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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Cost-effective rhamnolipid production by *Burkholderia thailandensis* E264 using agro-industrial residues

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Abstract

The agro-industrial by-products corn steep liquor (CSL) and olive mill wastewater (OMW) were evaluated as low-cost substrates for rhamnolipid production by *Burkholderia thailandensis* E264. In a culture medium containing CSL (7.5% (v/v)) as sole substrate, *B. thailandensis* E264 produced 175 mg rhamnolipid/L, which is about 1.3 times the amount produced in the standard medium, which contains glycerol, peptone, and meat extract. When the CSL medium was supplemented with OMW (10% (v/v)), rhamnolipid production further increased up to 253 mg/L in flasks and 269 mg/L in a bioreactor. Rhamnolipids produced in CSL + OMW medium reduced the surface tension up to 27.1 mN/m, with a critical micelle concentration of 51 mg/L, better than the values obtained with the standard medium (28.9 mN/m and 58 mg/L, respectively). However, rhamnolipids produced in CSL + OMW medium displayed a weak emulsifying activity when compared to those produced in the other media. Whereas di-rhamnolipid congeners represented between 90 and 95% of rhamnolipids produced by *B. thailandensis* E264 in CSL and the standard medium, the relative abundance of mono-rhamnolipids increased up to 55% in the culture medium containing OMW. The difference in the rhamnolipid congeners produced in each medium explains their different surface-active properties. To the best of our knowledge, this is the first report of rhamnolipid production by *B. thailandensis* using a culture medium containing agro-industrial by-products as sole ingredients. Furthermore, rhamnolipids produced in the different media recovered around 60% of crude oil from contaminated sand, demonstrating its potential application in the petroleum industry and bioremediation.

Key points

- *B. thailandensis produced RL using agro-industrial by-products as sole substrates*
- *Purified RL displayed excellent surface activity (minimum surface tension 27mN/m)*
- *Crude RL (cell-free supernatant) recovered 60% of crude oil from contaminated sand*

Keywords Bioremediation · Corn steep liquor · Olive mill wastewater · Circular economy · Biosurfactant · Emulsification

Introduction

Biosurfactants are amphiphilic surface-active compounds produced by various microorganisms, comprised of hydrophobic and hydrophilic moieties, that contribute to the reduction of surface and interfacial tensions. Their structure also promotes emulsification and demulsification, wetting, spreading, foaming, and solubilization of immiscible compounds (Gudiña and Teixeira 2022; Varjani et al. 2021). These biomolecules are attracting increasing interest over their chemical counterparts due to their advantages (lower toxicity and higher biodegradability) and potential applications in bioremediation, pharmaceuticals, cosmetics, agriculture, food, and petroleum industries, among others (Gudiña and Teixeira 2022; Jahan et al. 2020).

Rhamnolipids are among the most widely studied biosurfactants. They are composed of one or two rhamnose molecules, linked to one to three β-hydroxy fatty acids, either saturated or unsaturated, with a chain length of 8 to 16 carbon

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atoms (Varjani et al. 2021). In addition to their surface activity and emulsifying properties, rhamnolipids exhibit antimicrobial and antifungal activity (Ndlovu et al. 2017; Rodrigues et al. 2021). However, their main producer, *Pseudomonas aeruginosa*, is an opportunistic human pathogen, raising concerns regarding the safety of the rhamnolipids it produces and limiting their application in several fields (Toribio et al. 2010). Furthermore, the high operational costs associated to their production restrict their industrial-scale applications (Jahan et al. 2020). Several attempts to reduce the production costs of rhamnolipids have been conducted and include the use of low-cost agro-industrial wastes and by-products as substrates (Gudiña et al. 2015a; 2016; Varjani et al. 2021).

To overcome these concerns, alternative rhamnolipid-producing microorganisms are being studied. For example, *Lysinibacillus sphaericus* IITR51 and *Planococcus* spp. produce rhamnolipids with antimicrobial properties (Gaur et al. 2019; 2020). *Enterobacter cloacae* BAGM01 has been reported to produce rhamnolipids that are stable at high temperatures (up to 121 °C) and salinities (35 g NaCl/L), using different carbon sources, including diesel and sunflower oil (Curiel-Maciel et al. 2021). *Thermoanaerobacter* sp. CM-CNRG TB177 is also able to produce rhamnolipids using alternative carbon sources like molasses (Segovia et al. 2021). *Paraburkholderia* sp. C3 produces rhamnolipids that demonstrated to be useful in bioremediation applications using the biodiesel by-product glycerol as carbon source (Cao et al. 2021).

Burkholderia thailandensis E264 is another non-pathogenic rhamnolipid producer that has been widely studied in the past years. This microorganism produces mainly di-rhamnolipids with longer β-hydroxy fatty acid chains (C_{14}, C_{16}) when compared to *P. aeruginosa* (mainly C_{10}) (Dubeau et al. 2009). Two identical gene clusters, containing the genes *rhlA*, *rhlB*, and *rhlC*, are present in the genome of *B. thailandensis*, and both of them contribute to rhamnolipids biosynthesis (Dubeau et al. 2009). On the contrary, in *P. aeruginosa*, only a single copy of those genes exists, and the genes *rhlA* and *rhlB* are present in one operon, whereas the gene *rhlC* is present in a different location in the genome (Toribio et al. 2010). However, due to the low rhamnolipid yields of *B. thailandensis* comparing to *P. aeruginosa*, it is important to develop new strategies to improve their yield in a way that is cost-effective. In this work, corn steep liquor (CSL) was evaluated as a substrate for rhamnolipid production by *B. thailandensis* E264. CSL is the liquid by-product of the corn wet-milling industry and is used in biotechnological processes as a source of nitrogen (Gudiña et al. 2015a; Hofer et al. 2018). Besides amino acids and proteins, CSL is rich in vitamins and minerals, and contains variable amounts of carbohydrates, meaning that it can be also used as a low-cost carbon source (Gudiña et al. 2015a). Olive mill wastewater (OMW) is another interesting substrate that has been used as an inexpensive source of long-chain fatty acids for rhamnolipid production by *P. aeruginosa* (Gudiña et al. 2016). OMW is a liquid residue generated during the extraction of olive oil. It contains long-chain fatty acids, carbohydrates, phenolic compounds, organic acids, tannins, pectins, and minerals (Dermeche et al. 2013). Due to its low biodegradability, OMW is difficult to process and an environmentally hazardous residue. About 30 million m^3 of OMW is produced in the Mediterranean countries per year; consequently, the valorization of this residue is a crucial aspect for reaching circular economy within the olive oil sector (Dermeche et al. 2013; Hamimed et al. 2021).

The aim of this work was to optimize rhamnolipid production by *B. thailandensis* E264 using CSL and OMW as low-cost substrates, characterize the rhamnolipids produced, and study their applicability in bioremediation.

Materials and methods

Strain and culture conditions

B. thailandensis E264 (ATCC 700388) was used in this study. The strain was maintained in Luria–Bertani (LB) medium, supplemented with 20% (v/v) of glycerol at − 80 °C. Pre-cultures were prepared by inoculating 20 mL of standard (S) medium (glycerol 40 g/L, peptone 5 g/L, meat extract 3 g/L, pH 7.0) with 100 µL from a frozen stock, and were incubated overnight at 30 °C and 180 rpm.

Rhamnolipid production in flasks

Rhamnolipid production by *B. thailandensis* E264 was evaluated in 500-mL flasks, containing 150 mL of the different culture media. Each flask was inoculated with 1.5 mL of a pre-culture, and incubated at 30 °C and 180 rpm for different time intervals (between 96 and 240 h). S medium was used as control, as it has been previously used for rhamnolipid production by *B. thailandensis* (Dubeau et al. 2009; Elshikh et al. 2017; Funston et al. 2016; 2017). CSL (kindly provided by COPAM – Companhia Portuguesa de Amidos, S. A. (Portugal)) and OMW (obtained from an olive oil mill located in the north of Portugal) were evaluated as alternative substrates. Both substrates were characterized in our previous works (Gudiña et al. 2015a; 2016), and their composition is provided in Table S1. CSL was diluted with demineralized water at different concentrations (5–20% (v/v)) and used as culture medium, either alone or supplemented with OMW (5–25% (v/v)). All the media were adjusted to pH 7.0. Samples were taken at different time intervals to determine bacterial growth, rhamnolipid production, and substrates

consumption, as described in the following sections. All experiments were performed in triplicate.

Rhamnolipid production in bioreactor

The scale-up process was performed in a 3.7-L bioreactor (RALF Advanced, Bioengineering AG, Switzerland) with 2 L of the culture medium containing CSL (7.5% (v/v)) and OMW (10% (v/v)). Because this medium contains high amounts of precipitates, which can interfere with the bioreactor's performance, it was centrifuged (15,316 × *g*, 20 min) before being introduced in the reactor vessel. Two milliliters of silicon anti-foaming agent (Sigma-Aldrich) was added to the culture medium to avoid the formation of foam.

A pre-culture of *B. thailandensis* E264 was first prepared in S medium (20 mL), grown overnight at 30 °C and 150 rpm, and used to inoculate a second pre-culture, containing 100 mL of the same culture medium used in the bioreactor, which was incubated for 48 h at 30 °C and 150 rpm. Subsequently, the inoculum was centrifuged (5514 × *g*, 10 min) and the cells were resuspended in 5 mL of a phosphate-buffered saline (PBS) solution, which were used to inoculate the bioreactor.

The experiments were conducted under batch mode over 78 h at 30 °C. Stirring rate and air flow (previously optimized) were kept constant at 350 rpm and 0.3 vvm (volume of air per volume of cultivation broth per minute), respectively. Samples were taken at different time points and used to determine bacterial growth, substrate consumption, and rhamnolipid production. Experiments were performed in duplicate.

Rhamnolipid recovery

Rhamnolipids produced by *B. thailandensis* E264 were recovered through adsorption chromatography using the polystyrene resin Amberlite® XAD®-2 (Sigma-Aldrich). A 125-mL column was filled with Amberlite XAD-2 and equilibrated with two volumes of 0.1 M potassium phosphate buffer (pH 6.1). Subsequently, 100 mL of cell-free supernatant (centrifuged at 15,316 × *g* for 20 min) was adjusted to pH 6.1 and introduced into the column, which was then washed with four volumes of demineralized water to remove the non-adsorbed compounds. The column was further washed with 300 mL of solutions with increasing concentrations of methanol (25, 50, and 75% (v/v)), followed by 200 mL of pure methanol. The presence of rhamnolipids in the eluents was assessed by thin-layer chromatography (TLC), according to Rodrigues et al. (2017). After evaporation of the solvents, the recovered rhamnolipids were dissolved in 20 mL of demineralized water and freeze-dried. The products obtained were weighed and stored at − 20 °C. The amount of rhamnolipids produced was determined gravimetrically.

Analytical techniques

Bacterial growth determination

Bacterial growth was determined through the plate count technique. Samples (1 mL) were taken at different time points of fermentation and were serially diluted with NaCl-Tween buffer (9 g/L of NaCl and 100 mg/L of Tween 80); 100 µL of each dilution was plated on a LB agar plate and incubated at 30 °C for 48 h. The number of colony-forming units (CFU) at each dilution was counted and the average expressed as CFU/mL.

Substrate consumption determination

Substrate consumption was evaluated by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H (300 × 7.8 mm, Bio-Rad, USA) column coupled to a refractive index detector (RI-2031 Plus, JASCO) and an UV detector (K-2501, Knauer). Samples collected at different time points of fermentation were centrifuged (2450 × *g*,15 min) to remove the cells, and the supernatants were filtered and analyzed. H_2SO_4 (5 mM) was used as mobile phase at a flow rate of 0.6 mL/min, and the column was maintained at 60 °C. The concentrations of glucose, fructose, and lactic acid were calculated according to calibration curves prepared using pure compounds.

Surface tension measurement and critical micelle concentration (CMC)

Surface tension (ST) of cell-free supernatants and rhamnolipid solutions was measured using the Ring method described elsewhere (Gudiña et al. 2016). A KRÜSS K20 Tensiometer (KRÜSS GmbH, Germany) equipped with a 1.9-cm De Noüy platinum ring was used at room temperature (25 °C). All the measurements were done in triplicate. Critical micelle concentrations (CMC) were calculated by measuring the ST of rhamnolipid solutions prepared in 10 mM Tris–HCl buffer (pH 7.4) at different concentrations, as described elsewhere (Gudiña et al. 2016).

Emulsifying activity

Emulsifying activity (E_{24}) of rhamnolipid solutions was determined against *n*-hexadecane, as described elsewhere (Gudiña et al. 2016). All emulsification indexes were determined in duplicate.

Rhamnolipid characterization

Fourier transform infrared spectroscopy (FTIR)

The rhamnolipid mixtures previously obtained were characterized through FTIR spectroscopy. FTIR spectra were recorded using the IRSpirit FTIR spectrometer (Shimadzu, Kyoto, Japan) at room temperature (25 °C). The main functional groups of rhamnolipids were observed between 400 and 4000 wavenumbers (cm^{-1}) at a resolution of 2 cm^{-1}.

Identification of rhamnolipid congeners

Individual rhamnolipid congeners present in the different rhamnolipid mixtures were identified by electrospray ionization (ESI) mass spectrometry (MS) using a Compact™ Mass Spectrometer (Bruker Daltonics, Bremen, Germany) in negative electrospray ionization mode. The instrument parameters were as follows: electrospray voltage: − 5 kV; scan range: 50–3000 m/z; drying gas: nitrogen; flow rate: 4.0 L/min; temperature: 200 °C. For MS spectra analysis, a Bruker Compass Data Analysis 4.2 software was used.

Gas chromatography–mass spectrometry (GC–MS)

The different rhamnolipid mixtures (1 mg) were refluxed in 2 N HCl (0.5 mL) for 2 h. The 3-hydroxy fatty acids (3-OH-FAs) were extracted three times with hexane (10 mL). Next, the free 3-OH-FAs were derivatized using 2.5% sulfuric acid in methanol and resulting 3-hydroxy fatty acid methyl esters (3-OH-FAMEs) were analyzed by GC–MS (Shimadzu, Kyoto, Japan) using a Zebron ZB-FAME capillary column (30 m × 0.25 mm × 0.20 µm). Split injection mode was used for injecting the sample (1 µL at 250 °C) using helium (1 mL/min). All the experiments were performed in triplicate.

Oil recovery assays

Oil recovery assays were performed using artificially contaminated sand, containing 10% (w/w) of crude oil from the Potiguar oilfield (Brazil), provided by PARTEX Oil and Gas. The apparent viscosity of the crude oil (measured at 40 °C and with a shear rate of 1.4 s^{-1}) was 110 mPa s, and its density 910 g/L. To prepare the artificially contaminated sand, 40 g of dry sand was mixed with 4 g of crude oil in 100-mL flasks and allowed to age for 5 days at 50 °C. Afterwards, 40 mL of cell-free broth samples from cultures of *B. thailandensis* E264 performed in different culture media (S, CSL and CSL + OMW) was added to each flask. Control assays were performed using 40 mL of the corresponding uninoculated culture medium. The flasks were incubated at 150 rpm and 50 °C for 24 h. After the incubation period, the oil removed from the sand was recovered from the liquid surface and its volume was measured. In order to compare the performance of rhamnolipids produced by *B. thailandensis* E264 in the different culture media with commercial rhamnolipids (RL-90, Sigma-Aldrich, 90% of purity), they were dissolved in the uninoculated culture media (S, CSL and CSL + OMW) at a concentration of 200 mg/L, and oil recovery assays were performed as described above. All the experiments were carried out in triplicate.

Results

Rhamnolipid production by *B. thailandensis* E264 in flasks

Rhamnolipid production by *B. thailandensis* E264 was first evaluated using the standard (S) medium (Fig. 1). The rhamnolipids produced reduced the culture medium ST to 32.1 ± 1.4 mN/m after 96 h of growth. During this time, only about 25% of the total amount of glycerol provided (40 g/L) was consumed by *B. thailandensis* E264 to produce 139 ± 64 mg/L of rhamnolipids.

Aiming to reduce the production costs, rhamnolipid production by *B. thailandensis* E264 was evaluated using several low-cost media containing as sole ingredients agro-industrial residues (Table 1). CSL was evaluated as sole substrate for rhamnolipid production at different concentrations (5–20% (v/v)). The best results were obtained with a culture medium containing 7.5% (v/v) CSL, achieving ST values around 30 mN/m after 72 h of growth (Table 1; Fig. 2). Subsequently, the effect of supplementing the CSL medium with different concentrations of OMW (5–25% (v/v)) was studied. The lowest ST values (around 27.4 mN/m) were obtained with the medium containing 7.5% (v/v) CSL and 10% (v/v) OMW as sole substrates after 72–96 h of growth (Table 1). Evolution of ST, bacterial growth, and substrate consumption with this culture medium is illustrated in Fig. 2. For the

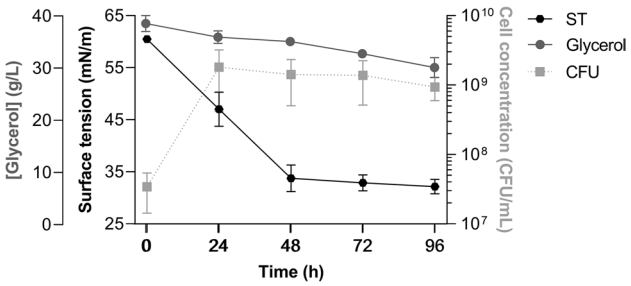

Fig. 1 Surface tension values (mN/m), glycerol concentration (g/L), and bacterial growth (CFU/mL) over time of *Burkholderia thailandensis* E264 grown in standard (S) medium in flasks at 30 °C and 180 rpm

Table 1 Surface tension values (mN/m) obtained with *Burkholderia thailandensis* E264 grown in CSL (5–20% v/v) and CSL (7.5% v/v) supplemented with OMW (5–25% v/v) at 30 °C and 180 rpm

Culture medium	Surface tension (mN/m)				
	0 h	24 h	48 h	72 h	96 h
5% CSL	50.2 ± 0.4	44.8 ± 1.2	36.2 ± 3.5	35.6 ± 1.2	38.2 ± 2.0
7.5% CSL	48.6 ± 0.6	47.2 ± 1.9	33.0 ± 1.7	30.3 ± 0.9	32.1 ± 0.9
10% CSL	48.3 ± 0.5	48.4 ± 0.5	37.6 ± 4.5	32.8 ± 1.3	32.5 ± 1.2
12.5% CSL	47.9 ± 0.8	47.1 ± 1.1	48.7 ± 1.8	46.3 ± 5.5	37.5 ± 0.8
15% CSL	47.5 ± 0.7	-	49.6 ± 0.7	50.0 ± 0.6	-
20% CSL	46.1 ± 0.3	-	48.6 ± 0.8	49.5 ± 0.7	-
7.5% CSL + 5% OMW	41.0 ± 0.5	-	37.4 ± 0.9	28.2 ± 0.4	27.7 ± 0.2
7.5% CSL + 10% OMW	41.8 ± 0.6	43.3 ± 0.2	29.8 ± 0.9	27.4 ± 0.4	27.5 ± 0.2
7.5% CSL + 15% OMW	39.7 ± 0.4	-	43.3 ± 0.3	42.5 ± 0.2	42.1 ± 0.2
7.5% CSL + 20% OMW	40.1 ± 0.4	-	42.6 ± 0.2	42.2 ± 0.2	41.5 ± 0.1
7.5% CSL + 25% OMW	40.2 ± 0.4	-	42.0 ± 0.3	41.8 ± 0.2	41.3 ± 0.3
Control (S medium)	60.5 ± 0.8	47.0 ± 3.3	33.7 ± 2.6	32.9 ± 1.6	32.1 ± 1.4

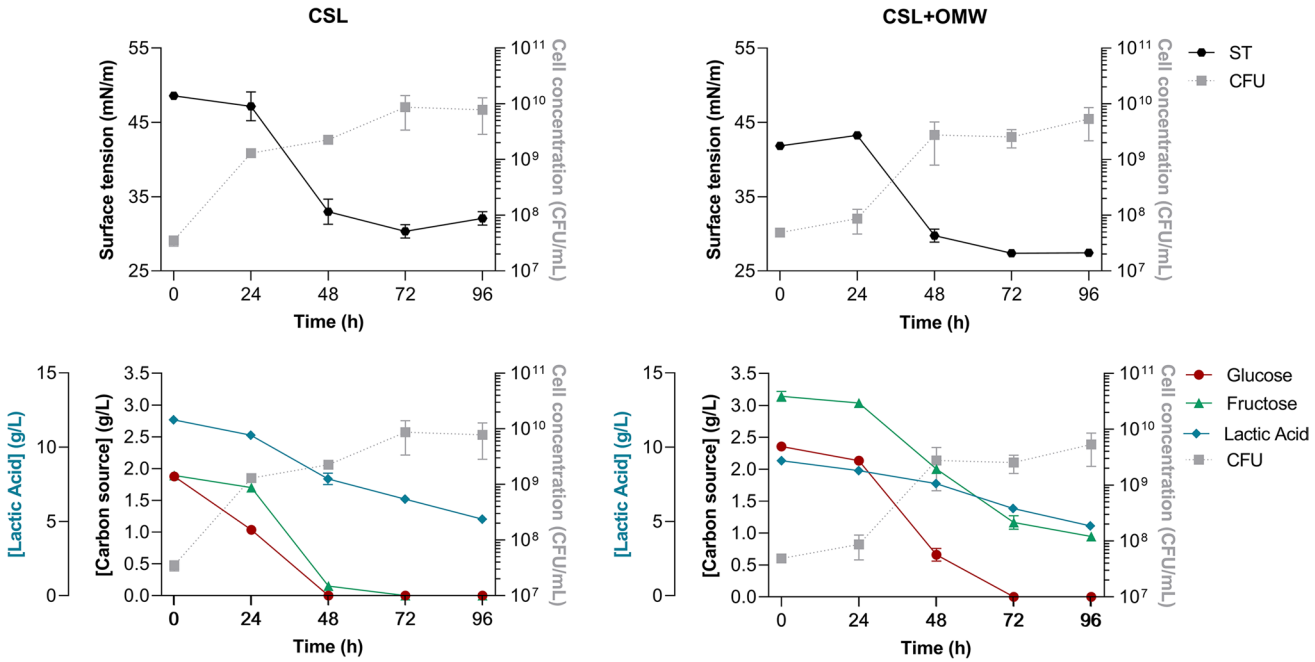

Fig. 2 Top: Surface tension values (mN/m) and bacterial growth (CFU/mL). Bottom: Carbon source and lactic acid concentrations (g/L), and bacterial growth (CFU/mL) over time of *Burkholderia thailandensis* E264 grown in flasks in CSL and CSL + OMW media at 30 °C and 180 rpm

other culture media presented in Table 1, no significant ST reductions were achieved even after 240 h of growth when compared with those observed at 96 h (*data not shown*). Consequently, they were not considered for rhamnolipid production.

HPLC analysis showed that in the CSL medium (7.5% (v/v) CSL), glucose (1.9 g/L) and fructose (1.9 g/L) were fully consumed within the first 48 h of growth. In the CSL + OMW medium (7.5% (v/v) CSL + 10% (v/v) OMW), which contains a higher concentration of both sugars (2.4 g glucose/L, 3.2 g fructose/L), fructose was not completely exhausted, even after 96 h. Moreover, 4.9 and 6.8 g/L of lactic acid present in CSL + OMW and CSL media, respectively, were consumed, which might be used as an additional carbon source (Fig. 2). The low-cost media also allowed a higher bacterial growth than the standard medium, although in the CSL + OMW medium there was almost no growth during the first 24 h, probably due to the presence of inhibitory compounds in OMW such as phenolic compounds (Gudiña et al. 2016) (Table S1; Fig. 2).

The amount of rhamnolipids produced in the CSL medium after 72 h of growth was 175 ± 3 mg/L, which is about 1.3 times the amount obtained in the standard medium. By supplementing the CSL medium with

OMW, rhamnolipid production further increased up to 253 ± 46 mg/L in 96 h. To the best of our knowledge, this is the first report of rhamnolipid production by *B. thailandensis* using a culture medium containing agro-industrial by-products as sole ingredients.

Rhamnolipid production by *B. thailandensis* E264 in a bioreactor

According to the results obtained in flask assays, the low-cost medium CSL + OMW was evaluated for rhamnolipid production in a 3.7-L bioreactor. As it can be seen in Fig. 3, the ST decreased faster when compared with flask assays, being the lowest ST value (26.3 ± 0.8 mN/m), achieved after 72 h of growth. The highest rhamnolipid production (269 ± 95 mg/L) was achieved after 54 h, which results in a better productivity when compared to flask assays, where the highest production was achieved after 96 h. Glucose and fructose consumption displayed similar profiles in flask and bioreactor assays, although in the last case, about 1 g glucose/L remained in the culture medium after 78 h of growth. The amount of lactic acid consumed in bioreactor (2.3 g/L) was also lower than in flasks (4.9 g/L) (Fig. 2; Fig. 3). Bacterial growth displayed a different behavior than the one observed in flasks. An abrupt decline in the number of cells was observed during the first hours of growth, which may be due to the mechanical stress applied to the cells in the bioreactor. In fact, in order to achieve good results regarding bacterial growth and rhamnolipid production, it was necessary to increase the size of the inoculum used for the bioreactor five times when compared with flask assays, as described in the "Materials and methods" section. Bacterial growth was slightly lower in the bioreactor (1.2×10^9 CFU/mL, compared to 5.4×10^9 CFU/mL in flask), and a decline phase was observed after 54 h (Fig. 2; Fig. 3).

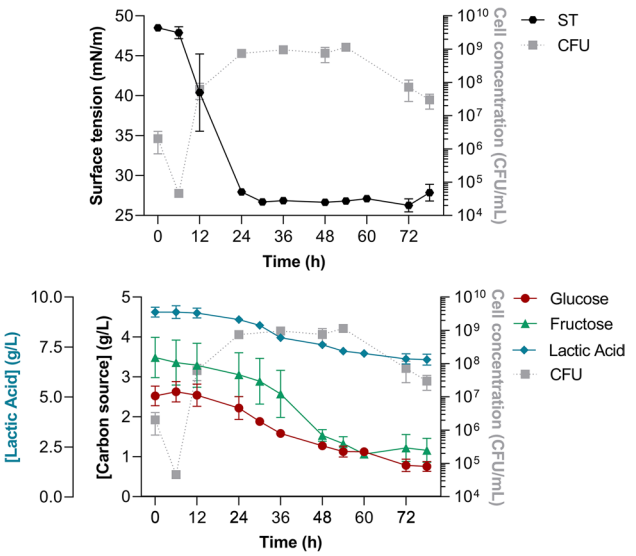

Fig. 3 Top: Surface tension values (mN/m) and bacterial growth (CFU/mL). Bottom: Carbon source and lactic acid concentrations (g/L), and bacterial growth (CFU/mL) over time of *Burkholderia thailandensis* E264 grown in the bioreactor with CSL + OMW medium at 30 °C, 350 rpm and 0.3 vvm

Rhamnolipid chemical characterization

FTIR analysis of the products obtained in S, CSL, and CSL + OMW media (Fig. S1) displayed similar functional groups to those reported for glycolipids in previous works (Borah et al. 2015; Gaur et al. 2020; Sen et al. 2020; Xu et al. 2020). The asymmetric and symmetric stretching of methylene (-CH) were detected at 2923 cm^{-1} and 2853 cm^{-1}, respectively, for rhamnolipids produced in S and CSL media, and at 2920 cm^{-1} and 2851 cm^{-1} for the CSL + OMW medium. The characteristic band of carbonyl groups (-C = O) was found at 1724 cm^{-1} and 1657 cm^{-1} for S medium, 1714 cm^{-1} and 1650 cm^{-1} for CSL, and 1714 cm^{-1} and 1658 cm^{-1} for CSL + OMW, confirming the presence of ester compounds. The carboxylic acid plane bending (-COH-) was detected at 1454 cm^{-1} and 1397 cm^{-1} for S medium, 1453 cm^{-1} and 1400 cm^{-1} for CSL, and 1445 cm^{-1} for CSL + OMW. The stretching band observed at 1040 cm^{-1}, 1052 cm^{-1}, and 1065 cm^{-1} for S, CSL, and CSL + OMW media, respectively, corresponded to the -C-O- bonds between the carbon atoms and the hydroxyl groups present in the rhamnose rings. Finally, the peaks detected at 704 cm^{-1}, 705 cm^{-1}, and 698 cm^{-1} for S, CSL, and CSL + OMW media related to the CH_2 rocking in the lipid structure (Fig. S1).

Subsequently, the rhamnolipid congeners produced by *B. thailandensis* E264 in the different media were characterized by ESI–MS operating in negative electrospray ionization mode. The same congeners were identified in all the media, but they were present in different relative abundances (Table 2). The most abundant congener in S and CSL media was the di-rhamnolipid Rha-Rha-C_{14}-C_{14} (62–64%), followed by Rha-Rha-C_{12}-C_{14}/C_{14}-C_{12} (18–22%). The hydrophilic–lipophilic balance (HLB) of these rhamnolipids ranges between 10.5 and 10.9 (calculated according to Griffin's method (Griffin 1954)), which characterizes them as oil/water emulsifiers. The supplementation of the CSL medium with OMW resulted in a substantial increase in the relative abundance of the mono-rhamnolipids Rha-C_{12}-C_{12} and Rha-C_{12}-C_{14}/C_{14}-C_{12} (Table 2). In fact, the relative abundance of mono-rhamnolipids increased from 5–10% in S and CSL media to 56% in the culture medium supplemented with OMW.

The mono-rhamnolipids Rha-C_{12}-C_{12}, Rha-C_{14}-C_{14}, and Rha-C_{12}-C_{14}/C_{14}-C_{12} have HLB values that range from 8.3 to 9.1, being characterized as wetting and spreading agents.

Table 2 Rhamnolipid congeners produced by *Burkholderia thailandensis* E264 and their relative abundance in the different culture media

Rhamnolipid congeners	Pseudomolecular ion (*m/z*)	Relative abundance (%)		
		S medium	CSL	CSL + OMW
Mono-rhamnolipids (total)		*5.35*	*9.81*	*55.82*
Rha-C_{12}-C_{12}	559.38	0.76	1.66	24.74
Rha-C_{12}-C_{14}/C_{14}-C_{12}	587.41	1.86	2.92	29.79
Rha-C_{14}-C_{14}	615.45	2.73	5.23	1.29
Di-rhamnolipids (total)		*94.64*	*90.19*	*44.18*
Rha-Rha-C_{12}-C_{12}	705.44	4.95	5.9	2.27
Rha-Rha-C_{12}-C_{14}/C_{14}-C_{12}	733.47	22.63	18.04	15.62
Rha-Rha-C_{14}-C_{14}	761.50	64.09	62.60	24.59
Rha-Rha-C_{14}-C_{16}/C_{16}-C_{14}	789.53	2.97	3.65	1.7

Table 3 The composition of 3-OH-FAMEs of rhamnolipids determined by GC–MS. The results represent the mean ± standard deviation of the analysis performed in triplicate

3-OH-FAMEs	Relative abundance (%)		
	S medium	CSL	CSL + OMW
3-OH-dodecanoic	18.00 ± 0.21	18.04 ± 0.14	49.74 ± 0.14
3-OH-tetradecanoic	80.55 ± 0.12	80.14 ± 0.25	49.41 ± 0.17
3-OH-hexadecanoic	1.45 ± 0.09	1.82 ± 0.11	0.85 ± 0.03

Table 4 Rhamnolipid titers obtained with *Burkholderia thailandensis* E264 in standard (S), CSL, and CSL + OMW (both in the flasks and bioreactor) media. Critical micelle concentration (CMC) and minimum surface tension values (ST_{min}) of rhamnolipid solutions prepared in 10 mM Tris–HCl buffer (pH 7.4)

Culture medium	RL titer (mg/L)	CMC (mg/L)	ST_{min} (mN/m)
S	139 ± 64	58	28.9 ± 0.4
CSL	175 ± 3	108	29.6 ± 0.4
CSL + OMW (flask)	253 ± 46	51	27.1 ± 0.1
CSL + OMW (bioreactor)	269 ± 95	48	27.1 ± 0.1

Because they have lower HLB values than di-rhamnolipids, they are also more hydrophobic, displaying more wetting properties than the latter.

The analysis of the fatty acid chains present in the produced rhamnolipids identified mainly tetradecanoic acid, followed by dodecanoic acid (Table 3). The fatty acids identified, and their abundance, are in line with the observed rhamnolipid congeners.

Critical micelle concentration of rhamnolipids produced by *B. thailandensis* E264

The rhamnolipids produced in the S medium had a CMC of 58 mg/L and reduced the ST of Tris–HCl buffer from 72.0 ± 0.2 up to 28.9 ± 0.4 mN/m (Table 4; Fig. 4). In CSL medium, the CMC of the rhamnolipids produced was 108 mg/L, meaning that they were less efficient when compared with those produced in S medium, even though their minimum ST values were similar (Table 4). The rhamnolipids produced in CSL + OMW medium were the most surface active, with a CMC of 51 mg/L and a minimum ST value of 27.1 ± 0.1 mN/m. Regarding the rhamnolipids produced in the bioreactor using this culture medium, the CMC (48 mg/L) and the minimum ST value (27.1 ± 0.1 mN/m) were similar to those obtained in the flasks (Table 4; Fig. 4). Furthermore, rhamnolipid production in the bioreactor was more efficient, since a similar amount of rhamnolipids, with similar surface-active properties, was produced in less time (54 h instead of 96 h in flasks).

Emulsification activity of the rhamnolipids produced by *B. thailandensis* E264

Aqueous solutions of purified rhamnolipids produced in S medium were able to stabilize emulsions with *n*-hexadecane at concentrations as low as 100 mg/L, with an E_{24} of 51 ± 4%, which increased up to 56–58% as the rhamnolipid concentration increased (Table 5). To note that, since the same volume of rhamnolipid solution and *n*-hexadecane was used to perform these studies, emulsifying indexes above 50% denote the complete emulsification of the oil phase. Regarding rhamnolipids produced in CSL medium, the emulsifying activity was similar to the one obtained in S medium with the highest rhamnolipid concentrations tested, although a weaker activity was obtained at 100 mg/L (Table 5). For rhamnolipids produced in CSL + OMW medium, the E_{24} was below 20% for all the concentrations tested (Table 5). These results can be explained by the structural differences of rhamnolipids produced in the different culture media. The mono-rhamnolipids produced mainly in CSL + OMW medium fall within the range of wetting and spreading agents in the HLB scale, while di-rhamnolipids, present in higher relative abundance in S and CSL media, are considered better emulsifying agents (Baccile et al. 2021).

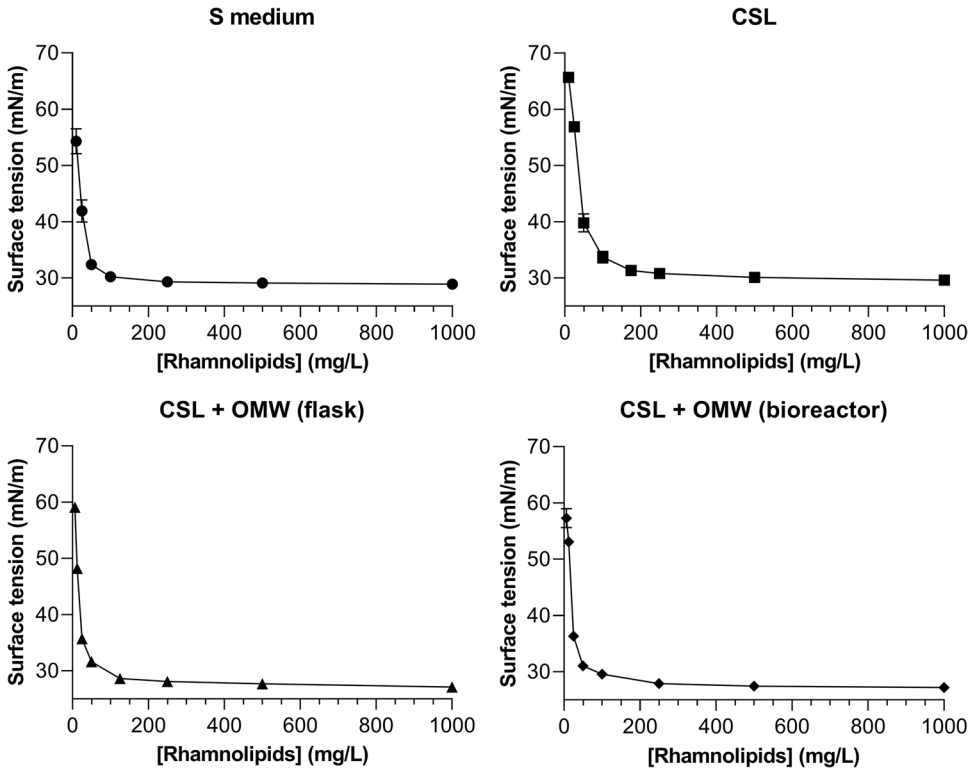

Fig. 4 Surface tension values (mN/m) of rhamnolipids produced by *Burkholderia thailandensis* E264 in standard, CSL, and CSL + OMW media (both in the flasks and bioreactor), dissolved in 10 mM Tris–HCl buffer (pH 7.4) at different concentrations

Table 5 Emulsification indexes (E_{24}) obtained with purified rhamnolipids produced by *Burkholderia thailandensis* E264 in standard (S), CSL, and CSL + OMW media

RL concentration (mg/L)	E_{24} (%)		
	S	CSL	CSL + OMW
500	56.0 ± 0.0	61.3 ± 1.8	17.4 ± 0.1
250	58.0 ± 2.8	63.3 ± 1.1	13.3 ± 0.4
100	51.1 ± 4.4	22.9 ± 2.9	4.4 ± 0.1

Oil recovery assays

The potential application of rhamnolipids produced by *B. thailandensis* E264 in microbial enhanced oil recovery (MEOR) and bioremediation was evaluated by studying their ability of recovering heavy crude oil from artificially contaminated sand. This methodology has been widely used in bioremediation studies, as it does not require specific equipment such as sand-pack columns or core flooding systems, commonly used in MEOR studies, and is less time-consuming (Bezza and Chirwa 2015; Chaprão et al. 2018; Gaur et al. 2022; Gudiña et al. 2015a; 2015b; Rufino et al. 2013; Soares da Silva et al. 2017; Teixeira Souza et al. 2018). However, it fails to reproduce the complex reservoir properties, like porosity, permeability, pressure, and injection flow rates, although it can still provide useful insights into possible MEOR applications (Ciurko et al. 2022; Datta et al. 2020; Rellegadla et al. 2019).

Table 6 Results obtained in oil recovery assays performed with the cell-free supernatants (CFS) from cultures of *Burkholderia thailandensis* E264 grown in standard (S), CSL, and CSL + OMW media, and commercial rhamnolipids (RL-90, Sigma-Aldrich, 90% of purity) dissolved in the same uninoculated culture media at a concentration of 200 mg/L. Control assays were performed using the uninoculated culture media

Treatment		ST (mN/m)	Oil recovery (%)
CFS	S	32.1 ± 1.4	61.4 ± 6.8
	CSL	30.3 ± 0.9	62.9 ± 2.5
	CSL + OMW	27.5 ± 0.2	60.7 ± 4.7
RL-90	S	30.0 ± 0.6	49.7 ± 2.6
	CSL	29.8 ± 0.3	50.6 ± 3.1
	CSL + OMW	29.5 ± 0.4	51.6 ± 2.8
Control	S	60.5 ± 0.8	9.1 ± 2.4
	CSL	48.6 ± 0.6	10.0 ± 2.0
	CSL + OMW	41.8 ± 0.6	11.3 ± 1.5

The cell-free supernatants obtained from the different culture media studied (S, CSL, and CSL + OMW) were able to recover about 60% of the crude oil present in the samples, whereas in the control assays, the corresponding uninoculated culture media recovered around 10% of crude oil (Table 6). In order to compare the performance of rhamnolipids produced by *B. thailandensis* E264 in the different

culture media with commercial rhamnolipids (RL-90, 90% of purity), oil recovery assays were performed using those rhamnolipids dissolved in the same uninoculated culture media at a concentration of 200 mg/L (similar to the rhamnolipid concentration present in the cell-free supernatants of *B. thailandensis* (Table 4)). The commercial rhamnolipids herein studied were characterized in our previous work as a mixture of two congeners: Rha-C_{10}-C_{10} (relative abundance 68%; HLB value 10.1), and Rha-Rha-C_{10}-C_{10} (relative abundance 32%; HLB value 12.3) (Gudiña et al. 2016). Oil recoveries around 50% were obtained in all the cases (Table 6). According to the results obtained, it can be concluded that rhamnolipids produced by *B. thailandensis* E264 displayed a good performance in oil recovery.

Discussion

In flask assays using S medium, which contains glycerol, peptone, and meat extract, *B. thailandensis* E264 produced 139 mg rhamnolipids/L. Contrary to previous studies that reported an extended stationary phase (216–264 h), associated to a continuous rhamnolipid production in *B. thailandensis* E264 (Funston et al. 2016; Irorere et al. 2018), in our case, the highest rhamnolipid production was achieved after 96 h of growth; after that, the surface tension of the culture medium increased, probably due to the degradation of the rhamnolipids previously produced (Fig. 1). Similarly to the results herein obtained, Funston et al. (2017) also reported that only 50% of the glycerol provided in the culture medium (40 g/L) was consumed by *B. thailandensis* E264 after 264 h of growth (Funston et al. 2017).

CSL and OMW proved to be good substrates for rhamnolipid production by *B. thailandensis* E264, achieving titers between 1.3 and 1.8 times higher than with the standard medium. The best results were obtained using a culture medium containing CSL (7.5% (v/v)) and OMW (10% (v/v)). The inductive effect of OMW on rhamnolipid production herein observed was previously reported for *P. aeruginosa*, and it was attributed to the presence of long-chain fatty acids (mainly oleic, palmitic, linoleic, and stearic acids) in this substrate, which can be used for rhamnolipid biosynthesis through the β-oxidation pathway, in addition to those provided by the fatty acid de novo synthesis (FAS II) (Gudiña et al. 2016). In *B. thailandensis*, it was suggested that the main supplier of lipid precursors for rhamnolipid biosynthesis is FAS II, although the β-oxidation pathway also contributes with a small percentage (less than 3%) (Irorere et al. 2018). Consequently, further studies are necessary to elucidate the effect of exogenous fatty acids on rhamnolipid production by *B. thailandensis*.

The higher rhamnolipid titer obtained in the CSL + OMW medium could also be explained by the effect of the different carbon sources in rhamnolipid production. In *B. thailandensis* E264 and *Burkholderia glumae* AU6208, rhamnolipid production significantly increased when the carbon source was changed from glycerol to canola oil (Costa et al. 2011; Dubeau et al. 2009). The authors suggested that rhamnolipid production in water-soluble carbon sources, such as glycerol, is generally lower than in water-immiscible substrates, such as vegetable oils (Costa et al. 2011). That can be explained because rhamnolipids reduce the interfacial tension between water and water-immiscible substrates, making them more readily available for uptake by the microorganism.

Rhamnolipid production by *B. thailandensis* E264 using the low-cost medium (CSL + OMW) was further validated in the bioreactor (Fig. 3). To the best of our knowledge, only a previous study evaluated rhamnolipid production by *B. thailandensis* E264 using an alternative carbon source (used cooking oil) in a bioreactor, producing 2.2 g rhamnolipid/L over 120 h in a 10-L bioreactor (Kourmentza et al. 2018). However, the minimum ST values obtained in that work (around 38 mN/m) were considerably higher than the ones herein presented (26.3 mN/m), and the methodologies used to purify the rhamnolipids produced were also different (as it will be discussed later), which does not allow to compare the results obtained in both works.

The chemical characterization revealed that *B. thailandensis* E264 produced mainly di-rhamnolipids when grown in S and CSL media (Rha-Rha-C_{14}-C_{14}: 62–64%; Rha-Rha-C_{12}-C_{14}/C_{14}-C_{12}: 18–22%) (Table 2). These results are in accordance with those obtained in previous studies, where the di-rhamnolipid Rha-Rha-C_{14}-C_{14} was the main congener produced by *B. thailandensis* E264 using glycerol as carbon source (between 40 and 82% of total rhamnolipid congeners) (Diaz de Rienzo et al. 2016; Dubeau et al. 2009; Elshikh et al. 2017; Funston et al. 2017; Irorere et al. 2018). However, when the CSL medium was supplemented with OMW, the relative abundance of mono-rhamnolipids increased from 10 up to 56% (Table 2). These results suggest that the addition of fatty acids to the culture medium results in the production of higher amounts of mono-rhamnolipids. A similar trend was observed by Irorere et al. (2018) growing *B. thailandensis* E264 in a mineral medium containing as carbon source glycerol (6% mono-rhamnolipids) or heptadecanoic acid (23% mono-rhamnolipids), although the effect of oleic acid was less evident (9% mono-rhamnolipids) (Irorere et al. 2018). Likewise, when growing *B. thailandensis* E264 in a medium containing olive mill pomace (OMP, another residue from the olive oil industry) as carbon source, only mono-rhamnolipids were produced, mainly Rha-C_{14}-C_{14} (Chebbi et al. 2021). In *B. glumae* AU6208, the relative abundance of mono-rhamnolipids was 2% when grown in glycerol, and 23% with canola oil (Costa et al. 2011). The authors suggested that glycerol favors di-rhamnolipid production because its carbon atoms are more readily

incorporated into rhamnose. An increase in the relative proportion of mono-rhamnolipids (from 53 to 70%) was also reported for *P. aeruginosa* #112 when a culture medium containing CSL + sugarcane molasses was supplemented with OMW (Gudiña et al. 2016).

Rhamnolipids produced by *B. thailandensis* E264 in the different culture media herein studied displayed lower CMC values (48–108 mg/L) (Table 4; Fig. 4) when compared with those reported for the same strain in similar media in previous studies (125–225 mg/L) (Diaz de Rienzo et al. 2016; Dubeau et al. 2009; Elshikh et al. 2017). The low CMC values obtained in our study, paired with ST reductions down to 26 mN/m, indicate that these rhamnolipids have a greater degree of purity than in other cases. This can be due to the different methodologies used to recover them. In previous works, rhamnolipids produced by *B. thailandensis* were recovered through solvent (ethyl acetate) extraction, followed by a solid-phase (silica column) purification (Chebbi et al. 2021; Diaz de Rienzo et al. 2016; Dubeau et al. 2009; Funston et al. 2016; 2017; Irorere et al. 2018; Kourmentza et al. 2018). In our case, rhamnolipid extraction and purification were performed using a methodology commonly used to recover the rhamnolipids produced by *P. aeruginosa*, and the impurities present in the culture medium were removed using increasing concentrations of methanol, allowing the recovery of a product with a higher degree of purity.

The surface-active properties of rhamnolipids are determined by their chemical structure (size of their hydrophilic head, length of the hydrophobic chains, and presence of unsaturated bonds), which also affects their solubility in either aqueous or oil phases (Jahan et al. 2020). Mono-rhamnolipids, because they only have one rhamnose molecule, are more hydrophobic than di-rhamnolipids with fatty acid chains of the same length, and they usually exhibit lower CMC values and are able to reduce the ST more efficiently than di-rhamnolipids (Costa et al. 2011; Gudiña et al. 2015a; Rodrigues et al. 2017). Accordingly, the differences observed in the surface activity of the rhamnolipids produced in the different culture media (Tables 2 and 4; Fig. 4) can be explained by their different congener distributions.

Other low-cost substrates have been studied as carbon sources for rhamnolipid production by *B. thailandensis*, such as non-fermented grape marcs (a winery residue) (Chebbi et al. 2021) and used cooking oil (Kourmentza et al. 2018), with production titers of 1.07 and 2.20 g rhamnolipid/L, respectively. However, the rhamnolipids produced in those media exhibited higher CMC values (500 and 225 mg/L) and minimum ST values (34.6 and 37.7 mN/m) than the ones achieved in this work. OMP was also evaluated as a low-cost substrate for rhamnolipid production by *B. thailandensis* E264 (Chebbi et al. 2021). After thermal and acid pre-treatments, OMP (2% (w/v)) was used as carbon source, yielding 270 mg rhamnolipid/L. However, as in the previous examples, those rhamnolipids exhibited a higher CMC (500 mg/L) and lower surface activity (reduced the ST of water up to 38 mN/m) when compared with the ones obtained in the present work.

Rhamnolipids produced by *B. thailandensis* E264 in S and CSL media exhibited an excellent emulsifying activity (Table 5). To the best of our knowledge, the emulsifying activity of purified rhamnolipids produced by *B. thailandensis* was not previously reported, and only in some cases, it was studied in the rhamnolipid-containing cell-free supernatants. However, the emulsifying activity was determined for the purified rhamnolipids produced by *B. glumae* in glycerol-containing medium, where the distribution of rhamnolipid congeners was similar to the ones obtained for *B. thailandensis* in S and CSL media (Costa et al. 2011). Similarly to the results herein presented, those rhamnolipids exhibited a good emulsification potential, displaying E_{24} values ranging from 44% with hexadecane to 100% with canola and motor oil.

The cell-free supernatants from cultures of *B. thailandensis* E264 performed in S, CSL, and CSL + OMW media recovered between 60 and 63% of heavy crude oil from contaminated sand. Although rhamnolipids produced in CSL + OMW medium displayed different congener distributions and different HLB values when compared with those produced in the other media (S and CSL), all of them proved to be effective in oil recovery. Regarding the commercial rhamnolipids, they allowed the recovery of approximately 50% of crude oil (Table 6), slightly lower than rhamnolipids produced by *B. thailandensis*. Although commercial rhamnolipids, which were produced by *P. aeruginosa*, contained a high percentage of mono-rhamnolipids (even higher than those produced by *B. thailandensis* in CSL + OMW medium), their HLB values were similar to those produced in S and CSL media, due to their shorter fatty acid chains (C_{10}).

Biosurfactants produced by different microorganisms (*Bacillus methylotrophicus*, *Bacillus subtilis*, *Bacillus tequilensis*, *Candida lipolytica*, *Pseudomonas cepacia*, *Wickerhamomyces anomalus*) recovered between 20 and 80% of crude oil or motor oil in similar assays (Bezza and Chirwa 2015; Chaprão et al. 2018; Ciurko et al. 2022; Datta et al. 2020; Gaur et al. 2022; Gudiña et al. 2015b; Rufino et al. 2013; Soares da Silva et al. 2017; Teixeira Souza et al. 2018). In a recent study, the cell-free supernatants of cultures of *B. subtilis* #309 performed in low-cost media (sunflower and rapeseed cake), containing between 1.2 and 1.4 g surfactin/L, recovered between 14 and 22% of motor oil from artificially contaminated sand after 24 h of treatment, increasing up to 30–33% after 168 h (Ciurko et al. 2022). Purified surfactin (200 mg/L) produced by *B. tequilensis* MK 729,017 and commercial rhamnolipids (RL-90, Sigma-Aldrich, 90% of purity, 200 mg/L)

recovered 80% of crude oil ($\eta_{30\ °C} = 13.6$ mPa s) from artificially contaminated sand after 48 h of treatment, slightly lower than the recoveries obtained with the chemical surfactants SDS (2350 mg/L, 86%) and CTAB (335 mg/L, 88%) (Datta et al. 2020). Detergent formulations containing commercial rhamnolipids (RL-90, Sigma-Aldrich, 90% of purity, 200 mg/L) recovered 91% of waste engine oil from contaminated sand after 3 h of treatment, while those containing purified rhamnolipids produced by *P. aeruginosa* gi (200 mg/L) allowed recoveries around 82% (Gaur et al. 2022). The cell-free supernatants of cultures of *P. cepacia* CCT6659 recovered 84% of motor oil from contaminated marine stones, whereas the recovery obtained with the purified biosurfactants produced by the same microorganism (3000 mg/L, 5 × CMC) was 72% (Soares da Silva et al. 2021).

Comparing with these reports, the results herein obtained proved to be satisfactory. Furthermore, in this study, a heavy crude oil ($\eta_{40\ °C,\ 1.4\ s\text{-}1} = 110$ mPa s) was used, which is more difficult to recover due to its viscosity. Moreover, the biosurfactants were used without purification as cell-free supernatants, which would significantly reduce the production costs associated to their application. Consequently, the results obtained are promising for the application of the rhamnolipids produced by *B. thailandensis* in bioremediation or MEOR, which, to the best of our knowledge, was not previously studied.

In conclusion, low-cost culture media, containing as sole ingredients agro-industrial residues (CSL and OMW), were developed for rhamnolipid production by *B. thailandensis* E264. These media allowed the production of higher amounts of rhamnolipids (175–269 mg/L) when compared with the standard medium (139 mg/L). Rhamnolipids produced in the low-cost media reduced the surface tension up to 27 mN/m (CMC 51 mg/L). These results were further validated in the bioreactor. Besides reducing the rhamnolipid production costs, these results can allow the development of strategies for the valorization of environmentally hazardous residues and promote the circular economy. Furthermore, the results obtained in the oil recovery assays, where these rhamnolipids (cell-free supernatants) recovered more than 60% of crude oil from contaminated sands, open the possibility for its application in microbial enhanced oil recovery and bioremediation.

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Author contribution JC, EJG, and JAT conceived and designed the study. JC, EJG, ZL, and TJ conducted the experiments. JC and EJG wrote the manuscript. ZL, TJ, and JAT reviewed the manuscript. All authors read and approved the manuscript.

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Data availability Data will be made available on request.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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