir is crucial for a successful MEOR process (Tatar, 2018). Successful field trials used mainly anaerobic or facultative anaerobic bacteria. Among the microorganisms used in the different field trials are *Bacillus* spp., *Clostridium* spp., *Pseudomonas* spp., sulphate-reducing bacteria and nitrate-reducing bacteria (Maudgalya et al., 2007).

1.4.2. Microbial bioproducts useful for application in MEOR

Microorganisms produce many metabolic compounds, such as gases, solvents, acids, biosurfactants and biopolymers, which together with the microbial biomass, can be used to enhance the

residual oil recovery from mature reservoirs (Bachmann et al., 2014; Safdel et al., 2017). In **Table 1** the main microbial bioproducts and their application in MEOR are presented, as well as some examples of the producing microorganisms.

Table 1. Microbial bioproducts, their application in MEOR and some examples of producing microorganisms (Safdel et al., 2017; Sen, 2008; Sheng, 2013; Tatar, 2018).

Bioproduct	Application in MEOR	Producing microorganisms
Acids (low molecular weight fatty acids, lactic acid, acetic acid, formic acid, propionic acid, butyric acid, etc.)	Carbonate rocks dissolution Porosity and permeability improvement CO_2 production due to the reactions between acids and carbonated minerals Oil emulsification	*Clostridium* Mixed acidogens *Enterobacter aerogenes*, *Desulfovibrio* *Bacillus*
Biomass	Selective plugging Wettability alteration Oil viscosity reduction Permeability reduction Oil degradation Oil emulsification	*Bacillus licheniformis* *Xanthomonas campestris* *Leuconostoc mesenteroides*
Biopolymers (xanthan gum, pullulan, levan, curdlan, dextran, scleroglucan, etc.)	Improve mobility control Injectivity profile and viscosity modification Permeability reduction in water-swept regions Selective plugging	*Xanthomonas* *Sclerotium* *Bacillus* *Aureobasidium* *Leuconostoc*
Biosurfactants and bioemulsifiers (emulsan, alasan, surfactin, rhamnolipids, lichenysin, viscosin, trehalolipids, etc.)	Oil emulsification Interfacial tension reduction Wettability alteration Pore scale displacement improvement	*Acinetobacter* *Arthrobacter* *Bacillus* *Pseudomonas* *Rhodococcus*
Gases (H_2, N_2, CH_4, CO_2)	Increase the reservoir pressure Permeability improvement Oil swelling Oil viscosity and interfacial tension reduction	*Clostridium* *Enterobacter* *Methanobacterium* Fermentative bacteria
Solvents (propanol, ethanol, butanol, acetone, propan-2-diol, etc.)	Rock dissolution for better permeability Oil viscosity reduction Wettability reduction Interfacial tension reduction Oil emulsification Removal of long chain hydrocarbons	*Clostridium acetobutylicum* *Clostridium pasteurianum* *Zymomonas mobilis* *Klebsiella*

Besides the biosynthesis of these bioproducts, several microorganisms are also capable of metabolizing the hydrocarbons present in crude oil, breaking down the long alkyl chains. As a result, the oil becomes lighter and less viscous, which facilitates its recovery (Patel et al., 2015; Sen, 2008).

1.4.2.1. Biosurfactants and bioemulsifiers

Biosurfactants and bioemulsifiers are amphipathic compounds consisting of hydrophilic and hydrophobic groups that allow their partition at the interface between fluid phases with different degrees of polarity (Banat et al., 2010; Tatar, 2018).

Biosurfactants are low molecular weight biodegradable compounds, produced mainly by aerobic microorganisms (Bachmann et al., 2014; De Almeida et al., 2016; Gudiña et al., 2012; Youssef et al., 2009). These compounds reduce the surface and the interfacial tension, forming stable emulsions between immiscible compounds (such as petroleum and water) that are important to increase oil recovery (Bachmann et al., 2014; Banat, 1995). Due to these properties, biosurfactants exhibit different applications, including emulsification, foaming, use as wetting agents and detergents, dispersion and solubilisation of hydrophobic compounds (Gudiña et al., 2016).

The main classes of biosurfactants include glycolipids and lipopeptides. Several microorganisms are reported to produce different types of biosurfactants. Most species of *Bacillus* and some *Pseudomonas* spp. produce lipopeptides. Rhamnolipids, a class of glycolipids, are produced mainly by species of *Pseudomonas*. Other microorganisms such as *Rhodococcus* spp. produce trehalose lipids (another class of glycolipids) (Banat et al., 2010; Youssef et al., 2009).

In many cases, biosurfactants perform similarly or better when compared with chemical surfactants. In addition, biosurfactants can be produced from renewable substrates and agro-industrial wastes, exhibit low toxicity, biodegradability and are effective under extreme temperatures, pH and salinities (Farias et al., 2017; Pacwa-Płociniczak et al., 2011).

As previously mentioned, one of the main markets for biosurfactants is the oil industry, with the aim of increasing oil production (Al-Sulaimani et al., 2011; Gudiña et al., 2016). Due to their various industrial applications, many patents have been issued for microorganisms producing biosurfactants,

namely species of *Pseudomonas*, *Bacillus* and *Candida*, for various types of biosurfactants, for the production processes and for industrial applications (Sachdev & Cameotra, 2013).

Bioemulsifiers are high molecular weight compounds produced by various types of microorganisms (Youssef et al., 2009). These compounds are effective in forming and stabilizing oil in water emulsions, and less commonly water in oil emulsions. However, they do not reduce the surface and the interfacial tension as in the case of biosurfactants (Bach & Gutnick, 2004; Banat et al., 2010).

Bioemulsifiers include, among others, emulsan produced by species of the genus *Acinetobacter*; protein complexes produced by *Methanobacterium thermoautotrophicus*; heteropolysaccharides produced by *Halomonas eurihalina* and *Pseudomonas tralucida*; carbohydrate-protein complexes (*e.g.,* liposan) produced by *Candida lipolytica*; protein-polysaccharide-lipid complex produced by *Bacillus stearothermophilus*; and mannan produced by *Saccharomyces cerevisiae* (Tatar, 2018; Youssef et al., 2009).

These compounds are considered more easily biodegradable and less toxic than the chemical emulsifiers, which makes them more compatible with the environment. Consequently, bioemulsifiers can be used in enhanced oil recovery, to prevent the formation of paraffin deposits in oil wells, to clean-up oil contaminated pipes and vessels, in bioremediation, as additives on cleaning products, and in the pharmaceutical, food and cosmetic industries as emulsion-stabilizing agents (Banat et al., 2010; Franzetti et al., 2012; Tatar, 2018; Youssef et al., 2009).

1.4.2.2. Biopolymers

Biopolymers are exopolysaccharides produced by different bacterial and fungal strains (**Table 2**), as well as by some plants and algae. These compounds confer protection against desiccation and predation to the producer microorganisms, and also aid in cell adhesion to the surfaces (Sen, 2008). Biopolymers are used in MEOR since they allow the selective blocking of high permeability channels, which modifies the permeability of the reservoir, directing the injected water to oil-rich areas (Al-Sulaimani et al., 2011; Sen, 2008). Moreover, these compounds improve the mobility rate and the displacement efficiency of the oil, since they increase the viscosity of the water injected during the water flooding process (Ramsay et al., 1989).

Table 2. Biopolymers used in MEOR and their producing microorganisms.

Biopolymer	Producing microorganisms	Reference
Curdlan	*Alcaligenes faecalis* *Agrobacterium* sp. *Pseudomonas* sp. QL212	Phillips et al. (1983) Ruffing et al. (2011) Yang et al. (2016)
Dextran	*Leuconostoc mesenteroides* *Weissella cibaria*	Ahmed et al. (2012) Aman et al. (2012)
Levan	*Bacillus* sp. *Zymomonas mobilis* *Halomonas smyrensi*	Ramsay et al. (1989) Shih & Yu (2005) Öner et al. (2016)
Pullulan	*Aureobasidium pullulans* *Pullularia pullulans*	Lazaridou et al. (2002) Taguchi et al. (1973)
Scleroglucan	*Sclerotium rolfsii*	Bo et al. (1987)
Xanthan gum	*Xanthomonas* sp.	Palaniraj & Jayaraman (2011) Rottava et al. (2009)

Xanthan gum is produced by the fermentation of carbohydrates by bacteria of the genus *Xanthomonas*, and it is used in the food, chemical, cosmetic and oil industry (Palaniraj & Jayaraman, 2011). This biopolymer alters the viscosity of the water injected into the reservoir, allowing a higher oil recovery. Due to its viscosifying activity and pseudoplastic behaviour, resistance to mechanical stress and tolerance to high salinities and temperatures, this biopolymer has become very popular for application in MEOR (Al-Sulaimani et al., 2011; Patel et al., 2015; Sen, 2008).

Levan is a homopolysaccharide of fructose produced by several microorganisms and some plant species. In microorganisms, this biopolymer contributes to the formation of the exopolysaccharide matrix and assists in the formation of microbial biofilms (Srikanth et al., 2015). Studies carried out with *Bacillus licheniformis* NRC 9012 demonstrated that this biopolymer allows the selective blocking of high permeability zones in oil reservoirs with temperatures below 50°C, pH values between 6 and 9, salt concentrations lower than 4% and pressure lower than 500 atm (Ramsay et al., 1989). Besides the oil industry, levan can be used in the food, cosmetic and pharmaceutical sectors (Srikanth et al., 2015).

Scleroglucan is a neutral polysaccharide produced by the fungus *Sclerotium rolfsii* that exhibits high viscosity at high temperatures and high salt concentrations (Bo et al., 1987). The tolerance of this biopolymer to high salinities is due to its non-ionic character. As this compound contains triple helix chains

in its structure, it behaves like a semi-rigid molecule in aqueous solution. This semi-rigid structure explains the high viscosity and shear strength properties of this biopolymer (Kulawardana et al., 2013). Scleroglucan is used in different applications, such as cosmetics, personal care products and drilling fluids in oil fields (Zentz et al., 1992).

Pullulan is a non-mutagenic, non-toxic, non-hygroscopic, odourless, water-soluble and edible biopolymer. This biopolymer can form thin films that are oil resistant, transparent and oxygen impermeable. In addition, pullulan is degraded at temperatures between 250°C and 280°C. Due to these characteristics, this biopolymer is used in various industrial applications including food, agriculture, cosmetics, pharmaceutical, lithography and oil recovery (Singh et al., 2008; Sugumaran & Ponnusami, 2017).

Curdlan is a neutral exopolysaccharide that modifies the permeability of the rocks that constitute the oil reservoir (Patel et al., 2015). This compound is insoluble in water and alcohols and is soluble in alkaline solutions (Zhan et al., 2012). Besides the oil industry, this biopolymer can be used in food, pharmaceutical and medical applications (Divyasri et al., 2014).

Dextran is a high molecular weight polymer of glucose which is obtained by fermentation of sucrose by bacteria of the genus *Leuconostoc* (Rehm, 2009). This compound is used in food, pharmaceutical and chemical industry. In the chemical industry, this biopolymer can be used as emulsifier, carrier and stabilizer (Sarwat et al., 2008).

1.5. Construction of efficient microorganisms for application in MEOR

The industrial production of bioproducts for application in MEOR is still limited by their high production costs. One strategy to reduce these costs consists in developing improved microorganisms by genetic manipulation, in order to increase the production yields (De Almeida et al., 2016). Furthermore, through the isolation of genetic material from extreme environments similar to the oil reservoirs it is possible the transference of those genes to other microorganisms in order to produce metabolites useful for application in MEOR.

One of the main drawbacks for the application of *in situ* MEOR is the difficulty to isolate microorganisms capable of growing and producing the desired bioproducts at the extreme environmental

conditions present in the oil wells. Since the environmental conditions cannot be changed, the number of reservoirs that exhibit the conditions that allow the use of microorganisms for *in situ* treatments is limited (Gudiña et al., 2012). One of the strategies to overcome this limitation is the construction of engineered microorganisms capable of surviving at the extreme conditions of the reservoirs and, at the same time, to produce the desired metabolites. For this purpose, the target DNA sequences can be cloned in appropriate plasmids and introduced into a host microorganism with the ability of growing at the oil reservoir conditions. Another alternative is the use of the protoplasts fusion technology (Xu & Lu, 2011).

1.5.1. Protoplasts fusion

Protoplasts fusion is an important strategy of genetic recombination that has been used to improve strains and to develop hybrid strains with desired properties through the combination of genomes from different organisms (M. Li et al., 2013; Liang et al., 2017; Xu & Lu, 2011). Protoplasts fusion is considered a simple, efficient and useful technique to create new hybrid strains (Xu & Lu, 2011).

Protoplasts result from cells which cellular wall was digested with lysozyme or other enzymes (depending on the microorganism), being this process carried out in the presence of osmotic stabilizers to guarantee their integrity (Biot-Pelletier & Martin, 2014; Peberdy, 1980). This process is more efficient in Gram-positive bacteria when compared with Gram-negative bacteria, due to the lack of the outer cell membrane which difficults the access of the enzymes to the peptidoglycan layer (Dai et al., 2005). The first attempts to form protoplasts from Gram-negative bacteria resulted in the formation of spheroplasts, which are corps that retain part of the cell wall (Peberdy, 1980). In 1976, Weiss developed a procedure for protoplasts formation in *Escherichia coli* which involved a treatment with lysozyme and ethylenediaminetetraacetic acid (EDTA) (Weiss, 1976). The success of protoplasts formation in Gram-negative bacteria depends of the concentration of enzyme used, the maintenance of a constant temperature or ionic conditions, and the presence of EDTA (Weiss, 1976).

The protoplasts formed can be inactivated through exposure to ultraviolet (UV) light or heat treatment before the protoplasts fusion. These protoplasts only can recover through fusion or recombination to repair their fatal lesions (Biot-Pelletier & Martin, 2014). Those lesions may occur in the same site of the chromosome, which difficults the regeneration; or they can occur in different sites of the chromosome, and in this case the information can be complementary, allowing a better screening of the

microorganisms resulting from the fusion. This multiple inactivation process of parental protoplasts can avoid the genetic marking processes and increases the screening efficiency of recombinants (Gong et al., 2009; Zhao et al., 2008).

The protoplasts fusion can be promoted through electric pulses or through incubation with fusogenic agents, which change the cellular membrane fluidity (Biot-Pelletier & Martin, 2014; Peberdy, 1980). Polyethylene glycol (PEG) is the most common fusogenic agent. PEG induces the protoplasts aggregation, and the fusion occurs after the fusogenic agent is washed or diluted (Peberdy, 1980). The concentration of PEG, its molecular weight, and the time of treatment are important factors for an efficient protoplasts fusion (Hopwood, 1981).

In fungi, protoplasts fusion induced by PEG is influenced by the presence and concentration of cations (Peberdy, 1980). The requisite of Ca^{2+} is well established, while the addition of Na^{+} or K^{+} decreases the fusion frequency. Besides that, the pH of the fusion media also influences the protoplasts fusion. A higher fusion frequency was obtained in alkaline conditions and in the presence of Ca^{2+}. On the other hand, the fusion frequency was better at lower pH values in the absence of Ca^{2+} (Anné & Peberdy, 1975; Ferenczy et al., 1976). PEG acts as a molecular bridge between the adjacent membranes involving directly hydrogen bonding in the membrane, or indirectly using the Ca^{2+} added to the fusion mixture. The linkages formed by hydrogen bonds could be promoted at lower pH values when Ca^{2+} is not present. Na^{+} and K^{+} decrease the protoplasts fusion due to their preferential binding to the plasma membrane, which reduces the stimulatory role of Ca^{2+} (Anné & Peberdy, 1975; Constabel & Kao, 1974).

After protoplasts fusion, the cells are plated on suitable culture media to allow their regeneration, being necessary their screening and selection (Biot-Pelletier & Martin, 2014).

1.5.2. Examples of strains constructed through protoplasts fusion

The main goal of modifying microorganisms for application in MEOR is to obtain strains with the ability of surviving in extreme environments (*e.g.,* extreme temperature, pH, salinity and pressure), and produce the desired bioproducts. However, there is still limited information about the genetic manipulation of microorganisms for application in MEOR (Patel et al., 2015).

Sun and co-workers (2011) constructed the transformant GW3-3.0 from *Enterobacter cloacae* JD and a *Geobacillus* strain by electrotransformation. *E. cloacae* JD produces a water insoluble biopolymer at 37°C, while the *Geobacillus* strain can grow at high temperatures. The strain GW3-3.0 produced up to 8.33 g/L of exopolysaccharide growing at 54°C. The transformant obtained exhibited a stable genetic phenotype, showing potential for application in MEOR (Sun et al., 2011). Using the same parental strains, the strain ZR3 was constructed through protoplasts fusion. This strain produced exopolysaccharide at optimal temperature of 35°C, but still produced a significant amount of exopolysaccharide at 45°C (7.5 g/L at pH values between 7 and 9). In sand-pack column assays performed with this strain at 40°C and two different permeability conditions, additional oil recoveries between 3.5% and 11.3% were obtained (Sun et al., 2013).

Xu & Lu (2011) used the strains *Enterobacter sakazakii* JD and *Bacillus subtilis* I to construct the transformants STP-1 and STP-5 by protoplasts fusion. *E. sakazakii* JD produced a water insoluble and highly viscous polysaccharide at temperatures below 30°C; *B. subtilis* I can survive in extreme environments. The strains STP-1 and STP-5 increased crude oil recovery by 25% and 17%, respectively, in assays performed in sand-pack columns. The strain STP-1 exhibited higher biopolymer production but lower biogas production when compared with the strain STP-5 (Xu & Lu, 2011).

More recently, the strain FA-2 was constructed from *Bacillus mojavensis* JF-2 and *Pseudomonas stutzeri* DQ. *B. mojavensis* JF-2 produces a water soluble lipopeptide biosurfactant under anaerobic conditions, while *P. stutzeri* DQ exhibits a fast growth under anaerobic conditions. The resulting strain FA-2 produces the lipopeptide under anaerobic conditions, at temperatures up to 50°C, in a range of pH values between 4.5 and 10, and in the presence of NaCl concentrations up to 10%. Through the simulation of oil reservoir conditions, an additional oil recovery of 5.22% was obtained. These experimental results suggest that the strain FA-2 exhibits potential for application in MEOR (Liang et al., 2017).

2. OBJECTIVE

The main goal of this Thesis was the construction of engineered microorganisms with the ability of growing and producing biopolymers at the oil reservoir conditions towards the development of effective MEOR processes. For this purpose, the protoplasts fusion technology was used. The specific aims of the work were:

- Optimization of protoplasts formation, regeneration and fusion for *Bacillus subtilis* and *Rhizobium viscosum*;
- Study the ability of the engineered strains of growing and producing biopolymers.

Additionally, other studies were performed:

- Screening biopolymer producers among different *Paenibacillus* sp. strains;
- Evaluate the applicability of new biopolymers in *ex situ* MEOR using sand-pack columns;
- Study the effect of biopolymer-biosurfactant cocktails in *ex situ* MEOR using sand-pack columns.

3. MATERIALS AND METHODS

3.1. Strains and standard culture conditions

R. viscosum CECT 908 (previously classified as *Arthrobacter viscosus* CECT 908 (Flores-Félix et al., 2017)) and *B. subtilis* PX573 were used as parental strains for the protoplasts fusion experiments. *R. viscosum* CECT 908 is a biopolymer-producing strain, and its potential application in MEOR has been recently demonstrated (Couto et al., 2019). The strain *B. subtilis* PX573, isolated from a crude oil sample obtained from a Brazilian oil field, has the capacity of growing under the extreme conditions usually found in oil reservoirs (high pressure and temperature, and oxygen-limiting conditions (Gudiña et al., 2012)). *R. viscosum* CECT 908 was grown at 30°C (and 150 rpm for liquid cultures), whereas *B. subtilis* PX573 was grown at 37°C (and 180 rpm for liquid cultures).

R. viscosum CECT 908 and *B. subtilis* PX573 were also used to produce biopolymer and biosurfactant in the same conditions described above. Both bioproducts were produced to study their effect in oil recovery assays (alone and combined).

The strains *Paenibacillus* sp. #210, #510, #521 and #540 were isolated from a crude oil sample obtained from a Brazilian oil field. These strains were characterized as bioemulsifier producers (*unpublished results*) and they were grown at 37°C.

3.2. Culture media and solutions

The composition of the different culture media used is presented in **Table 3**. All media were sterilized at 121°C for 15 min. To prepare solid media, agar was added to a final concentration of 20 g/L unless indicated otherwise.

The MA medium and the synthetic medium were used to grow *R. viscosum* CECT 908. The LB medium was used to grow *B. subtilis* PX573. The MSS-S medium and the ML9 medium were used to grow the *Paenibacillus* sp. strains (Liu et al., 2009). Furthermore, all media were used for the regeneration of fusants.

Table 3. Composition of the culture media used in this work.

Culture medium	Composition	pH
MA	20 g/L glucose, 5 g/L peptone, 3 g/L malt extract, 3 g/L yeast extract	7.0
Luria-Bertani (LB)	10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract	7.0
Synthetic medium	25 g/L glucose, 3 g/L yeast extract, 2 g/L K_2HPO_4, 0.1 g/L $MgSO_4.7H_20$	7.0
Low-cost medium	60 g/L sugarcane molasses, 1% (v/v) corn steep liquor (CSL), 2 g/L K_2HPO_4	7.0
SS plating medium	171.1 g/L sucrose (pH 7.3), 3.5 g/L K_2HPO_4, 1.5 g/L KH_2PO_4, 2 g/L glucose, 1.2 g/L $MgSO_4.7H_2O$, 1 g/L NH_4NO_3, 7.5 g/L agar, 20 g/L gelatine	–
MSS	10 g/L NaCl, 5 g/L Na_2HPO_4, 2 g/L NH_4NO_3, 2 g/L KH_2PO_4, 0.02 g/L $MgSO_4.7H_2O$	7.0
MSS-S	MSS medium supplemented with 200 g/L sucrose	7.0
ML9	160 g/L sucrose, 10 g/L yeast extract, 3 g/L K_2HPO_4, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$	6.9

To prepare 1 L of SS plating medium (**Table 3**), 530 mL of demineralised water containing 7.5 g of agar and 1 g of NH_4NO_3 were brought to boiling and poured on 20 g of gelatine. When the gelatine was completely dissolved, the mixture was sterilized at 121°C for 15 min. Subsequently, it was cooled to 52°C, and sterile, pre-warmed (at 52°C) concentrated stock solutions of various reagents (**Table 4**) were added, in the order listed: sucrose (340 mL), K_2HPO_4 (10 mL), KH_2PO_4 (10 mL), glucose (100 mL) and $MgSO_4.7H_2O$ (10 mL). The plates prepared with this medium were allowed to dry for 3 days at room temperature (Clive & Landman, 1970).

Table 4. Concentrated stock solutions used in this work.

Solution	Concentration
Sucrose *	513.4 g/L; pH 7.3
K_2HPO_4 **	350 g/L
KH_2PO_4 **	150 g/L
Glucose *	20 g/L
$MgSO_4.7H_2O$ **	123.4 g/L
$MgCl_2$ **	47.6 g/L
EDTA **	2.9 g/L

* Sterilized at 121°C for 15 min.
** Filter-sterilized (0.2 μm pore-size filter).

The composition of the different buffers and solutions used is shown in **Table 5**. All solutions were sterilized at 121°C for 15 min (except for the DFC dilution fluid), and they were stored at 4°C until use.

The DFC dilution fluid was prepared by adding sterile concentrated stock solutions to sterile demineralised water in the order listed: sucrose, K_2HPO_4, KH_2PO_4, $MgCl_2$ and EDTA (**Table 4**) (Clive & Landman, 1970).

Table 5. Composition of solutions used in this work.

Solution	Composition
Tris-HCl	1.2 g/L Tris-HCl; pH 8.0
SMM buffer	171.1 g/L sucrose, 1.9 g/L $MgCl_2$, 2.3 g/L maleic acid; pH 6.5
PBS buffer	1.7 g/L K_2HPO_4, 1.4 g/L KH_2PO_4, 8.8 g/L NaCl; pH 6.0
Hypertonic phosphate buffer	PBS buffer containing 145.7 g/L mannitol
Fusogenic agent	SMM buffer containing 40% (w/v) PEG4000
PEG buffer	SMM buffer containing 40% (w/v) PEG6000, 1.1 g/L $CaCl_2$, 5% (v/v) DMSO
DFC dilution fluid	171.1 g/L sucrose (pH 7.3), 3.5 g/L K_2HPO_4, 1.5 g/L KH_2PO_4, 0.48 g/L $MgCl_2$, 0.15 g/L EDTA

The concentrated antibiotic stock solutions were prepared according to **Table 6,** and filter-sterilized using 0.2 μm pore-size filters.

Table 6. Concentrated antibiotic stock solutions.

Antibiotic	Concentration	Diluent
Ampicillin	100 mg/mL	Water
Chloramphenicol	25 mg/mL	Ethanol (95-99%, v/v)
Erythromycin	25 mg/mL	Ethanol (95-99%, v/v)
Kanamycin	50 mg/mL	Water
Spectinomycin	100 mg/mL	Water
Streptomycin	50 mg/mL	Water
Tetracycline	10 mg/mL	Ethanol (70%, v/v)

3.3. Preparation of heat-killed intact-organism suspensions

R. viscosum CECT 908 was grown in 500 mL Erlenmeyer flasks containing 250 mL of synthetic medium for 5 days. *B. subtilis* PX573 was grown in 500 mL Erlenmeyer flasks containing 250 mL of LB medium for 24 h. The flasks were incubated at the conditions described in the Section 3.1 for each microorganism. Afterwards, the cells were harvested by centrifugation (2600 × g, 20 min) and resuspended in a minimal volume of the respective culture medium. The cell suspensions were

autoclaved at 121°C for 20 min and after that washed three times with a sterile NaCl solution (8.5 g/L). Finally, the cell pellets were resuspended in DFC buffer, steamed at 100°C for 10 min, and stored at -20°C. The heat-killed cell suspensions were used without dilution in the following experiments (Clive & Landman, 1970).

3.4. Preparation of SS plating medium with membrane filters and inactive cells

A sterile cellulose acetate membrane filter (45 mm diameter, 0.45 µm pore-size; Sartorius Stedim Biotech, Germany) was placed on the surface of each petri dish (60 mm diameter) containing SS plating medium. The membrane filter was allowed to dry, and subsequently 100 µL of the inactive cell suspension of *R. viscosum* CECT 908 or *B. subtilis* PX573 were spread on the membrane filter. The plates were stored at 4°C until use (Clive & Landman, 1970).

3.5. Antibiotics selection

The antibiotics tested to be used as limiting factors for the growth of the parental strains after protoplasts fusion were ampicillin (100 µg/mL), kanamycin (50 µg/mL), chloramphenicol (25 µg/mL), spectinomycin (100 µg/mL), tetracycline (10 µg/mL), streptomycin (50 µg/mL) and erythromycin (25 µg/mL). The assays were performed in petri dishes containing the appropriate medium (MA for *R. viscosum* CECT 908 and LB for *B. subtilis* PX573) supplemented with one of the antibiotics above mentioned at the indicated concentration. The plates were incubated at the appropriate temperature for each microorganism (Section 3.1) for 5 days.

3.6. Construction of engineered microorganisms

For the construction of engineered microorganisms, two different strategies were followed.

3.6.1. Strategy 1

Protoplasts formation and fusion was performed following the methodology previously reported by Sun and co-workers (2013) with some modifications.

3.6.1.1. Protoplasts formation

R. viscosum CECT 908 and *B. subtilis* PX573 were grown in Erlenmeyer flasks (250 mL) containing 100 mL of MA or LB medium, respectively. The flasks were inoculated with 1% (v/v) of pre-culture and incubated at the appropriate conditions for each microorganism (Section 3.1). When the cultures reached an optical density ($OD_{600\ nm}$) between 0.6 and 0.9, the cells were harvested by centrifugation (3700 × g, 10 min, 4°C) and washed twice with PBS buffer (pH 6.0) (in this case, the centrifugations were performed at 1700 × g for 10 min at 4°C). The cells obtained from 6 mL of culture were resuspended in 6 mL of SMM buffer containing lysozyme from chicken egg white (Sigma-Aldrich, USA) at two different concentrations (3 and 4 g/L). The cells were incubated at 30°C and 150 rpm to allow the digestion of the peptidoglycan layer. The release of protoplasts was monitored periodically through light microscopy observation. When most of the cells were protoplasts, they were centrifuged (1700 × g, 10 min, 4°C), washed twice with hypertonic phosphate buffer, and resuspended in 3 mL of the same buffer. Serial dilutions of the *R. viscosum* CECT 908 and *B. subtilis* PX573 protoplasts suspensions (100 µL) were plated on MA or LB medium, respectively, to determine the number of protoplasts. The plates were incubated at the appropriate temperature for each microorganism until the colonies became visible.

3.6.1.2. Protoplasts fusion

1 mL of each protoplasts suspension were mixed in a 15 mL tube and incubated at 30°C and 150 rpm. After 5 min, the cells were collected by centrifugation (650 × g, 10 min). The protoplasts mixture was resuspended in a solution containing 0.2 mL of SMM buffer and 1.8 mL of fusogenic agent, and then incubated at 30°C and 150 rpm for 5 min. Subsequently, the cells were collected by centrifugation (650 × g, 10 min) and resuspended in 2 mL of SMM buffer. Aliquots (50 µL) of the protoplasts suspensions were plated on MA and LB medium containing erythromycin (25 µg/mL). The plates were incubated at 37°C.

3.6.1.3. Protoplasts regeneration and screening of fusants

The fusants obtained were transferred to different solid regeneration media to evaluate their morphology: MA; MA supplemented with erythromycin (12.5 μg/mL); LB; and LB supplemented with erythromycin (12.5 μg/mL). The plates were incubated at 37°C.

The fusants that were able to grow in solid medium were inoculated in 50 mL Erlenmeyer flasks containing 20 mL of different liquid media to evaluate the production of biopolymer. The media used were MA, LB, synthetic medium and low-cost medium. The assays were performed at 37°C and 150 rpm.

3.6.2. Strategy 2

Protoplasts formation and fusion was performed following the methodology described by Dai et al. (2005) with some modifications.

3.6.2.1. Protoplasts formation

R. viscosum CECT 908 was grown in 30 mL of MA medium for 48 h, and *B. subtilis* PX573 was grown in 30 mL of LB medium for 24 h. When the cultures reached an $OD_{600\ nm}$ between 0.8 and 1.2, the cells of the parental strains were harvested by centrifugation (4400 × g, 10 min, 4°C). The cells were washed three times with Tris-HCl (pH 8.0) and centrifuged at 3500 × g for 10 min at 4°C. Finally, the cells were resuspended in 30 mL of Tris-HCl (pH 8.0) containing 171.1 g/L of sucrose.

In the case of *R. viscosum* CECT 908, a treatment with EDTA was performed to remove the outer cell membrane. An EDTA solution (29.2 g/L, pH 8.0) was added slowly to the cell suspension over a period of 20 min, to achieve a final concentration of 2.9 g/L. The cells were incubated at 150 rpm and 30°C in the presence of EDTA for different times (**Table 7**). Subsequently, the cells were harvested by centrifugation (6000 × g, 20 min, 4°C) and washed with SMM buffer. For *B. subtilis* PX573, when the EDTA treatment was not performed, the cells were harvested by centrifugation (6000 × g, 20 min, 4°C) and washed with SMM buffer. When the EDTA treatment was performed, the procedure was the same described above for *R. viscosum* CECT 908 (**Table 7**).

Table 7. Conditions tested for EDTA and lysozyme treatments for *Rhizobium viscosum* CECT 908 and *Bacillus subtilis* PX573 protoplasts formation.

Assay	EDTA treatment (incubation time, min)		Lysozyme treatment					
			R. viscosum CECT 908			*B. subtilis* PX573		
	R. viscosum CECT 908	*B. subtilis* PX573	Concentration (g/L)	Incubation time (h)	Incubation temperature (°C)	Concentration (g/L)	Incubation time (h)	Incubation temperature (°C)
1	20	–	1	1	30	1	1	30
2	20	–	1	1	30	1	1	30
3	20	–	1	1	30	1	1	30
4	20	–	1	1	30	1	1	30
5	20	–	1	1	30	1	1	30
6	20	–	1	1	30	1	1	30
7	20	–	1	1	30	1	1	30
8	20	20	1	1	30	1	1	30
9	20	–	2	1	30	2	1	30
10	20	–	3	1	30	3	1	30
11	20	–	2	1.5	37	3	1.5	37
12	20	–	2	2	37	3	2	37
13	20	–	2	1.5	37	3	1.5	37
14	20	–	2	2	37	3	2	37
15	20	20	2	21	37	3	21	37
16	40	40	2	21	37	3	21	37
17	60	60	2	1	37	3	1	37
18	120	120	2	1	37	3	1	37
19	60*	60*	2	1	37	3	1	37
20	60**	60**	2	1	37	3	1	37

* A Triton X-100 solution (1%, v/v) was added to the cell suspension to achieve a final concentration of 0.1% (v/v) instead of EDTA.
** After the addition of EDTA, a Triton X-100 solution (1%, v/v) was added to the cell suspension to achieve a final concentration of 0.1% (v/v). After that the cells were incubated in the conditions indicated in the table.

Subsequently, the cells from both strains were resuspended in 30 mL of SMM buffer containing different concentrations of lysozyme from chicken egg white (Sigma-Aldrich, USA). The cells were incubated at 30°C or 37°C and 150 rpm to allow the digestion of the peptidoglycan layer (**Table 7**). After the incubation time, the protoplasts were centrifuged at 6000 × g and 4°C for 20 min and washed once or twice with SMM buffer. The protoplasts formed were resuspended in a variable volume of SMM buffer

(between 2 and 30 mL). The number of washing steps and the volume of SMM used depended on the stability of the pellet.

Aliquots (100 µL) of serial dilutions of the *R. viscosum* CECT 908 and *B. subtilis* PX573 protoplasts suspensions were plated on MA or LB medium (with 20 g/L or 50 g/L of agar), respectively, supplemented or not with one of the following osmotic stabilizers: sucrose (171.1 g/L), sodium succinate (135 g/L), mannitol (91 g/L), sorbitol (91 g/L), KCl (37.3 g/L), NaCl (29.2 g/L) and $MgCl_2$ (47.6 g/L). The use of these osmotic stabilizers has been reported by different authors (Gao et al., 2011; Singhvi et al., 2010; Yu et al., 2015). In all the cases, these concentrations correspond to 0.5 M. The plates were incubated at the appropriate temperature for each microorganism (Section 3.1) for 1 day for *B. subtilis* PX573 and at least 3 days for *R. viscosum* CECT 908.

3.6.2.2. Protoplasts lysis

1 mL of each protoplasts suspension was centrifuged at 3700 × g for 10 min, washed twice with sterile demineralised water, and resuspended in 1 mL of sterile demineralised water. Aliquots (100 µL) of serial dilutions of the lysed protoplasts suspensions from *R. viscosum* CECT 908 and *B. subtilis* PX573 were plated on MA medium or LB medium, respectively. The plates were incubated at the appropriate temperature for each microorganism (Section 3.1) for 1 day for *B. subtilis* PX573 and at least 3 days for *R. viscosum* CECT 908.

3.6.2.3. Protoplasts fusion

The protoplasts suspensions obtained from both parental strains (1 mL of each) were mixed in a 15 mL tube and incubated at 30°C and 150 rpm. After 10 min of incubation, the protoplasts were harvested by centrifugation (2000 × g, 20 min, 4°C) and resuspended in 1 mL of PEG buffer. After incubation at 30°C for 10 or 20 min, 2 mL of SMM buffer were added and the protoplasts were harvested by centrifugation at 2000 × g and 4°C for 20 min. The protoplasts were resuspended in 1 mL of LB medium containing 171.1 g/L of sucrose. Aliquots of the protoplasts suspension (50 µL) were spread on MA plates and LB plates containing erythromycin (12.5 µg/mL or 25 µg/mL) and 171.1 g/L of sucrose. The plates were incubated at 37°C.

3.6.2.4. Protoplasts regeneration and screening of fusants

The fusants obtained were transferred to different solid regeneration media to evaluate their morphology. The different media used are presented in **Table 8**.

Table 8. Solid regeneration media used in this work.

Medium	Agar concentration	Supplements
MA	20 g/L	———
		12.5 µg/mL erythromycin
		25 µg/mL erythromycin
		12.5 µg/mL erythromycin, 171.1 g/L sucrose
		25 µg/mL erythromycin, 171.1 g/L sucrose
		91 g/L mannitol
		135 g/L sodium succinate
	50 g/L	———
		171.1 g/L sucrose
		91 g/L mannitol
LB	20 g/L	———
		12.5 µg/mL erythromycin
		25 µg/mL erythromycin
	50 g/L	———
		171.1 g/L sucrose
SS plating medium	7.5 g/L	———
		Membrane filters Inactive cells

The fusants that were able to grow in solid medium were inoculated in liquid medium to evaluate the production of biopolymer. The fusants were inoculated in 50 mL Erlenmeyer flasks containing 20 mL of MA medium or LB medium (supplemented or not with 171.1 g/L of sucrose). The fusants were incubated at 37°C and 110 rpm.

3.7. Production of biosurfactants and biopolymers

3.7.1. Biosurfactant production by *B. subtilis* PX573

The strain *B. subtilis* PX573 was used for biosurfactant production. Erlenmeyer flasks (500 mL) containing 250 mL of LB medium were inoculated with 1% (v/v) of a pre-culture of *B. subtilis* PX573 and incubated at 37°C and 180 rpm for 24 h. At the end of the fermentation, the cells were harvested by centrifugation (1700 × g, 15 min). To recover the biosurfactant, the cell-free supernatant was subjected to acid precipitation. Briefly, the supernatant was adjusted to pH 2.0 with HCl (18%, v/v) and incubated overnight at 4°C to promote the precipitation of the biosurfactant. Afterwards, the precipitate was collected by centrifugation (4400 × g, 20 min, 4°C) and washed twice with acidified water (pH 2.0). The crude biosurfactant was dissolved in a minimal amount of demineralised water by adjusting the pH to 8.0 using NaOH (40 g/L). Finally, the crude biosurfactant solution was freeze-dried and stored at -20°C for further use (Gudiña et al., 2012).

3.7.2. Biopolymer production by *R. viscosum* CECT 908

The strain *R. viscosum* CECT 908 was used for biopolymer production. Erlenmeyer flasks (500 mL) containing 250 mL of synthetic medium were inoculated with 1% (v/v) of a pre-culture of *R. viscosum* CECT 908 and incubated at 30°C and 150 rpm for 5 days. At the end of the fermentation, the biopolymer was purified following the procedure described by Novak et al. (1992). The *R. viscosum* cultures were diluted with two volumes of demineralised water and the cells were removed by centrifugation at 2600 × g for 20 min. Afterwards, hexadecyltrimethylammonium bromide (CTAB) was added to the cell-free supernatants at a final concentration of 0.175% (w/v) to precipitate the biopolymer. The crude biopolymer was recovered through centrifugation (2600 × g, 20 min) and subsequently it was resuspended in a NaCl solution (58.4 g/L). The biopolymer was precipitated by the addition of three volumes of ethanol (99%, v/v). The supernatant was carefully removed by decantation and the remaining ethanol was evaporated by incubation at 37°C. Subsequently, the biopolymer was resuspended in a minimal volume of demineralised water. To remove the quaternary ammonium salts formed during the process, the biopolymer solution was dialysed against demineralised water at 4°C in a Cellu-Sep membrane (molecular weight cut-off 12-14 kDa; Membrane Filtration Products, Inc., USA) for three days with twice

replacement of demineralised water per day. Finally, the purified biopolymer was freeze-dried, weighed and stored at -20°C for further studies.

3.7.3. Biopolymer production by *Paenibacillus* sp. strains

Four *Paenibacillus* sp. strains (#210, #510, #521 and #540) were evaluated for biopolymer production. Erlenmeyer flasks (50 mL) containing 20 mL of MSS-S or ML9 medium were inoculated with 1% (v/v) of a pre-culture of each *Paenibacillus* sp. strain and incubated at 37°C and 120 rpm for 13 days. At different time intervals, an Erlenmeyer flask was collected to evaluate biopolymer production by measuring the viscosity of the cultures (as described in Section 3.8.1).

In other set of experiments, 250 mL Erlenmeyer flasks containing 100 mL of ML9 medium were inoculated with 1% (v/v) of a pre-culture of the corresponding *Paenibacillus* sp. strain and incubated at 37°C and 100 or 120 rpm. Samples (20 mL) were collected at different time intervals to evaluate biopolymer production as described below (Section 3.8.1).

At the end of the assays, the cells were harvested by centrifugation (8400 × g, 30 min) and the biopolymer was recovered from the cell-free supernatants through precipitation by the addition of two volumes of ethanol (99%, v/v). The mixture was incubated overnight at -20°C, the precipitate was collected by centrifugation (8400 × g, 30 min, 4 °C) and resuspended in 5 mL of demineralised water (crude biopolymer). Subsequently, the crude biopolymer solution was dialysed against demineralised water at 4°C in a Cellu-Sep membrane (molecular weight cut-off 12-14 kDa; Membrane Filtration Products, Inc., USA) for three days with twice replacement of demineralised water per day. Finally, the purified biopolymer was freeze-dried, weighed and stored at -20°C for further studies.

3.8. Bioproducts characterization

3.8.1. Study of rheological properties

The rheological properties of the different culture samples, biopolymer solutions, biosurfactant-biopolymer mixtures and crude oil samples were studied using a hybrid rheometer (Discovery HR1, TA

Instruments, USA) equipped with a cone-plate geometry (diameter 60 mm; angle 2.006°; gap 0.064 mm). All the measurements were performed at 40°C.

The shear stress (Pa) and apparent viscosity (mPa s) were measured at different shear rates (0.1-300 s^{-1}) through three successive flow ramps (0.1 → 300 s^{-1}; 300 → 0.1 s^{-1}; 0.1 → 300 s^{-1}) (Couto et al., 2019). For the crude oil samples, only one ramp (0.1 → 300 s^{-1}) was performed. Each sample was analysed in duplicate.

3.8.2. Surface and interfacial tension

Surface and interfacial tension measurements of culture broth samples, biosurfactant and biopolymer solutions and biosurfactant-biopolymer mixtures were performed according to the De Noüy Ring method using a KRÜSS K20 Tensiometer (KRÜSS GmbH, Hamburg, Germany) equipped with a 1.9 cm De Noüy platinum ring. For the interfacial tension measurements, *n*-hexadecane was used as the hydrocarbon phase. The values presented represent the average ± standard deviation of five measurements. All the measurements were performed at room temperature (25°C).

3.8.3. Critical micelle concentration (*cmc*)

Critical micelle concentration is defined as the concentration of an amphiphilic compound in solution at which the formation of micelles is initiated. Therefore, it is important for several biosurfactant applications to establish the *cmc*, as above this concentration it is not expected to observe any additional effect in the surface activity. Concentrations ranging from 0.001 to 0.8 g/L of the crude biosurfactant produced by *B. subtilis* PX573 were prepared in demineralised water and the surface tension of each sample was determined as described above. 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 (Gudiña et al., 2010).

3.8.4. Emulsifying activity

The emulsifying activity was determined by the addition of 2 mL of *n*-hexadecane to the same volume of bioproduct solution in 20 mL glass tubes. The tubes were mixed with vortex at high speed for 2 min and then incubated at 25°C for 24 h. The emulsifying indexes at 24 hours (E_{24}) were calculated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm) (Gudiña et al., 2013). Control assays were performed with demineralised water at the same conditions. All the measurements were performed at least in triplicate.

3.9. MEOR assays

The performance of the different bioproducts in oil recovery was studied using a sand-pack column model through an *ex situ* approach as described by Couto et al. (2019). The assays were performed using a heavy crude oil ($\eta_{40°C}$= 545 ± 41 mPa s). The viscosity of the oil was determined as described above (Section 3.8.1).

The oil recovery assays were performed using vertically oriented acrylic columns with a volume of 280 mL, at 40°C and a constant flow rate of 2 mL/min. The columns were provided with a sieve and cap fixed at the bottom. After packing the columns tightly with sand (previously sifted with a 0.45 mm sieve), a top sieve and cap were fixed. The caps on both the ends of the column were provided with holes for insertion of inlet and outlet tubes, and rubber 'O' rings surrounded the caps to hermetically seal the column.

The columns were flooded with demineralised water. The pore volume (PV, mL) was calculated by measuring the volume of water required to saturate each column and the porosity (%) was determined by dividing the PV by the total volume of the column (280 mL). After that, the crude oil was injected into the columns and the original oil in place (OOIP, mL) was calculated as the volume of crude oil retained into the columns. The initial oil saturation (S_{oi}, %) was calculated as follows:

$$S_{oi} = \left(\frac{OOIP}{PV}\right) \times 100 \qquad \text{Eq. 1}$$

The columns were incubated overnight at 40°C. Afterwards, they were flooded again with demineralised water to remove the excess of crude oil, until crude oil was no longer observed in the effluent. The amount of crude oil recovered, called oil recovered after water flooding (S_{orwf}, mL), was determined volumetrically and the residual oil saturation (S_{or}, %) was calculated as:

$$S_{or} = \left(\frac{OOIP - S_{orwf}}{OOIP}\right) \times 100 \qquad \textbf{Eq. 2}$$

The residual oil was subjected to the tertiary oil recovery processes. 200 mL of the different bioproducts solutions were injected into the columns. Afterwards, the columns were flooded with 250 mL of demineralised water. The volume of crude oil recovered (oil recovered after bioproduct flooding (S_{orbf}, mL)) was measured and the additional oil recovery (AOR, %) was calculated as:

$$AOR = \left(\frac{S_{orbf}}{OOIP - S_{orwf}}\right) \times 100 \qquad \textbf{Eq. 3}$$

Control assays were performed by injecting 450 mL of demineralised water into the columns at the same conditions. All the assays were performed at least in triplicate.

4. RESULTS AND DISCUSSION

4.1. Construction of engineered microorganisms

4.1.1. Selection of selective markers

The use of selective markers in protoplasts fusion is advantageous for selecting successful fusants (M. Li et al., 2013). In order to choose two antibiotics to be used as limiting factors for the growth of the parental strains (*B. subtilis* PX573 and *R. viscosum* CECT 908) after their fusion, several antibiotics were tested.

All the antibiotics tested (Materials and Methods Section 3.5) completely inhibited the growth of *B. subtilis* PX573 in solid medium, except for streptomycin, which was not completely lethal. On the contrary, *R. viscosum* CECT 908 was able to grow in the presence of all the antibiotics tested, being the highest growth observed in erythromycin-containing plates. Consequently, erythromycin was chosen as selective marker for *B. subtilis* PX573, and it was used at two different concentrations in the different assays, namely 12.5 μg/mL and 25 μg/mL (both of them completely inhibit the growth of *B. subtilis* PX573). As it was not found an antibiotic at which *B. subtilis* PX573 was resistant and *R. viscosum* CECT 908 was sensitive, the temperature (37°C) was chosen as a selective marker for *R. viscosum* CECT 908. 37°C is the optimum temperature for the growth of *B. subtilis* PX573, whereas *R. viscosum* CECT 908 does not grow at this temperature.

4.1.2. Strategy 1

4.1.2.1. Protoplasts formation

The protoplasts formation process is influenced by many factors, including the concentration of lysozyme and the time of incubation with lysozyme. **Table 9** shows the lysozyme concentrations used in each assay, as well as the optimum incubation times (*i.e.*, when the majority of the cells observed were protoplasts) for each microorganism, determined by observation of protoplasts formation through light microscopy.

Table 9. Lysozyme concentrations and incubation times tested for the formation of protoplasts from the parental strains.

Assay	Lysozyme concentration (g/L)	Incubation time (h)	
		R. viscosum CECT 908	*B. subtilis* PX573
1	3	2.5	1.5
2	4	2	1

As expected, the optimum incubation time for the formation of protoplasts decreased with the increase of lysozyme concentration, from 2.5 h to 2 h in the case of *R. viscosum* CECT 908 and from 1.5 h to 1 h for *B. subtilis* PX573, with an increase of lysozyme concentration from 3 g/L to 4 g/L.

After protoplasts formation, serial dilutions of the *R. viscosum* CECT 908 and *B. subtilis* PX573 protoplasts suspensions were plated on MA and LB medium, respectively, and incubated at the appropriate temperature for each microorganism. In **Table 10** the number of viable cells per mL obtained after protoplasts formation is presented.

Table 10. Number of viable cells/mL obtained after protoplasts formation.

Assay	*R. viscosum* CECT 908 (cells/mL)	*B. subtilis* PX573 (cells/mL)
1	5.57×10^8	2.51×10^7
2	2.62×10^7	1.45×10^7

The number of viable cells (**Table 10)** includes the protoplasts formed and the parental cells that did not generate protoplasts. In both assays, the number of viable cells obtained for *R. viscosum* CECT 908 was higher than the one for *B. subtilis* PX573, being that difference lower in the assay 2.

4.1.2.2. Protoplasts fusion

The protoplasts suspensions obtained from each parental strain were mixed and subjected to the fusion protocol. In the assay 1, three fusants were obtained in plates containing MA medium supplemented with erythromycin (25 µg/mL) after 5-7 days of incubation at 37°C. In the assay 2, five fusants were obtained in LB medium supplemented with sucrose (171.1 g/L) and 12.5 µg/mL of erythromycin, one fusant in MA supplemented with 25 µg/mL of erythromycin, and 25 fusants in MA supplemented with 12.5 µg/mL of erythromycin (all of them after 6-7 days of incubation at 37°C). In this assay, sucrose was added to the culture medium as an osmotic stabilizer, to provide a hypertonic

environment and stabilize the osmotically fragile protoplasts until the cell wall was regenerated (Baehman, 1986).

4.1.2.3. Protoplasts regeneration and screening of fusants

The three fusants obtained in the assay 1 were transferred to new MA plates supplemented with erythromycin (25 μg/mL). Only one of the fusants exhibited growth after 2 days of incubation at 37°C, while the other two fusants did not grow even with a longer incubation time. This fusant was inoculated in liquid medium (MA and LB medium) at 37°C to evaluate the production of biopolymer. Although the fusant was able to grow in these media, it did not exhibit the ability of producing the envisaged biopolymer.

Regarding the assay 2, the five fusants obtained in LB plates supplemented with sucrose and 12.5 μg/mL of erythromycin were transferred to new LB plates with and without erythromycin (12.5 μg/mL). The fusant obtained in MA plates supplemented with 25 μg/mL of erythromycin and four of the 25 fusants obtained in MA plates supplemented with 12.5 μg/mL of erythromycin were transferred to new plates of MA and MA supplemented with erythromycin (12.5 μg/mL). In the new plates, eight fusants grew after 2 days of incubation at 37°C: four fusants in MA plates supplemented with 12.5 μg/mL of erythromycin and four fusants in LB plates supplemented with 12.5 μg/mL of erythromycin.

Subsequently, the ability of the eight fusants of growing in liquid medium and producing biopolymer was evaluated by growing them in MA and LB medium at 37°C. After 4 days of growth, the fusants inoculated from the MA plates grew faster in MA medium than in LB medium. The cultures of the fusants inoculated from the LB plates formed aggregates, which were higher in MA medium than in LB medium. After 10 days of growth, the culture media did not appear viscous, thus indicating that the fusants obtained did not produce biopolymers in these conditions. The eight fusants were also inoculated in other liquid media (synthetic medium and low-cost medium) to evaluate the production of biopolymer. The fusants were able to grow in these media but they did not have the ability of producing biopolymers, even after 6 days of growth.

As the fusants obtained in both assays using this strategy did not exhibit the ability to produce biopolymers, thus a different strategy for the formation and fusion of protoplasts was evaluated.

4.1.3. Strategy 2

4.1.3.1. Protoplasts formation

As *R. viscosum* is a Gram-negative bacterium, the lysozyme treatment was not enough to allow the formation of protoplasts due to the presence of the outer cell membrane. A preliminary assay was performed to evaluate the effect of the EDTA treatment (final concentration 2.9 g/L and incubation time 20 min) on *R. viscosum* CECT 908 and *B. subtilis* PX573. The number of viable cells/mL obtained after the protoplasts formation for *B. subtilis* PX573 was considerably low (2.6×10^3 cells/mL) comparing with the results obtained in the assays performed in the strategy 1. For *R. viscosum* CECT 908, the number of viable cells obtained (3.3×10^8 cells/mL) was similar to the assays performed in the strategy 1. In the case of *B. subtilis* PX573, the EDTA treatment caused a significant decrease in the number of viable cells, which indicates that this treatment was very aggressive for this microorganism.

According to the previous results, an initial EDTA treatment was used to remove the outer membrane of *R. viscosum* CECT 908. Subsequently, a lysozyme treatment was used to digest the peptidoglycan layer of *R. viscosum* CECT 908 and *B. subtilis* PX573. The different conditions tested are shown in **Table 7**. After protoplasts formation, serial dilutions of the *R. viscosum* CECT 908 and *B. subtilis* PX573 protoplasts suspensions were plated on different culture media, and the number of viable cells obtained is presented in **Table 11**.

Table 11. Number of viable cells/mL obtained after protoplasts formation.

Assay	*R. viscosum* CECT 908 (cells/mL)	*B. subtilis* PX573 (cells/mL)
1	3.9×10^9	1.8×10^6
2	1.9×10^8	1.0×10^3
3	1.8×10^9	2.7×10^4
4	———	1.6×10^6
5	8.0×10^8	3.0×10^6
6	2.0×10^9	1.3×10^4

The number of viable cells presented in **Table 11** includes the protoplasts formed and the parental cells that did not generate protoplasts. In all the assays, the number of viable cells obtained was higher for *R. viscosum* CECT 908 when compared with *B. subtilis* PX573.

In the assays 1, 2 and 3, the protoplasts suspensions were plated on MA medium (*R. viscosum* CECT 908) or LB medium (*B. subtilis* PX573). Due to the low number of viable cells obtained for *B. subtilis* PX573, the culture media were modified. In the assay 4, sucrose (171.1 g/L) was added to both culture media as osmotic stabilizer, and a higher concentration of agar (50 g/L) was added to the LB medium. It has been reported that the use of a high concentration of agar (hard agar regeneration medium) is favourable for the regeneration of the protoplasts (Gao et al., 2011). However, the addition of a high concentration of sucrose to the culture medium inhibited the growth of *R. viscosum* CECT 908; consequently, it was not used in the following assays for this microorganism. However, in the case of *B. subtilis* PX573, an increase in the number of viable cells was observed when compared with the assays 2 and 3. For that reason, these media were used to plate the protoplasts suspensions in the following assays. However, in some cases (assay 5), the use of LB medium supplemented with sucrose and 50 g/L of agar led to a fast and widespread growth that did not allow a proper counting of the colonies, hence being necessary to plate the suspensions again on the same culture medium containing 20 g/L of agar.

In order to evaluate the efficiency of the protoplasts formation, the protoplasts formation frequency and the regeneration frequency were calculated as described by Dai et al. (2005) in the assay 7 (**Table 12**).

Table 12. Protoplasts formation and regeneration frequencies obtained in the assay 7.

Parental strain	Cells/mL before protoplasts formation (A)	Viable cells/mL after protoplasts formation (B)	Viable cells/mL after lysis of protoplasts (C)	Protoplasts formation frequency (%) *	Regeneration frequency (%) **
R. viscosum CECT 908	2.1×10^8	1.6×10^8	1.4×10^8	13.7	10.49
B. subtilis PX573	6.5×10^6	1.8×10^6	1.7×10^6	6.6	1.85

A: Assessed by counting colonies in MA medium or LB medium before addition of EDTA and lysozyme.
B: Assessed by counting colonies in MA medium or LB medium supplemented with sucrose (171.1 g/L) and 50 g/L of agar.
C: Assessed by counting colonies in MA medium or LB medium after dilution of protoplasts suspensions in demineralised water.
*Protoplasts formation frequency was calculated using the following formula: (B-C)/B.
**Regeneration frequency was calculated using the following formula: (B-C)/A.

As it can be observed in **Table 12**, the protoplasts formation and regeneration frequencies were higher for *R. viscosum* CECT 908. The protoplasts formation frequencies obtained for *R. viscosum* CECT 908 and *B. subtilis* PX573 were considerably lower as compared to those reported in the literature. Dai

et al. (2005) reported protoplasts formation frequencies between 93% and 99%, and regeneration frequencies between 45% and 75% for two auxotrophic strains of *Escherichia coli*. Wang et al. (2012) reported protoplasts formation frequencies above 90% for *Acinetobacter johnsonii* LP28 in three different conditions tested to optimize the formation and regeneration of protoplasts. However, the regeneration frequencies obtained were below 2% (Wang et al., 2012). In the case of *Bacillus polymyxa*, protoplasts formation efficiencies above 70% were reported for the different conditions tested (Raymundo at al., 1991). Sun et al. (2013) reported the fusion of a Gram-negative (*Enterobacter cloacae* JD) and a Gram-positive (*Geobacillus* sp. AJ) bacterium, being the regeneration frequencies 55.8% and 66.1%, respectively.

4.1.3.2. Protoplasts fusion

To promote the fusion of the protoplasts obtained from *B. subtilis* PX573 and *R. viscosum* CECT 908, the protoplasts suspensions were incubated together in the presence of the fusogenic agent (PEG buffer) for 10 min (assays 1 and 2) or 20 min (assays 3-7). Aliquots of the protoplasts suspensions subjected to the fusion process were plated on MA and LB medium containing sucrose (171.1 g/L) as osmotic stabilizer and two different concentrations of erythromycin: 12.5 µg/mL or 25 µg/mL. The plates were incubated at 37°C to allow the growth of those fusants that were resistant to both selective markers (antibiotic and temperature) and to avoid the growth of the parental strains.

Fusants were observed in all the assays performed after 3-7 days of incubation. The limited number of assays and the variability observed did not allow to establish which incubation time (10 or 20 min) offered better results. However, it could be concluded that in most of the cases, a higher number of fusants was obtained in MA plates containing 12.5 µg/mL of erythromycin when compared with the other media tested. The highest number of fusants was achieved in the assays 7 (27 fusants), 5 (89 fusants) and 6 (136 fusants).

In all the assays, the fusants obtained formed small and transparent colonies, as it can be observed in **Figure 1**. These colonies, formed by bacterial cells without cell wall, are referred in the literature as L colonies, and in most cases they are not able to regenerate the cell wall and revert to normal cells (Gao et al., 2011; Mallonee & Speckman, 1989).

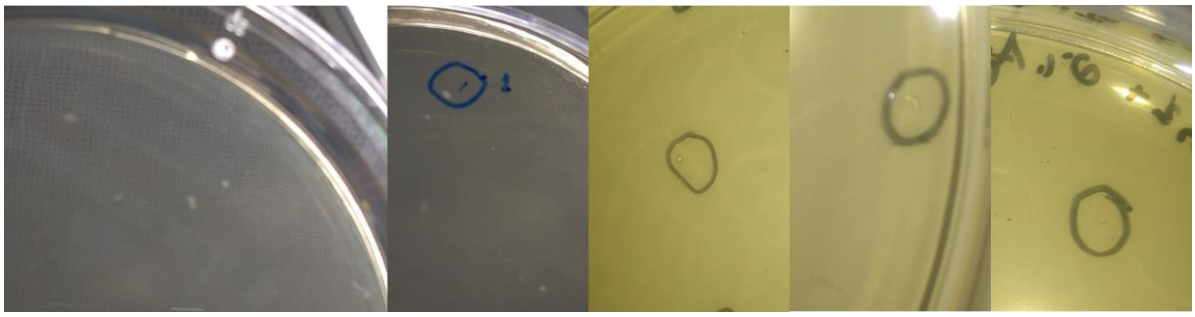

Figure 1. Different fusants obtained in the protoplasts fusion assays.

4.1.3.3. Protoplasts regeneration and screening of fusants

In order to allow their regeneration, the fusants obtained in the different assays were transferred to new agar plates. The different media used are shown in **Table 8**. In a first approach, the regeneration media used were LB and MA, both supplemented with sucrose (171.1 g/L) as an osmotic stabilizer and erythromycin (12.5 µg/mL or 25 µg/mL). However, most of the fusants did not grow in these media (even after 8 days of incubation) or they exhibited a limited growth. For that reason, the same fusants were further inoculated on solid LB and MA medium supplemented only with sucrose or with erythromycin; neither in these media the fusants were able to regenerate.

Due to these negative results, new alternatives for the regeneration of the fusants obtained were evaluated, including the use of MA medium supplemented with mannitol as an alternative osmotic stabilizer, and the use of LB and MA medium with a higher concentration of agar (50 g/L) and different osmotic stabilizers. Neither using these culture media, which did not contain erythromycin as selective marker, it was possible to allow the regeneration and the growth of the fusants.

Seven fusants obtained in the assay 5 were able of growing on MA medium with 50 g/L of agar after 2-6 days of incubation. These fusants were inoculated in liquid medium (MA and LB medium) to evaluate the production of biopolymer. However, after 8 days, only four fusants grew, but they did not produce biopolymer. Also, LB and MA supplemented with sucrose (liquid media) were evaluated but with the same result.

Finally, a more complex medium (SS plating medium containing membrane filters and inactive cells of *B. subtilis* PX573 or *R. viscosum* CECT 908) was evaluated. Five fusants obtained in the assay 5 grew in this culture medium after 8 days of incubation (two in the medium containing inactive cells of *B. subtilis* PX573 and three in the medium containing inactive cells of *R. viscosum* CECT 908).

Subsequently, these fusants were able of growing when they were transferred to MA plates and MA plates supplemented with sucrose and 50 g/L of agar (**Figure 2**).

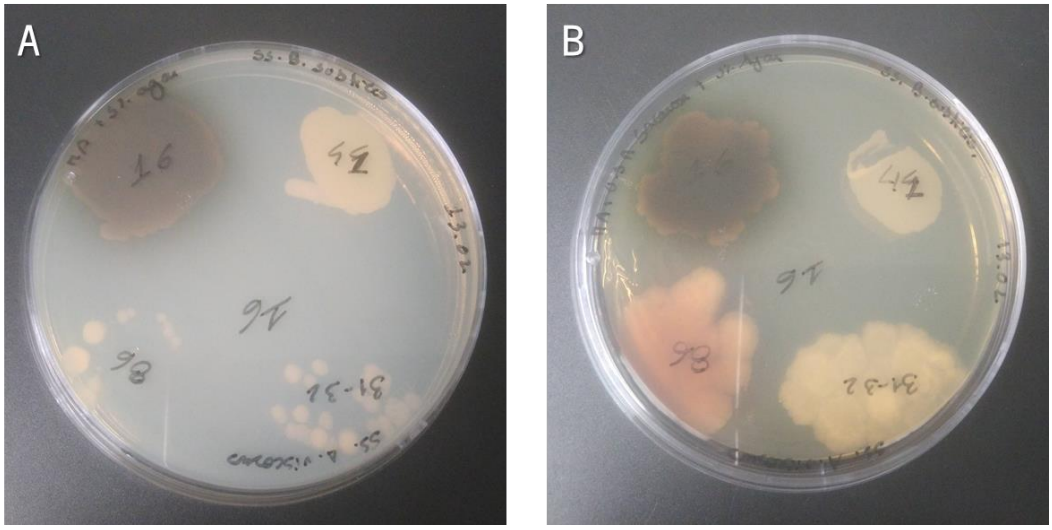

Figure 2. Fusants obtained in the assay 5 growing in MA medium (**A**) and MA supplemented with sucrose (171.1 g/L) and 50 g/L of agar (**B**) after 2 days of incubation at 37°C.

In order to evaluate the production of biopolymer by these fusants, they were grown in liquid medium (MA). After 4 days of incubation, three fusants exhibited growth in liquid medium (**Figure 3**). The apparent viscosity of the cultures from these fusants was measured; however, the values obtained (55-60 mPa s) are not indicative of the production of biopolymer (the apparent viscosity of cultures of *R. viscosum* CECT 908 grown in the same culture medium for 4 days was 739 ± 30 mPa s (Couto et al., 2019)).

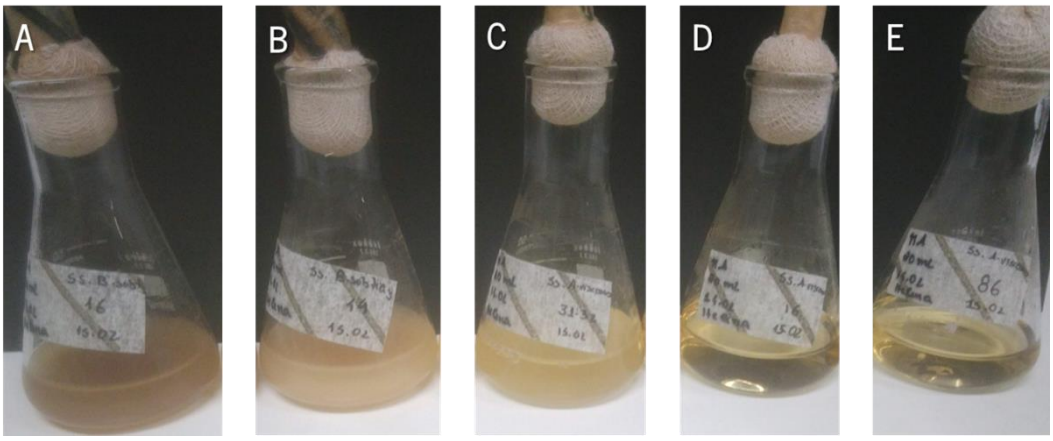

Figure 3. Fusants obtained in the assay 5 growing in MA liquid medium, at 37°C and 150 rpm for 4 days. Fusants 16 (**A**) and 44 (**B**) from the plates containing inactive cells of *Bacillus subtilis* PX573; and fusants 31 (**C**), 16 (**D**) and 86 (**E**) from the plates containing inactive cells of *Rhizobium viscosum* CECT 908.

The 136 fusants obtained in the assay 6 were distributed in plates of SS plating medium containing membrane filters and inactive cells of *B. subtilis* PX573 or *R. viscosum* CECT 908. After 7 days of incubation, all the fusants exhibited some growth. These fusants were transferred to plates of MA medium with 50 g/L of agar (supplemented or not with sucrose), and MA supplemented with mannitol or sodium succinate as osmotic stabilizers. All fusants grew in most of the solid media tested (**Figure 4**).

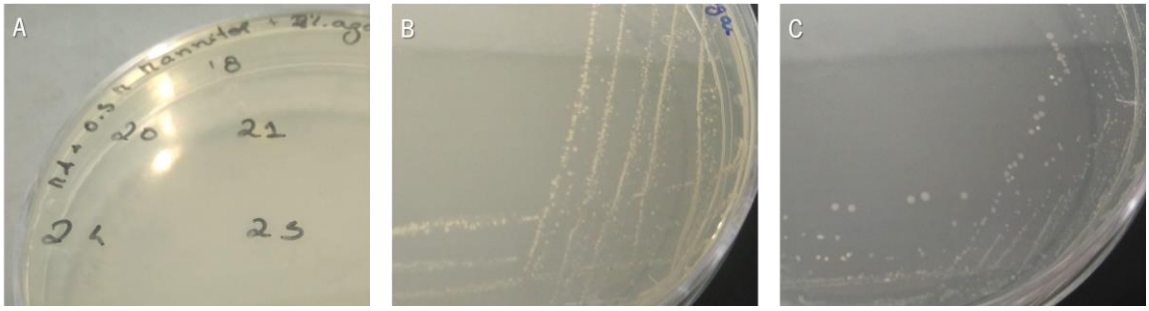

Figure 4. Example of fusants obtained in the assay 6 growing in MA medium supplemented with mannitol (**A** and **B**) and MA medium (**C**).

However, after transferring the fusants several times to new plates containing different culture media, their capacity of growing was progressively lost. The capacity of growing and producing biopolymer by these fusants was evaluated in MA liquid medium and MA liquid medium containing inactive cells of *B. subtilis* PX573 or *R. viscosum* CECT 908. However, after 7 days of incubation, only three fusants exhibited some growth, but they did not produce biopolymer.

Regarding the assay 7, none of the 27 fusants obtained were able to grow when transferred to these solid media.

Several strategies were evaluated to improve the regeneration of the fusants obtained, including the use of different osmotic stabilizers (sucrose, mannitol and sodium succinate (Dai et al., 2005; Gao et al., 2011)), the use of a higher concentration of agar (Gao et al., 2011), and the use of gelatin (SS plating medium), membrane filters and inactive cells of both parental strains (Clive & Landman, 1970). These strategies allowed the regeneration and the growth of several fusants in solid medium. However, when these fusants were inoculated in liquid medium, some of them did not grow, and none of them produced biopolymer.

The protoplasts fusion is affected by many factors, including PEG concentration, the temperature and time of incubation, and the formation of protoplasts. The protoplasts formation process is an essential step for the subsequent fusion, and it is affected by the microorganisms used, the cell age, the presence and the concentration of EDTA, the concentration of enzymes and the time of incubation (Dai et al., 2005;

Weiss, 1976). Based on the negative results obtained in the previous assays, a further optimization of the conditions to generate protoplasts from the parental strains was performed.

4.1.3.4. Optimization of protoplasts formation

In order to optimize the protoplasts formation protocol, different assays were performed to establish the optimum lysozyme concentration, incubation time with lysozyme, osmotic stabilizers and detergent treatments for each parental strain.

4.1.3.4.1. Lysozyme concentration

The effect of different lysozyme concentrations in the protoplasts formation and regeneration frequencies (for *R. viscosum* CECT 908) are shown in **Table 13**.

Table 13. Protoplasts formation and regeneration frequencies obtained with different lysozyme concentrations for *Rhizobium viscosum* CECT 908. The incubation time and temperature were 1 h and 30°C, respectively.

Assay	EDTA treatment (incubation time, min)	Lysozyme concentration (g/L)	Cells/mL before protoplasts formation (A)	Viable cells/mL after protoplasts formation (B)	Viable cells/mL after lysis of protoplasts (C)	Protoplasts formation frequency (%) *	Regeneration frequency (%) **
8	20	1	2.5×10^9	3.3×10^8	8.9×10^7	73.3	9.64
9		2	2.5×10^9	1.1×10^8	1.9×10^7	82.4	3.54
10		3	2.5×10^9	2.1×10^8	9.6×10^7	54.2	4.47

A: Assessed by counting colonies in MA medium before addition of EDTA and lysozyme.

B: Assessed by counting colonies in MA medium after protoplasts formation.

C: Assessed by counting colonies in MA medium after dilution of protoplasts suspensions with demineralised water.

*Protoplasts formation frequency was calculated using the following formula: (B-C)/B

**Regeneration frequency was calculated using the following formula: (B-C)/A

Similar assays were performed for *B. subtilis* PX573, and the results obtained are presented in **Table 14**.

Table 14. Protoplasts formation and regeneration frequencies obtained with different lysozyme concentrations for *Bacillus subtilis* PX573. The incubation time and temperature were 1 h and 30°C, respectively.

Assay	EDTA treatment (incubation time, min)	Lysozyme concentration (g/L)	Cells/mL before protoplasts formation (A)	Viable cells/mL after protoplasts formation (B)	Viable cells/mL after lysis of protoplasts (C)	Protoplasts formation frequency (%) *	Regeneration frequency (%) **
8	20	1	2.0×10^8	2.3×10^5	1.8×10^5	21.7	0.03
9	———	2	2.0×10^8	1.1×10^5	4.2×10^5	-284.8	-0.16
10		3	2.0×10^8	3.6×10^5	8.5×10^4	76.4	0.14

A: Assessed by counting colonies in LB medium before addition of EDTA and lysozyme.

B: Assessed by counting colonies in LB medium after protoplasts formation.

C: Assessed by counting colonies in LB medium after dilution of protoplasts suspensions with demineralised water.

*Protoplasts formation frequency was calculated using the following formula: (B-C)/B

**Regeneration frequency was calculated using the following formula: (B-C)/A

As it can be observed in **Tables 13** and **14**, the protoplasts formation frequencies obtained in these assays for both parental strains were higher when compared with those obtained in the assay 7 (**Table 12**). However, the regeneration frequencies were lower. According to the results, the concentration of lysozyme chosen for protoplasts formation for *R. viscosum* CECT 908 and *B. subtilis* PX573 were 2 g/L and 3 g/L, respectively.

4.1.3.4.2. Osmotic stabilizers

The addition of osmotic stabilizers to the culture medium provides the hypertonic environment necessary to stabilize the osmotically fragile protoplasts until the cell wall is regenerated (Baehman, 1986). Different osmotic stabilizers (for details see the Materials and Methods Section 3.6.2.1) were evaluated to choose the most appropriate for the regeneration of protoplasts from *R. viscosum* CECT 908 and *B. subtilis* PX573.

In the case of *B. subtilis* PX573, the cells obtained after protoplasts formation grew in the presence of all the osmotic stabilizers tested (**Figure 5**). However, for *R. viscosum* CECT 908, none of the osmotic stabilizers tested allowed its growth. Consequently, the osmotic stabilizer chosen to supplement the culture media for the regeneration of protoplasts from *B. subtilis* PX573 was sorbitol.

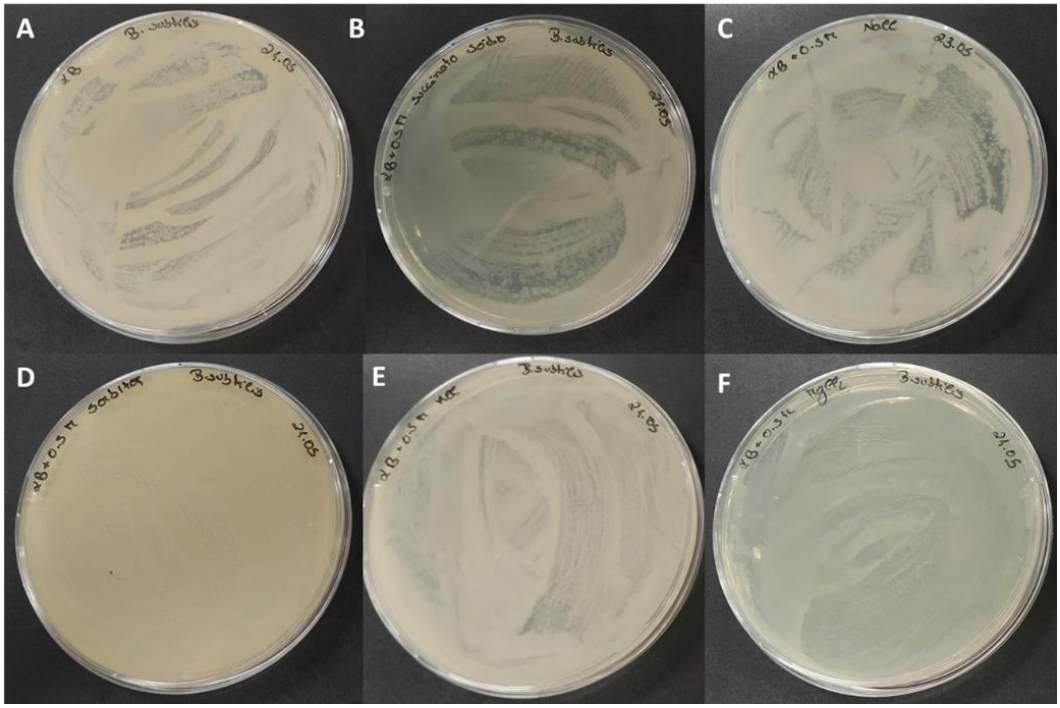

Figure 5. Agar plates inoculated with cell suspensions obtained after protoplasts formation from *Bacillus subtilis* PX573, after incubation at 37°C: LB medium (control; **A**), LB supplemented with sodium succinate (**B**), LB supplemented with NaCl (**C**), LB supplemented with sorbitol (**D**), LB supplemented with KCl (**E**) and LB supplemented with $MgCl_2$ (**F**). All the osmotic stabilizers were evaluated at a concentration 0.5 M.

4.1.3.4.3. Detergent treatment and incubation time with lysozyme

The effect of the incubation time with EDTA, the incubation with Triton X-100, the combination of both detergents, and the incubation time with lysozyme in the protoplasts formation and regeneration frequencies were evaluated for *R. viscosum* CECT 908 (**Table 15**) and *B. subtilis* PX573 (**Table 16**).

Regarding *R. viscosum* CECT 908, a great variability was observed in the results obtained in the different experiments (**Table 15**), which is mainly due to the difficulty of growing this strain in the presence of an osmotic stabilizer. However, it can be observed an increase in the protoplasts formation frequency as the incubation time with EDTA increases, and also with the use of Triton X-100 and the combination of EDTA and Triton X-100. However, the combination of EDTA and Triton X-100 resulted in a decrease in the protoplasts regeneration frequency. Taking into account the protoplasts formation and regeneration frequencies obtained with the different treatments, the conditions used in the assay 17 were selected as the most favourable for protoplasts formation for *R. viscosum* CECT 908, as they were also less aggressive when compared with other conditions tested that offered similar results.

Table 15. Protoplasts formation and regeneration frequencies obtained with different treatments for *Rhizobium viscosum* CECT 908. The temperature of incubation with the detergent(s) was 30°C and the lysozyme concentration and incubation temperature were 2 g/L and 37°C, respectively.

Assay	Detergent treatment (incubation time, min)	Incubation time with lysozyme (h)	Cells/mL before protoplasts formation (A)	Viable cells/mL after protoplasts formation (B)	Viable cells/mL after lysis of protoplasts (C)	Protoplasts formation frequency (%) *	Regeneration frequency (%) **
11	EDTA (20)	1.5	3.2×10^9	6.1×10^7	2.9×10^7	52.9	1.02
12		2	3.2×10^9	5.5×10^7	1.6×10^7	71.6	1.24
13		1.5	2.2×10^9	1.6×10^8	1.6×10^8	-0.2	-0.02
14		2	2.2×10^9	7.0×10^7	1.4×10^8	-94.4	-2.95
15		21	1.7×10^9	——	6.3×10^6	——	——
16	EDTA (40)	21	1.7×10^9	——	1.4×10^7	——	——
17	EDTA (60)	1	5.3×10^9	2.0×10^8	6.3×10^6	96.9	3.74
18	EDTA (120)	1	5.3×10^9	2.2×10^8	3.3×10^6	98.5	4.03
19	Triton X-100 (60)	1	3.5×10^9	1.4×10^8	1.6×10^7	88.4	3.50
20	EDTA + Triton X-100 (60)	1	3.5×10^9	4.2×10^6	0.0	100.0	0.12

A: Assessed by counting colonies in MA medium before addition of EDTA and lysozyme.

B: Assessed by counting colonies in MA medium after protoplasts formation.

C: Assessed by counting colonies in MA medium after dilution of protoplasts suspensions with demineralised water.

*Protoplasts formation frequency was calculated using the following formula: (B-C)/B

**Regeneration frequency was calculated using the following formula: (B-C)/A

EDTA is used to remove the outer cell membrane of Gram-negative bacteria, thus making easier the access of lysozyme to the peptidoglycan layer. Although *B. subtilis* PX573 is a Gram-positive bacterium, the effect of EDTA in the protoplasts formation and regeneration frequencies was also studied for this strain. As in the case of *R. viscosum* CECT 908, a great variability was observed in the results obtained (**Table 16**). Furthermore, the protoplasts formation frequencies were considerably lower when compared with those obtained for that strain. The highest protoplasts formation frequencies were obtained in the assays 13 (EDTA treatment: 20 min; lysozyme treatment: 1.5 h) and 18 (EDTA treatment: 120 min; lysozyme treatment: 1 h). However, taking into account all the assays performed, in the case of *B. subtilis* PX573 the conditions selected were those from the assay 10: incubation with 3 g/L of lysozyme for 1 h without EDTA treatment.

Table 16. Protoplasts formation and regeneration frequencies obtained with different treatments for *Bacillus subtilis* PX573. The temperature of incubation with the detergent(s) was 30°C and the lysozyme concentration and incubation temperature were 3 g/L and 37°C, respectively.

Assay	Detergent treatment (incubation time, min)	Incubation time with lysozyme (h)	Cells/mL before protoplasts formation (A)	Viable cells/mL after protoplasts formation (B)	Viable cells/mL after lysis of protoplasts (C)	Protoplasts formation frequency (%) *	Regeneration frequency (%) **
11	EDTA (20)	1.5	3.1×10^8	3.8×10^6	3.7×10^6	2.6	0.03
12		2	3.1×10^8	3.4×10^6	3.2×10^6	5.9	0.06
13		1.5	1.5×10^8	3.3×10^8	1.4×10^8	56.9	122.77
14		2	1.5×10^8	3.0×10^8	2.2×10^8	25.9	50.83
15		21	4.7×10^8	1.3×10^5	1.5×10^5	-20.0	-0.01
16	EDTA (40)	21	4.7×10^8	1.0×10^5	8.0×10^4	20.0	0.00
17	EDTA (60)	1	3.7×10^8	1.6×10^6	2.9×10^6	-81.3	-0.35
18	EDTA (120)	1	3.7×10^8	4.6×10^6	2.3×10^6	50.0	0.61
19	Triton X-100 (60)	1	4.5×10^8	2.1×10^6	2.3×10^6	-9.8	-0.05
20	EDTA + Triton X-100 (60)	1	4.5×10^8	4.0×10^6	4.8×10^6	-20.3	-0.18

A: Assessed by counting colonies in LB medium before addition of EDTA and lysozyme.

B: Assessed by counting colonies in LB medium supplemented with sodium succinate (assays 11 and 12), mannitol (assays 13 and 14) or sorbitol (assays 15 to 20) after protoplasts formation.

C: Assessed by counting colonies in LB medium after dilution of protoplasts suspensions with demineralised water.

*Protoplasts formation frequency was calculated using the following formula: (B-C)/B

**Regeneration frequency was calculated using the following formula: (B-C)/A

4.1.3.4.4. Protoplasts formation and fusion using the optimized conditions

A new protoplasts formation assay was performed using the optimum conditions selected for each microorganism in the previous assays. The protoplasts formation and regeneration frequencies obtained are shown in **Table 17**.

As it can be observed, the protoplasts formation frequencies obtained in this assay for both parental strains were lower when compared with those obtained in the optimization assays. The protoplasts regeneration frequency for *R. viscosum* CECT 908 was higher, while the regeneration frequency for *B. subtilis* PX573 was lower when compared with the previous assays.

Table 17. Protoplasts formation and regeneration frequencies obtained after optimization of protoplasts formation.

Parental strain	Cells/mL before protoplasts formation (A)	Viable cells/mL after protoplasts formation (B)	Viable cells/mL after lysis of protoplasts (C)	Protoplasts formation frequency (%) *	Regeneration frequency (%) **
R. viscosum CECT 908	2.0×10^9	4.3×10^8	7.3×10^7	83.1	17.78
B. subtilis PX573	7.9×10^7	2.8×10^5	2.2×10^5	21.4	0.08

A: Assessed by counting colonies in MA medium or LB medium before addition of EDTA and lysozyme.
B: Assessed by counting colonies in MA medium or LB medium supplemented with sorbitol (91 g/L).
C: Assessed by counting colonies in MA medium or LB medium after dilution of protoplasts suspensions in demineralised water.
*Protoplasts formation frequency was calculated using the following formula: (B-C)/B.
**Regeneration frequency was calculated using the following formula: (B-C)/A.

The protoplasts suspensions obtained from each parental strain were incubated together in the presence of the fusogenic agent (PEG buffer) for 20 min. Aliquots of the protoplasts suspensions obtained after the fusion process were plated on MA medium containing sucrose (171.1 g/L) as osmotic stabilizer and two different concentrations of erythromycin (12.5 µg/mL or 25 µg/mL) and incubated at 37°C. In this assay was obtained 28 fusants. In order to allow their regeneration, the fusants obtained were transferred to new agar plates of MA medium containing sucrose (171.1 g/L) and 50 g/L of agar. However, after 7 days of incubation, none of the fusants was able of growing.

4.2. Production of biosurfactants and biopolymers

4.2.1. Biosurfactant production by *B. subtilis* PX573

After 24 h of growth in LB medium, biosurfactant production by *B. subtilis* PX573 was evaluated by measuring the surface tension of the cell-free supernatant (**Table 18**).

Table 18. Surface tension values (mN/m) of the cell-free supernatant and amount of crude biosurfactant produced by *Bacillus subtilis* PX573 grown in LB medium at 37°C and 180 rpm for 24 h.

	1st Production	2nd Production
Surface tension (mN/m)	26.9 ± 0.0	26.5 ± 0.1
Crude biosurfactant (g/L)	0.73	0.44

The biosurfactant was recovered by acid precipitation and subsequently freeze-dried (**Figure 6**). The amount of crude biosurfactant recovered in each assay is shown in **Table 18**. Although the surface tension values of the cell-free supernatants were quite similar in the two assays performed, the amount of crude biosurfactant produced in the second assay was lower when compared with the first one, which can be due to the different purity of the product obtained.

Figure 6. Freeze-dried crude biosurfactant produced by *Bacillus subtilis* PX573 recovered through acid precipitation.

4.2.1.1. Critical micelle concentration

To calculate the *cmc* of the crude biosurfactant produced by *B. subtilis* PX573, the relationship between biosurfactant concentration and surface tension was determined. The most important surfactant activities, such as detergency and solubilisation, are affected by the presence of micelles in the solution (El-Sukkary et al., 2008). Furthermore, the *cmc* is widely used as index to evaluate the efficiency of a given surfactant (Vaz et al., 2012).

The freeze-dried crude biosurfactant obtained from the first production was dissolved in demineralised water at concentrations ranging from 0.001 to 0.8 g/L. As it can be seen in **Figure 7**, the surface tension decreased with the increase of biosurfactant concentration. For biosurfactant concentrations higher than 0.2 g/L, the surface tension became stable and there were no significant reductions even at the highest biosurfactant concentration tested. The surface tension *versus* crude biosurfactant concentration plot was used to determine the *cmc* as the point of intersection between the two lines that best fit the decline and the constant plateau of surface tension (**Figure 8**). The *cmc* value obtained was 0.064 g/L with a minimum surface tension value of 27.6 mN/m.

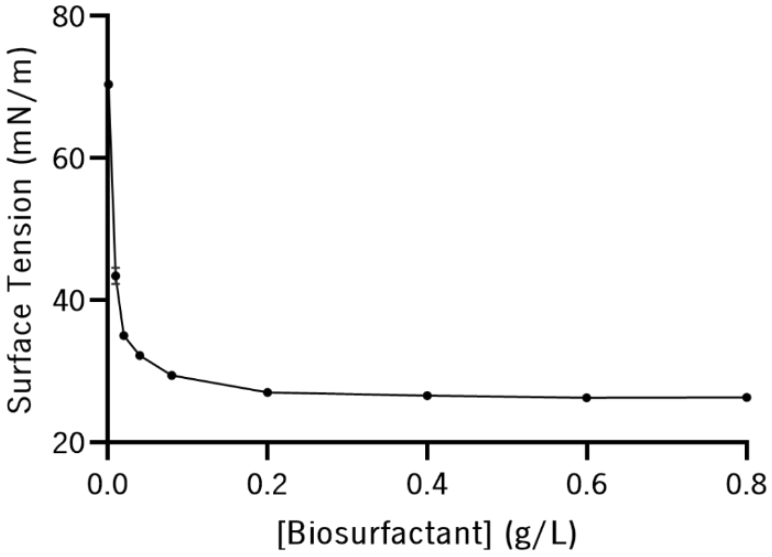

Figure 7. Surface tension values (mN/m) of the crude biosurfactant obtained from *Bacillus subtilis* PX573 dissolved in demineralised water at different concentrations. The measurements were performed at room temperature (25°C). The results represent the average of five measurements, and the error bars represent the standard deviation.

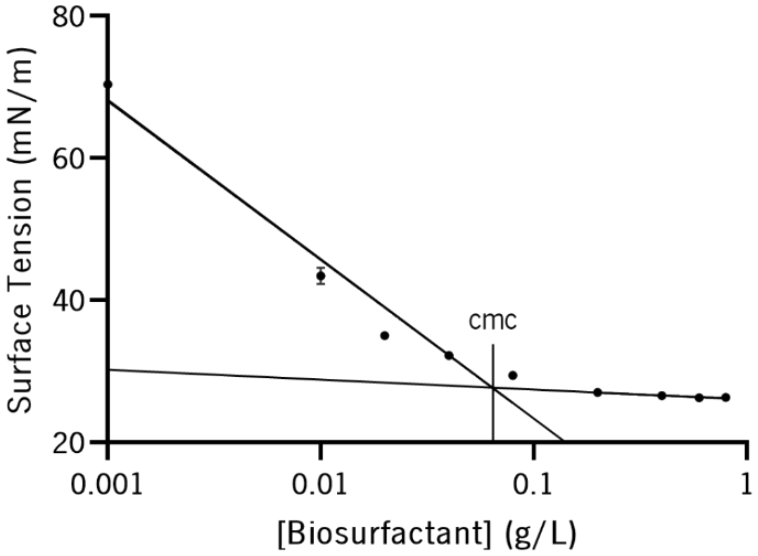

Figure 8. Surface tension (mN/m) *versus* logarithm of crude biosurfactant concentration (g/L). The *cmc* was determined from the point of intersection of the regression lines that better describe the two parts of the curve, below and above *cmc*. The measurements were performed at room temperature (25°C). The results represent the average of five measurements, and the error bars represent the standard deviation.

Different *cmc* values have been reported for biosurfactants produced by *B. subtilis* strains by several authors. The biosurfactant produced by the same *B. subtilis* strain used in this work grown in corn steep liquor exhibited a *cmc* of 0.16 g/L (Gudiña et al., 2015). Gudiña et al. (2012) reported *cmc* values between 0.02 and 0.13 g/L for biosurfactants produced by different *B. subtilis* isolates (#191,

#309, #311 and #573); those biosurfactants were produced in LB medium and the *cmc* values were obtained with the freeze-dried biosurfactants dissolved in PBS buffer (pH 7.0). Vaz et al. (2012) reported a *cmc* of 0.04 g/L for the biosurfactant produced by *B. subtilis* EG1 grown in LB medium, with a minimum surface tension value of 29.0 ± 0.2 mN/m. The biosurfactant produced by *B. subtilis* R1 grown in a molasses-based medium exhibited a *cmc* of 0.0395 g/L (Joshi & Desai, 2013); however, when this strain was grown in glucose-based minimal medium, the *cmc* value was 0.02 g/L (Jha et al., 2016).

The surface tension values obtained in this work are in accordance with the above mentioned studies. However, the *cmc* value obtained was higher comparing to some of the *cmc* values reported in the literature. These differences may be due to the purity of the crude biosurfactant herein studied, which was recovered through acid precipitation without further purification.

For the MEOR assays, the crude biosurfactant concentrations used were 0.25 g/L (first production) and 0.2 g/L (second production), which corresponded to surface tension values between 27.3 and 28.0 mN/m.

4.2.2. Biopolymer production by *R. viscosum* CECT 908

Biopolymer production by *R. viscosum* CECT 908 was evaluated after 5 days of growth in synthetic medium by measuring the apparent viscosity of the cultures (**Table 19**).

Table 19. Apparent viscosity values (mPa s) of the culture medium and amount of purified biopolymer produced by *Rhizobium viscosum* CECT 908 grown in synthetic medium, at 30°C and 150 rpm for 5 days. The measurements were performed at 40°C and the values presented correspond to a shear rate of 1.4 s^{-1}.

	1st Production	2nd Production
Apparent viscosity (mPa s)	556 ± 2	389 ± 49
Purified biopolymer (g/L)	2.34	1.85

The apparent viscosity of the culture medium in the first production achieved a higher value when compared with the second production (**Table 19**). The rheograms of the culture media from both productions exhibited a similar profile (**Figure 9**).

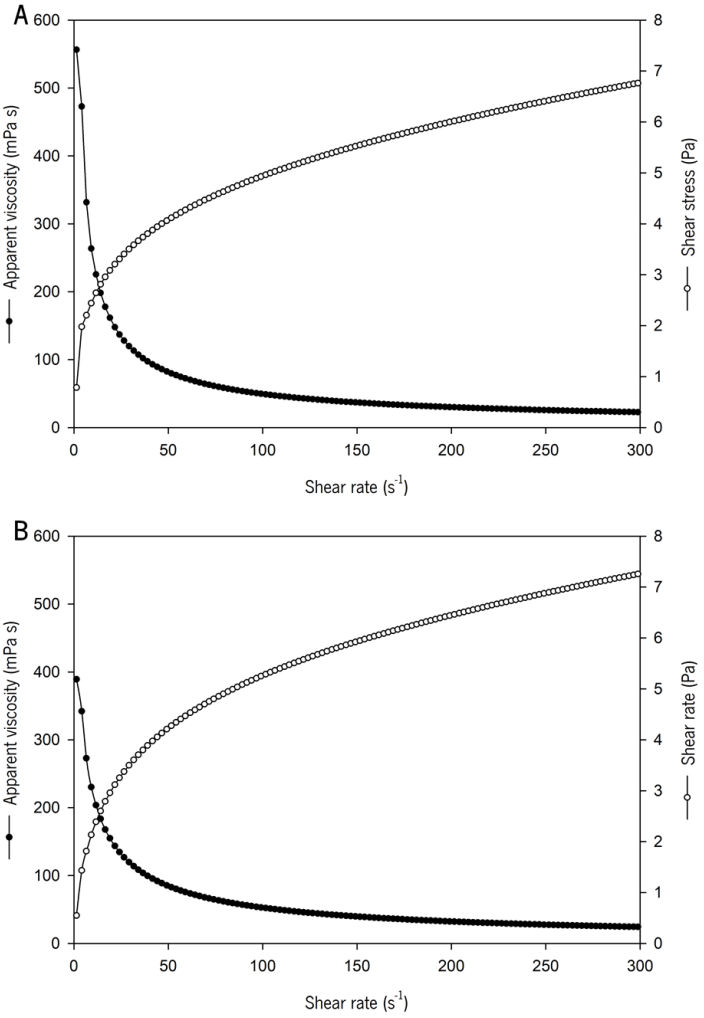

Figure 9. Rheograms of cultures of *Rhizobium viscosum* CECT 908 grown in synthetic medium in flasks incubated at 30°C and 150 rpm for 5 days, corresponding to the first production (**A**) and the second production (**B**). The measurements were performed at 40°C.

The biopolymer produced in both fermentations was purified as described in the Materials and Methods (Section 3.7.2), and subsequently freeze-dried (**Figure 10**).

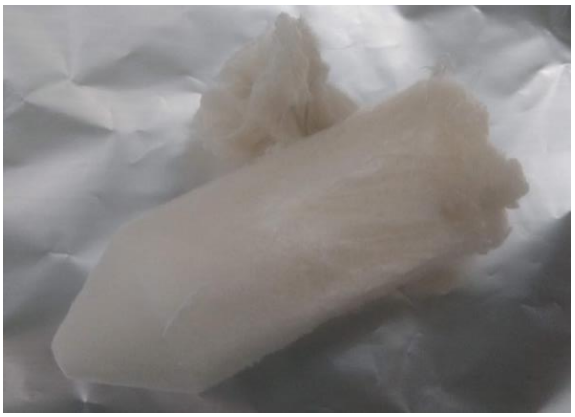

Figure 10. Freeze-dried purified biopolymer produced by *Rhizobium viscosum* CECT 908.

The amount of purified biopolymer recovered after freeze-drying was higher in the first production than in the second production, which is in accordance with the apparent viscosity values of the cultures (**Table 19**).

The rheological properties of biopolymers play an important role in their application in MEOR (Hashmet et al., 2011). The characterization of the rheological behaviour of biopolymer solutions at different concentrations is usually performed through the study of the shear stress and the apparent viscosity as a function of the shear rate. In this case, the shear stress and the apparent viscosity of an aqueous solution of the purified biopolymer produced by *R. viscosum* CECT 908 at a concentration of 2 g/L were measured over a range of shear rates (0.1-300 s^{-1}) at 40°C (**Figure 11**).

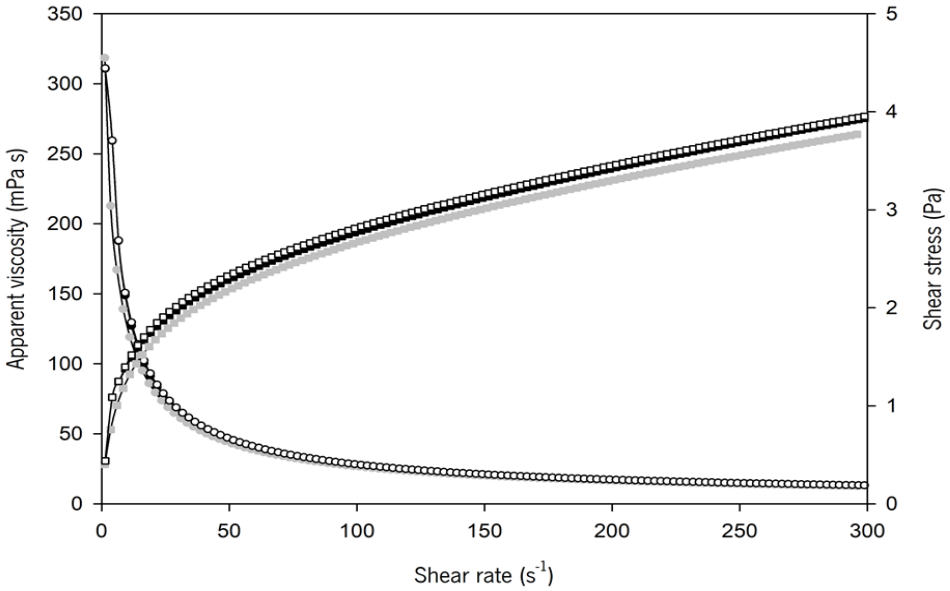

Figure 11. Rheograms of an aqueous solution of the purified biopolymer produced by *Rhizobium viscosum* CECT 908 at a concentration of 2 g/L. The circles correspond to the apparent viscosity (mPa s) as a function of the shear rate (s^{-1}), while the squares represent the shear stress (Pa) as a function of the shear rate (s^{-1}). The first flow ramp (0.1 → 300 s^{-1}) is represented in black, the second (300 → 0.1 s^{-1}) in grey, and the third (0.1 → 300 s^{-1}) in white. The measurements were performed at 40°C.

The biopolymer produced by *R. viscosum* CECT 908 exhibited a non-Newtonian behaviour, as the shear stress varied non-linearly with the shear rate. Given that the apparent viscosity decreased as the shear rate increased, this biopolymer is classified as pseudoplastic or shear-thinning fluid (as previously reported by Couto et al., 2019). The shear-thinning property is related to the molecular aggregation or disperse states in the shear flow. Generally, the polysaccharides exist as aggregates at low shear rates. With the increase in the shear rate, the shear forces gradually dissociate the aggregates and

the individual molecules rearrange along the flow direction, which results in a reduction of the apparent viscosity (Chagas et al., 2004; Li et al., 2017).

As shown in **Figure 11**, the initial apparent viscosity values were recovered after decreasing the shear rate, and the three successive flow ramps (0.1 → 300 s^{-1}; 300 → 0.1 s^{-1}; 0.1 → 300 s^{-1}) were coincident, which indicates that the *R. viscosum* biopolymer was able to recover its structure after being exposed to high shear rates (as previously reported by Couto et al., 2019).

Subsequently, the rheological properties of the purified biopolymer produced in both assays were studied at different concentrations. The rheograms of the biopolymer solutions at the different concentrations studied exhibited a similar profile to the biopolymer solution at a concentration of 2 g/L (*data not shown*). **Table 20** shows the apparent viscosity values obtained for the different biopolymer solutions at a shear rate of 1.4 s^{-1}.

Table 20. Apparent viscosity values (mPa s) obtained for solutions of the purified biopolymer produced by *Rhizobium viscosum* CECT 908 at different concentrations. The measurements were performed at 40°C and the values presented correspond to a shear rate of 1.4 s^{-1}.

1ª Production		2ª Production	
[Biopolymer] (g/L)	Apparent viscosity (mPa s)	[Biopolymer] (g/L)	Apparent viscosity (mPa s)
1	117 ± 5	1.5	155 ± 7
2	311 ± 1	2	269 ± 5
2.5	474 ± 13	2.5	371 ± 6
3	640 ± 13		

As expected, the apparent viscosity values (at a shear rate of 1.4 s^{-1}) increased with the increase of biopolymer concentration. However, the apparent viscosity values of the biopolymer solutions from the second production were lower when compared with those prepared at the same concentration from the first production. This may be due to the lower apparent viscosity of the culture in the second production.

For the MEOR assays it was chosen an apparent viscosity value (at shear rate of 1.4 s^{-1}) around 300 mPa s, which corresponded to a biopolymer concentration of 2 g/L in the first production and 2.3 g/L in the second production.

4.2.3. Study of biopolymer production by *Paenibacillus* sp. strains

The strains *Paenibacillus* sp. #210, #510, #521 and #540 were previously identified as bioemulsifier producers (*unpublished results*). Several *Paenibacillus* sp. strains produce biopolymers when they are grown in culture media containing high concentrations of sucrose as the sole carbon source (O. Li et al., 2013; Liu et al., 2009, 2010). For that reason, these strains were evaluated regarding their ability of producing biopolymers.

4.2.3.1. Study of biopolymer production using MSS-S medium

The *Paenibacillus* sp. strains were grown in MSS-S medium to evaluate their ability of producing biopolymers. After 6 days of growth, the culture medium did not appear viscous as it usually occurs when biopolymers are produced. Nevertheless, the biopolymer was recovered, and the apparent viscosity of the culture medium and the precipitate recovered was measured (**Table 21**).

Table 21. Apparent viscosity values (mPa s) of the culture medium and the crude biopolymer produced by the *Paenibacillus* sp. strains grown in 20 mL of MSS-S medium at 37°C and 120 rpm for 6 days. The measurements were performed at 40°C and the values presented correspond to a shear rate of 1.4 s^{-1}.

Paenibacillus sp. strain	Apparent viscosity (mPa s)	
	Culture medium	Crude biopolymer
#210	58.4	51.5
#510	54.0	62.3
#521	54.1	52.9
#540	52.1	57.3

The apparent viscosity values obtained for the culture medium were very similar for all the *Paenibacillus* sp. strains (52-58 mPa s). However, these values are very low when compared with those achieved with *R. viscosum* CECT 908. The apparent viscosity values obtained for the crude biopolymer were similar or even lower when compared with the culture medium. Consequently, the *Paenibacillus* sp. strains did not produce biopolymers with high viscosifying activity when they were grown in MSS-S medium. In this medium, sucrose is the carbon source while the nitrogen source is ammonium nitrate. It has been reported in previous studies that inorganic nitrogen sources resulted in lower exopolysaccharides and biomass production comparing to organic nitrogen sources (Kim et al., 2005;

Pokhrel & Ohga, 2007). For that reason, a culture medium containing an organic nitrogen source was used to study the production of biopolymers by these strains.

4.2.3.2. Study of biopolymer production using ML9 medium

The *Paenibacillus* sp. strains were grown in ML9 medium to evaluate their capacity to produce biopolymers. This culture medium contains an organic nitrogen source (yeast extract). After 2, 5 and 6 days of growth, samples were collected and the apparent viscosity of the cell-free supernatants was measured (**Table 22**).

Table 22. Apparent viscosity values (mPa s) of the cell-free supernatant and the crude biopolymer obtained from cultures of *Paenibacillus* sp. strains grown in 100 mL of ML9 medium at 37°C and 120 rpm for different time intervals. The measurements were performed at 40°C and the values presented correspond to a shear rate of 1.4 s^{-1}.

Paenibacillus sp. strain	Time (days)	Apparent viscosity (mPa s)	
		Culture medium	Crude biopolymer
#210	2	52.9	_____
	5	52.4	_____
	6	56.5	90 ± 6
	8	_____	62 ± 4
	13	_____	70 ± 20
#510	2	55.0	_____
	5	55.4	_____
	6	56.6	213 ± 27
	8	_____	141 ± 78
	13	_____	59 ± 10
#521	2	53.6	_____
	5	54.6	_____
	6	54.0	452 ± 22
#540	2	53.3	_____
	5	51.3	_____
	6	58.7	247 ± 44
	8	_____	69 ± 4
	13	_____	68 ± 9

As the highest apparent viscosity values for the crude biopolymers were obtained after 6 days of growth (the first point studied), in order to find the optimum time for biopolymer production for each strain, a new set of experiments was performed. In this case, the biopolymer produced was recovered every 24 h from the beginning of the fermentation, and the apparent viscosity was measured (**Figure 12**).

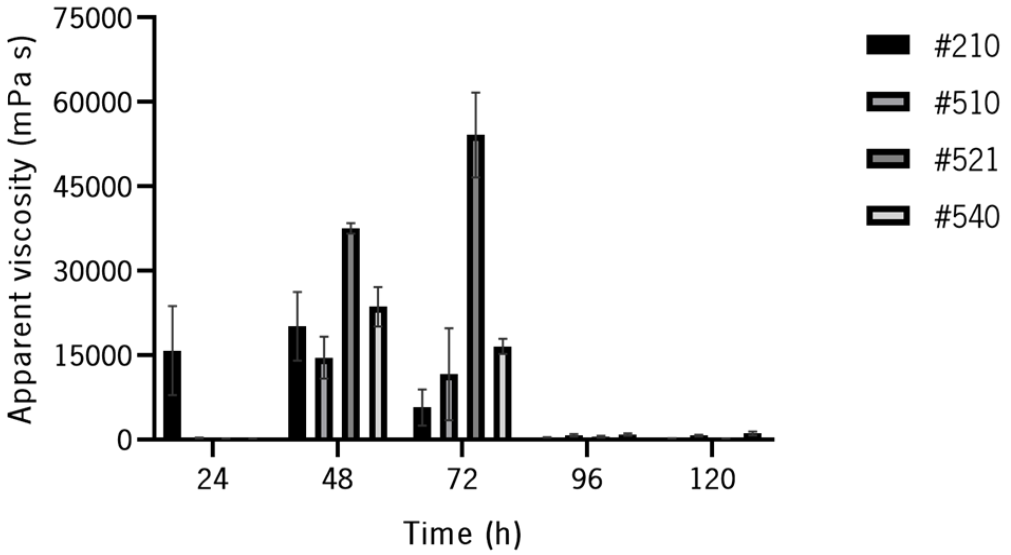

Figure 12. Apparent viscosity values (mPa s) of the crude biopolymers produced by *Paenibacillus* sp. #210, #510, #521 and #540 grown in 20 mL of ML9 medium at 37°C and 120 rpm for 120 h. The measurements were performed at 40°C and the values presented correspond to a shear rate of 1.4 s^{-1}.

As it can be seen from the results obtained (**Figure 12**), the highest apparent viscosity values of the crude biopolymers were obtained after 48 h of growth for all the strains, except for *Paenibacillus* sp. #521 (72 h). After that, the apparent viscosity of the crude biopolymers decreased reaching minimum values between 100 and 1300 mPa s after 120 h. The decrease of the apparent viscosity of the crude biopolymers may be due to their degradation.

It is important to highlight that the apparent viscosity values achieved with the crude biopolymers produced by the four *Paenibacillus* sp. strains (between 14549 and 54140 mPa s) were considerably higher when compared with the highest value obtained with the purified biopolymer produced by *R. viscosum* CECT 908 (640 mPa s). In **Figure 13** it can be observed the aspect of the crude biopolymers produced by the *Paenibacillus* sp. strains.

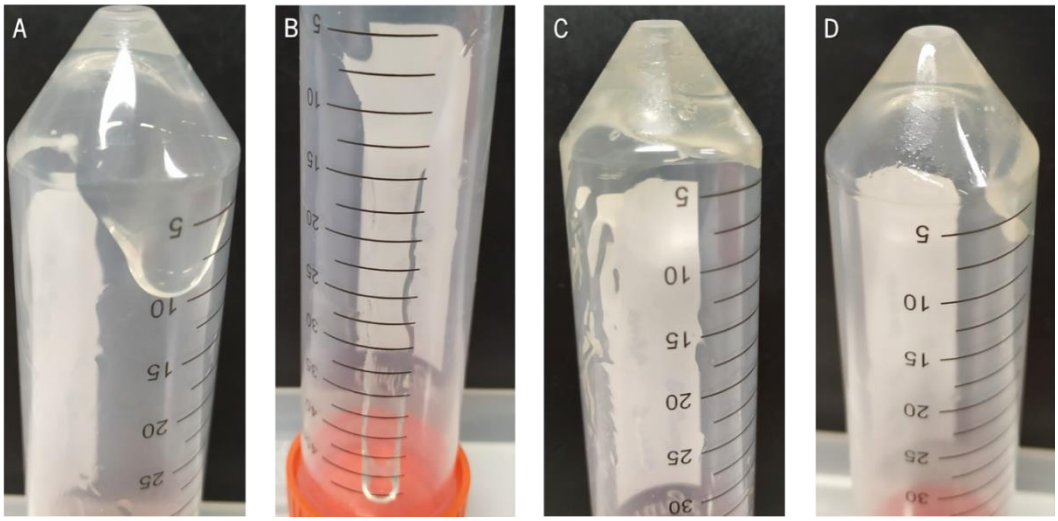

Figure 13. Crude biopolymers produced by *Paenibacillus* sp. #210 (**A**), #510 (**B**), #521 (**C**) and #540 (**D**) grown in 20 mL of ML9 medium at 37°C and 120 rpm for 48 h (#210) or 72 h (#510, #521 and #540).

The previous assays were performed in 50 mL Erlenmeyer flasks containing 20 mL of ML9 medium. Subsequently, it was studied the production of biopolymer by the same strains in 250 mL Erlenmeyer flasks containing 100 mL of the same culture medium at the same operational conditions. However, as it can be seen from the results obtained (**Figure 14**), the apparent viscosity values achieved were considerably lower when compared with those achieved at small scale for all the strains.

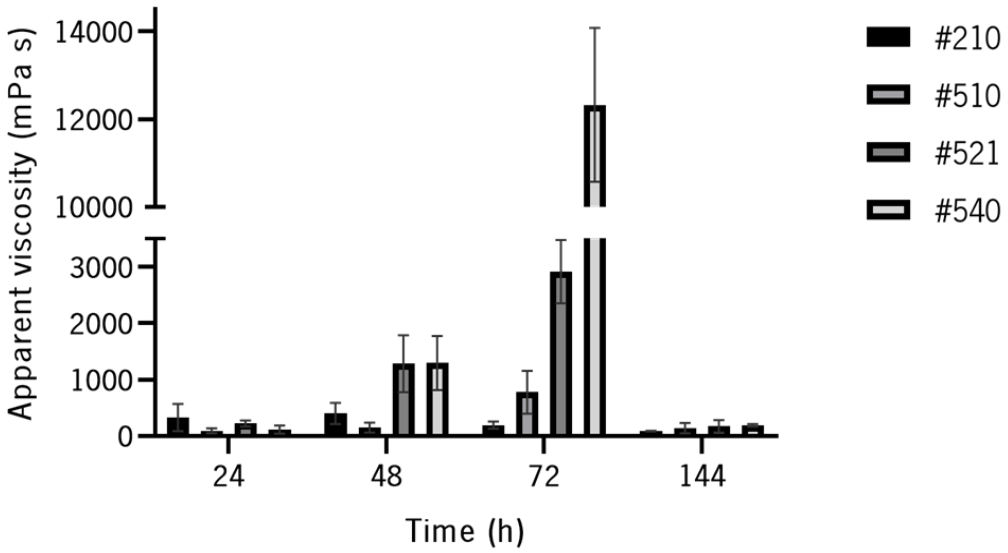

Figure 14. Apparent viscosity values (mPa s) of the crude biopolymers produced by *Paenibacillus* sp. #210, #510, #521 and #540 grown in 100 mL of ML9 medium at 37°C and 120 rpm for 144 h. The measurements were performed at 40°C and the values presented correspond to a shear rate of 1.4 s^{-1}.

In order to study the effect of the agitation speed on biopolymer production by the *Paenibacillus* sp. strains, a new assay was performed decreasing the agitation speed to 100 rpm. However, as it can be seen from the results obtained, the decrease of the agitation speed from 120 rpm to 100 rpm resulted in a decrease of the apparent viscosity of the crude biopolymers produced (**Figure 15**). The apparent viscosity of the crude biopolymers produced by the strains #521 and #540 was not evaluated due to the contamination of the cultures.

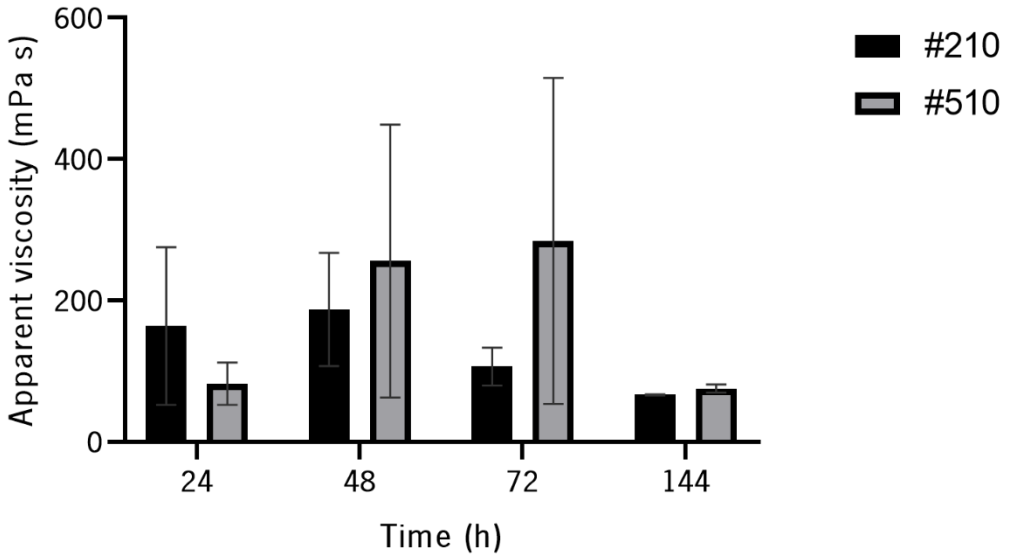

Figure 15. Apparent viscosity values (mPa s) of the crude biopolymers produced by *Paenibacillus* sp. #210 and #510 grown in 100 mL of ML9 medium at 37°C and 100 rpm for 144 h. The measurements were performed at 40°C and the values presented correspond to a shear rate of 1.4 s^{-1}.

As it can be observed in **Figure 14** and **Figure 15**, the apparent viscosity values obtained in cultures performed in 100 mL of ML9 medium were lower when compared with those obtained when the strains were grown in 20 ml of the same medium. This decrease in the apparent viscosity may be due to the agitation profile. The agitation rate is a crucial parameter for the growth of the microorganisms, improving the characteristics of mass transfer with respect to substrates, products and oxygen (García-Ochoa et al., 2000).

Although the apparent viscosity of the crude biopolymer produced by *Paenibacillus* sp. #521 was higher when compared with the other strains studied, it also exhibited a high variability in the different assays performed. On the other hand, the strain #540 exhibited less variability when compared with the other strains. For that reason, the crude biopolymer produced by *Paenibacillus* sp. #540 was selected to be used in the oil recovery trials using sand-pack columns.

4.2.3.3. Biopolymer production by *Paenibacillus* sp. #540

According to the results obtained in the previous section, *Paenibacillus* sp. #540 was grown in 50 mL Erlenmeyer flasks containing 20 mL of ML9 medium for 48 h. Afterwards, the biopolymer produced in 200 mL of culture medium was recovered and resuspended in 50 mL of demineralised water. From this solution (crude biopolymer stock solution), several dilutions were prepared with demineralised water and the apparent viscosity was measured (**Figure 16**).

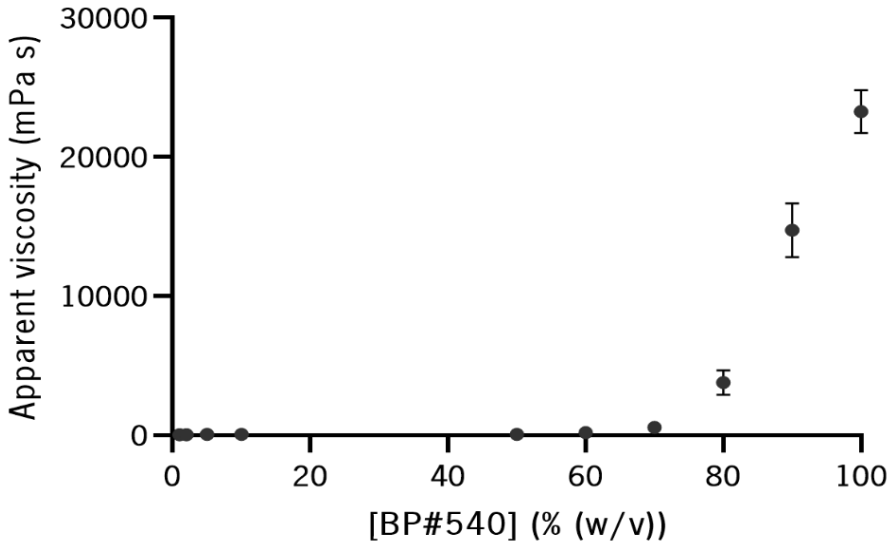

Figure 16. Apparent viscosity values (mPa s) of solutions containing different percentages (w/v) of the stock solution of the crude biopolymer produced by *Paenibacillus* sp. #540. The measurements were performed at 40°C and the values presented correspond to a shear rate of 1.4 s^{-1}.

The apparent viscosity values remained below 60 mPa s for solutions containing up to 50 % (w/v) of the crude biopolymer stock solution. However, a substantial increase in the apparent viscosity was observed for concentrations higher than 60% (**Figure 16**). The apparent viscosity values obtained for solutions containing 60% and 80% of the stock solution were 175 ± 89 mPa s and 3794 ± 876 mPa s, respectively. The apparent viscosity of the stock solution of crude biopolymer was 23251 ± 1569 mPa s, which is in accordance with the apparent viscosity values obtained previously for the biopolymer produced by *Paenibacillus* sp. #540 (**Figure 12**).

In order to purify the biopolymer produced by *Paenibacillus* sp. #540, new productions were performed as described above and, in this case, the stock solutions of crude biopolymer obtained through precipitation were dialysed and freeze-dried. The amount of purified biopolymer recovered was 30.6 ±

1.7 g/L, and its aspect is shown in **Figure 17**. The freeze-dried purified biopolymer recovered its viscosifying activity when it was dissolved in demineralised water (*data not shown*). When this purification process was applied to crude biopolymer stock solutions from cultures performed in 100 mL of culture medium, the amount of purified biopolymer obtained was lower (16.4 g/L), which is in accordance with the results obtained in the previous section.

Figure 17. Freeze-dried purified biopolymer produced by *Paenibacillus* sp. #540.

4.3. Biopolymer-biosurfactant cocktails

In order to study the interaction between the crude biosurfactant produced by *B. subtilis* PX573 and the purified biopolymer produced by *R. viscosum* CECT 908, mixtures of these bioproducts at different concentrations were prepared in demineralised water. The concentrations used were selected according to those that will be used in the MEOR assays. Subsequently, the apparent viscosity and the surface tension of those solutions were measured (**Table 23**).

Table 23. Apparent viscosity (mPa s) and surface tension (mN/m) values of solutions of purified biopolymer produced by *Rhizobium viscosum* CECT 908, crude biosurfactant produced by *Bacillus subtilis* PX573 and mixtures of both bioproducts at different concentrations. The measurements of apparent viscosity were performed at 40°C and the values presented correspond to a shear rate of 1.4 s^{-1}. The measurements of surface tension were performed at 25°C.

[Biosurfactant] (g/L)	[Biopolymer] (g/L)	Apparent viscosity (mPa s)	Surface tension (mN/m)
0	1	114 ± 5	59.2 ± 0.22
	2	301 ± 18	55.8 ± 0.48
0.25	0	52 ± 8	28.4 ± 0.38
	1	84 ± 5	27.2 ± 0.10
	2	235 ± 5	26.8 ± 0.55

As it can be seen from **Table 23**, the crude biosurfactant at a concentration of 0.25 g/L reduced the apparent viscosity of the purified biopolymer solutions; this effect seems to be higher as the biopolymer concentration increases. Contrary to these results, Machale et al. (2019) reported an increase in the apparent viscosity of xanthan gum solutions when they were mixed with a biosurfactant obtained from water hyacinth.

As expected, the biopolymer exhibited a low surface activity when compared with the biosurfactant. However, the combination of biosurfactant and biopolymer resulted in surface tension values slightly lower when compared with the aqueous solution of biosurfactant.

In summary, the combination of the biosurfactant produced by *B. subtilis* PX573 and the biopolymer produced by *R. viscosum* CECT 908 has a positive effect in the surface activity but a negative effect in the apparent viscosity.

4.4. MEOR assays

Laboratory studies on MEOR are usually performed using sand-pack columns. This is an inexpensive model where multiple columns can be configured simultaneously simulating the crude oil recovery operations commonly conducted in oil reservoirs (Gudiña et al., 2013). In this work, a sand-pack column model was used to study the effect of the biosurfactant produced by *B. subtilis* PX573, the biopolymer produced by *R. viscosum* CECT 908, the mixture of this two bioproducts and the biopolymer produced by *Paenibacillus* sp. #540 in oil recovery.

4.4.1. Bioproducts characterization

The different solutions of bioproducts used in the MEOR studies performed in the sand-pack columns were characterized regarding their rheological properties (apparent viscosity) and their surface activity (surface tension, interfacial tension and emulsifying activity) (**Table 24**).

Table 24. Apparent viscosity values (mPa s), surface and interfacial tension values (mN/m) and emulsifying indexes (E_{24}, %) obtained for solutions of the biosurfactant produced by *Bacillus subtilis* PX573 (BS), the biopolymer produced by *Rhizobium viscosum* CECT 908 (BP 908), the mixture of both bioproducts (BS + BP 908) and the biopolymer produced by *Paenibacillus* sp. #540 (BP #540). Demineralised water was also included as it was used in the control assays. The results represent the average of at least of two independent experiments ± standard deviation.

Bioproduct	Apparent viscosity (mPa s)	Surface tension (mN/m)	Interfacial tension (mN/m)	E_{24} (%)
Water	_____	70.9 ± 0.1	45.3 ± 0.1	0 ± 0
BS	52 ± 8	27.8 ± 0.7	2.9 ± 0.3	57 ± 2
BP 908	336 ± 57	43.5 ± 0.5	45.5 ± 0.4	32 ± 9
BS + BP 908	286 ± 38	26.7 ± 1.0	< 1*	18 ± 7
BP #540	283 ± 36	49.0 ± 0.8	28.6 ± 1.5	0 ± 0

[BS]: 0.20-0.25 g/L. [BP 908]: 2.0-2.3 g/L. [BS + BP 908]: 0.25 g/L BS + 2.0 g/L BP 908 or 0.20 g/L BS + 2.3 g/L BP 908. [BP #540]: 78.7 g/L.

* Value below the limit of detection of the equipment.

The surface tension and the apparent viscosity values determined for the solutions used in the MEOR assays were similar to those obtained in the preliminary characterization of these solutions (**Table 23**). The apparent viscosity value obtained for the biopolymer produced by *Paenibacillus* sp. #540 was similar to that obtained for the combination of biosurfactant (PX573) and biopolymer (CECT 908), while the surface tension value was similar to the biopolymer produced by *R. viscosum* CECT 908.

Regarding the interfacial tension against *n*-hexadecane, similar values were obtained for the biopolymer produced by *R. viscosum* CECT 908 and demineralised water, indicating the lack of surface activity of this bioproduct. The biosurfactant produced by *B. subtilis* PX573 reduced the interfacial tension from 45.3 ± 0.1 mN/m to 2.9 ± 0.3 mN/m. The combination of biosurfactant and biopolymer decreased even more the interfacial tension, as it was previously observed for the surface tension. The interfacial tension achieved with this mixture was lower than 1 mN/m, and it was below the limit of detection of the equipment. The biopolymer produced by *Paenibacillus* sp. #540 reduced the interfacial tension to 28.6 ± 1.5 mN/m.

Regarding the emulsifying indexes, the highest value was obtained for the biosurfactant produced by *B. subtilis* PX573, followed by the biopolymer from *R. viscosum* CECT 908. Contrary to what happened with the surface and the interfacial tension, the mixture of both bioproducts exhibited a lower emulsifying activity when compared with each bioproduct alone. The biopolymer produced by *Paenibacillus* sp. #540 did not exhibit emulsifying activity (**Figure 18**).

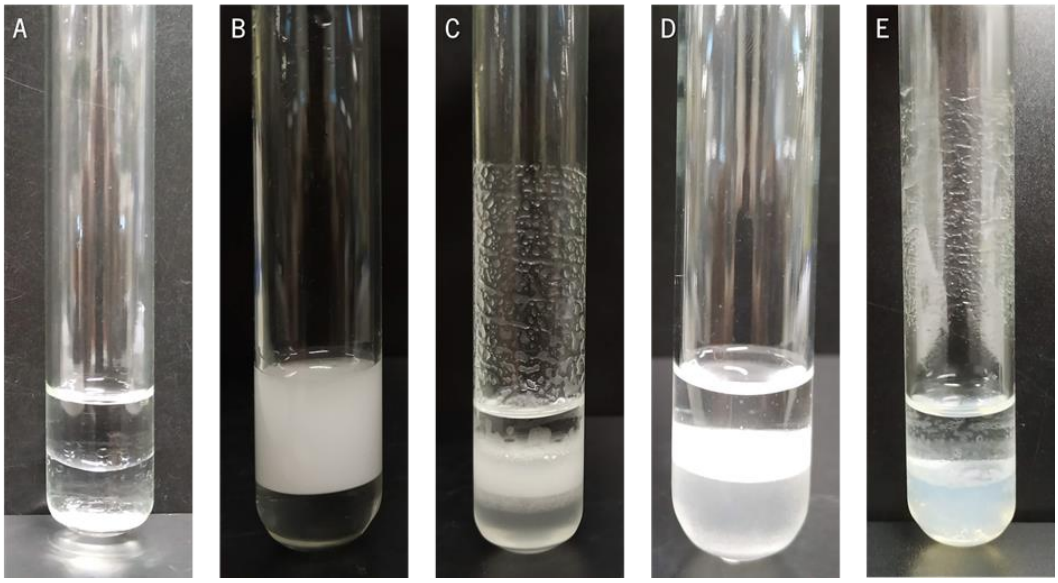

Figure 18. Emulsification assays performed with *n*-hexadecane and demineralised water (**A**), the biosurfactant produced by *Bacillus subtilis* PX573 (**B**), the biopolymer produced by *Rhizobium viscosum* CECT 908 (**C**), the mixture of the biosurfactant produced by *Bacillus subtilis* PX573 and the biopolymer produced by *Rhizobium viscosum* CECT 908 (**D**) and the biopolymer produced by *Paenibacillus* sp. #540 (**E**).

In summary, the combination of the biosurfactant (PX573) and the biopolymer (CECT 908) resulted in a positive effect in the surface and the interfacial tension. However, this mixture had a negative effect in the apparent viscosity and the emulsifying activity.

4.4.2. *Ex situ* MEOR assays

The effect of the different bioproducts in the mobilization of the entrapped oil was evaluated, for application in MEOR, using sand-pack columns and an *ex situ* approach. The aspect of the sand-pack columns during the process is shown in **Figure 19**.

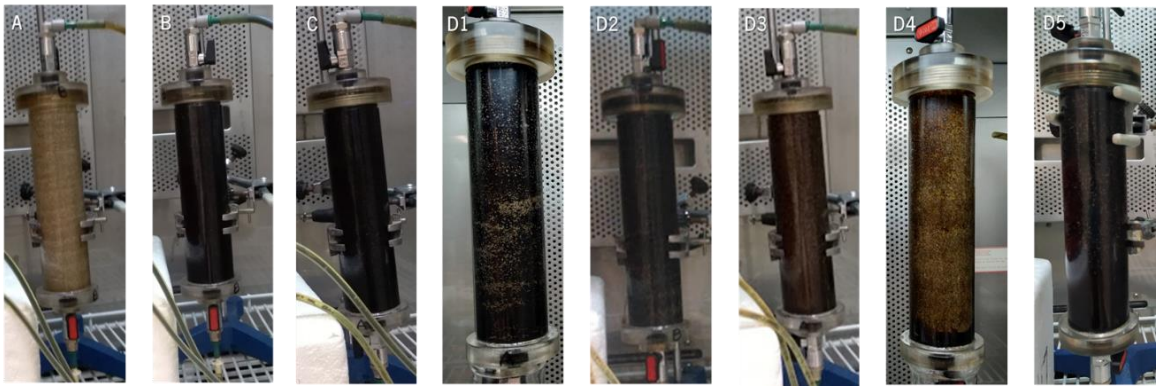

Figure 19. Schematic representation of the oil recovery assays performed in sand-pack columns. Sand-pack column after saturation with water (**A**), after saturation with crude oil (**B**), after water flooding (**C**), and after the different treatments: control (**D1**), biosurfactant produced by *Bacillus subtilis* PX573 (**D2**), biopolymer produced by *Rhizobium viscosum* CECT 908 (**D3**), mixture of biosurfactant (*Bacillus subtilis* PX573) and biopolymer (*Rhizobium viscosum* CECT 908) (**D4**), and biopolymer produced by *Paenibacillus* sp. #540 (**D5**).

Looking at the pictures, the most relevant differences can be observed between the control column (**Figure 19 D1**) and the column treated with the mixture of biosurfactant and biopolymer (**Figure 19 D4**). The results obtained in the *ex situ* MEOR assays are shown in **Table 25**.

Table 25. Results obtained in *ex situ* MEOR sand-pack column assays performed with the biosurfactant produced by *Bacillus subtilis* PX573 (BS), the biopolymer produced by *Rhizobium viscosum* CECT 908 (BP 908), the mixture of both bioproducts (BS + BP 908), and the biopolymer produced by *Paenibacillus* sp. #540 (BP #540). The results represent the average of at least three independent experiments (two in the case of BP #540) ± standard deviation.

Oil Recovery Parameter	Control	BS	BP 908	BS + BP 908	BP #540
PV (mL)	91.7 ± 7.6	95.0 ± 8.7	93.3 ± 5.8	95.2 ± 5.3	92.5 ± 3.5
Porosity (%)	32.7 ± 2.7	33.9 ± 3.1	33.3 ± 2.1	34.0 ± 1.9	33.0 ± 1.3
OOIP (mL)	89.7 ± 3.7	89.7 ± 6.3	88.2 ± 4.1	92.0 ± 6.3	91.5 ± 1.1
S_{oi} (%)	98.1 ± 4.1	94.8 ± 7.9	94.6 ± 2.3	96.7 ± 2.0	99.0 ± 2.6
S_{orwf} (mL)	46.7 ± 2.9	38.3 ± 2.9	44.3 ± 1.2	45.7 ± 4.0	45.0 ± 0.0
S_{or} (%)	47.9 ± 4.1	57.9 ± 6.0	49.7 ± 1.5	50.3 ± 3.7	50.8 ± 0.6
S_{orbt} (mL)	3.6 ± 0.8	3.9 ± 0.9	20.2 ± 0.3	21.9 ± 2.7	19.5 ± 0.1
AOR (%)	8.4 ± 0.7	7.8 ± 0.9	46.2 ± 3.8	47.2 ± 0.7	41.9 ± 0.7

The displacement efficiency of the biosurfactant produced by *B. subtilis* PX573 was lower when compared with the solutions of biopolymers (BP 908 and BP #540) and the mixture of biosurfactant and biopolymer (BS + BP 908) (**Table 25**). The low oil recovery obtained with the biosurfactant treatment (similar to the control) may be due to the high apparent viscosity ($\eta_{40^{\circ}C}$ = 545 ± 41 mPa s) of the heavy crude oil used in these studies. In this case it seems to be more important the improvement of the mobility ratio between the injected water and the oil inside the columns than the reduction of the interfacial tension. Accordingly, the highest AOR values were obtained with the formulations containing the biopolymer from *R. viscosum* CECT 908 (BP 908 and BS + BP 908), followed by the biopolymer produced by *Paenibacillus* sp. #540 (**Table 25**). Regarding the assays performed with this biopolymer, only 150 mL of solution were injected into the columns (instead 200 mL).

In order to evaluate if the differences in the AOR values obtained for the different treatments were statistically significant, the results were analysed by one-way ANOVA followed by the Tukey's Multiple Comparison Test. The results obtained are shown in **Figure 20**.

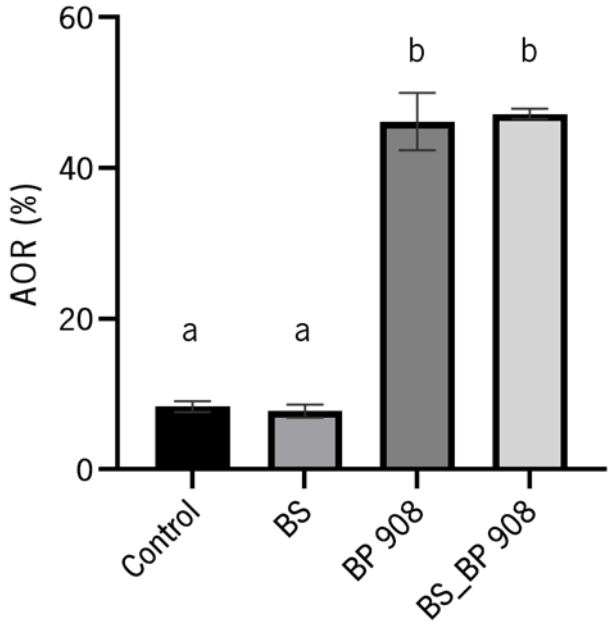

Figure 20. Results obtained from the analysis by one-way ANOVA followed by the Tukey's Multiple Comparison Test of the Additional Oil Recovery values obtained in the *ex situ* MEOR sand-pack column assays reported in Table 25. BS: biosurfactant from *Bacillus subtilis* PX573; BP 908: biopolymer from *Rhizobium viscosum* CECT 908; BS + BP 908: mixture of both bioproducts. Different letters indicate statistically significant differences ($p < 0.0001$).

As it can be observed in **Figure 20**, no statistically significant differences were found between the control and the treatments performed with the single biosurfactant (*p*-value = 0.9820). It was expected that the mixture of the biosurfactant and the biopolymer produced by *R. viscosum* CECT 908 resulted in AOR values higher when compared with the treatments performed with the biopolymer alone, due to the combination of the viscosifying activity and the reduction of the interfacial tension. However, no statistically significant differences (*p*-value = 0.9193) were observed between both treatments. This may be due to the low efficiency observed in the assays conducted with the single biosurfactant. The differences observed between the AOR values obtained in the treatments where biopolymers were used and the other treatments were found to be statistically significant (*p*-value < 0.0001).

According to the AOR values obtained, the biopolymer produced by *R. viscosum* CECT 908 exhibited the best performance in oil recovery, being even better than previous reports. Couto and co-workers reported AORs of 14% and 26% using the biopolymer produced by *R. viscosum* CECT 908 in sand-pack column assays performed at 40°C using two different crude oils with viscosities of 81 and 167 mPa s, respectively (Couto et al., 2019). These results suggest that the performance of this biopolymer in oil recovery increases as the viscosity of the oil increases, which is an advantage for the recovery of

heavy crude oils with high viscosity. Additional oil recoveries of 18% and 25% were reported for xanthan and welan gum, respectively, in core-flood assays performed at 50°C using a heavy crude oil with a viscosity of 458 mPa s. In this case, the viscosity of the solutions used was 600 mPa s for xanthan gum and 2000 mPa s for welan gum (both of them measured at 25°C) (Xu et al., 2014).

5. CONCLUSIONS AND FUTURE PERSPECTIVES

5.1. Conclusions

The aim of the present work was the construction of engineered microorganisms with the ability to grow and produce biopolymers at the oil reservoir conditions towards the development of effective MEOR processes. To reach these purposes several subjects were studied, and different applications were tested. The main conclusions drawn from this work are the following:

- Different conditions were evaluated to improve the protocols for protoplasts formation and regeneration for *B. subtilis* PX573 and *R. viscosum* CECT 908. For *R. viscosum* CECT 908, the most favourable condition was an incubation with EDTA for 60 min at 30°C followed by a treatment with 2 g/L of lysozyme for 1 h at 37°C. For *B. subtilis* PX573, the most favourable condition was an incubation with 3 g/L of lysozyme for 1 h at 37°C without EDTA treatment.
- After the protoplasts fusion, most of the fusants were not able of growing in the different regeneration media evaluated, most probably because they could not regenerate the cell wall and revert to normal cells.
- None of the fusants obtained was found to produce biopolymers.
- Four *Paenibacillus* sp. strains were identified as promising biopolymer producers, leading to apparent viscosity values more than 80 times higher than the ones reported for *R. viscosum* CECT 908.
- The biopolymer produced by *Paenibacillus* sp. #540 allowed oil recoveries around 42% in MEOR assays performed using a heavy crude oil.
- The combination of surfactin produced by *B. subtilis* PX573 and the biopolymer produced by *R. viscosum* CECT 908 resulted in a positive effect in the surface activity but a negative effect in the apparent viscosity when compared with the respective biomolecules alone.
- The combination of surfactin and the *R. viscosum* biopolymer did not improve the efficiency of the biopolymer in oil recovery (47.2 ± 0.7% *versus* 46.2 ± 3.8%).
- The low interfacial tension values achieved with surfactin are not enough to promote the recovery of heavy crude oils.

5.2. Future perspectives

The results herein gathered showed that the fusants obtained did not have the ability to produce the envisaged biopolymers at the reservoir conditions. On the other hand, the ability of four *Paenibacillus* sp. strains to produce biopolymers with potential application in MEOR was demonstrated. Also, the effect of biopolymer-biosurfactant cocktails in *ex situ* MEOR was studied using sand-pack columns. Despite of these interesting results, further research should be done as suggested bellow:

- Since it was not possible to obtain recombinant strains with the desired properties, further optimizations are necessary (*e.g.*, study of different concentrations of fusogenic agent, different incubation times and temperatures, use of PEG with different molecular weights, and the study of the effect of different cations in the solution containing the fusogenic agent).
- As the strategies applied to allow the regeneration of fusants were not conclusive, different culture media (containing different combinations of trace elements) should be tested.
- Optimization of the production of biopolymers by the *Paenibacillus* sp. strains herein reported, using low-cost substrates to reduce the production costs and study their production in bioreactor.
- Study of the chemical composition and the structure of the biopolymers produced by *R. viscosum* CECT 908 and *Paenibacillus* sp. #540 in order to understand their mechanisms of action in oil recovery, their biosynthesis, and evaluate other possible applications. For this purpose, it will be necessary to optimize the purification techniques.
- Due to the promising results obtained with the biopolymer produced by *Paenibacillus* sp. #540 in the *ex situ* MEOR assays, further studies are necessary to determine its real potential for this application (*e.g.*, stability studies, oil recovery assays using different types of crude oil).
- Regarding the combination of surfactin produced by *B. subtilis* PX573 and the biopolymer produced by *R. viscosum* CECT 908, the results obtained in the oil recovery assays were not favourable. Therefore, different ratios of surfactin (or other biosurfactants) and biopolymer should be evaluated using crude oils with different viscosities.

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