Exploiting the Biocatalytic Potential of Marine Bacteria

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Abstract

Marine bacteria are often under extreme conditions of temperature, pressure, salinity and depletion of nutrients. To survive, these microorganisms developed mechanisms of adaptation and produce biologically active compounds. This work aimed to assess and exploit the potential of marine bacteria to produce interesting compounds for biotechnological processes.

Strains isolated from samples harvested in harbour of Ponta Delgada (Azores) and Aveiros beach in Albufeira (Algarve) were identified by their fatty acid profile using the Sherlock® Microbial ID System. The identified isolates from Azores were screened to produce biosurfactants and lipases. Among the tested strains, *Bacillus pumilus* and *Micrococcus luteus* were found to be potential biosurfactant and lipase producers, respectively. *B. pumilus* produced biosurfactants and exopolysaccharides able to reduce superficial tension of supernatant until 21.3 mN/m and 12.4 mN/m by using *n*-hexadecane and raffinose as carbon source, respectively. Furthermore, *B. pumilus* degraded *n*-alkanes while changing the cell membrane to overcome substrate toxicity, which suggests that this bacterium is suitable for bioremediation. *M. luteus* produced extracellular lipases with enzymatic activities of 0.09 and 0.06 U/mL in presence and absence of the tributyrin, respectively.

The adaptation of marine bacteria from Aveiros beach to different NaCl concentrations (5, 35, 50, 100 and 150 g/L) was studied. The results showed that almost all bacteria could grow until 150 g/L by adapting their fatty acids of cell membrane, especially in the presence of 100 and 150 g/L of salt.

Keywords: marine bacteria, screening, biosurfactants, lipases, fatty acids, bacterial adaptation

1. Introduction

The oceans represent a source of a varied type of organisms due to the several environments offered by the different oceanic zones [1]. The ecological resources offered by the seas have been explored since ancient times by man, from the use of marine animals such as fish, and algae preparations for medicine, to compounds with suitable properties to be used in pharmaceutical products [1]. However, the oceans and seas, that occupy around 70% of Earth, continue largely undiscovered [2]. It is estimated that there are 10^9 taxa of bacteria on the Earth with 10^6 of bacterial taxa from the oceans [3].

With the advancement and improvement of techniques, like culturing technique, small ribosomal RNA (rRNA) analysis and metagenomics approach, it is possible to study marine microbial diversity [4].

Marine bacteria can be found in all oceans, e.g. from the seafloor to fish stomachs, and they are present in a wide variety of habitats, like deep-sea sediments, seamounts and sometimes outside of algal cells [5], and they can survive on more diverse environmental conditions than terrestrial bacterial owing to ocean currents that can transport these organisms substantial distances [2]. Marine environments are considered one of the most adverse due to fluctuations in pH, salinity, temperature, sea surface temperature, pressure, radiation, tides, precipitation regimes and sea level rise [2, 6]. The ability of marine bacteria to endure and utilize the resources found in these habitats has an important role of distribution of bacteria in the sea [6].

Relatively to the functions performed by marine microorganisms: they are responsible for the uptake of large part of the carbon dioxide that human society emits into the atmosphere by fixation; they are the basis of the ocean food web due to fix some of the dissolved inorganic carbon and provide the particulate and dissolved organic carbon [7, 8]. Nevertheless, the survival mechanisms developed by the marine bacteria have made them a promise source for new biologically active substances with potential uses in industrial processes, medicine, food and bioremediation [5].

Some reports refer that many heterotrophic bacteria are known to carry genetic and metabolic potential to synthesize and control extracellular enzymes, which can modify and degrade many natural polymers in water basins [2]. Other important bioactive compounds produced by marine microbes include exopolysaccharides (EPS), biosurfactants and antibacterial compounds [2].

The aim of this study was to assess and exploit the potential of marine bacteria to produce interesting compounds for biotechnological processes.

2. Material and Methods

2.1. Identification of marine bacteria

The strains used in this work were isolated from samples collected (i) at the harbour of Ponta Delgada, Azores (AZ1-C16), as described by de *Rodrigues et al.* (2017) [9], (ii) and at Aveiros beach, Albufeira, Algarve (AV-SALT). The sample

from Aveiros beach used in the present study was collected in a small pool way from the seawater where the water had evaporated, and a salt deposited could be collected. The samples were diluted in water and then plated under different conditions to promote the isolation of cultures in a previous study performed in the IBB-IST. The cells from the sampling site of the Azores, were isolated following an enrichment culture approach by using hexadecane as carbon source, and the cells from the Algarve were isolated using for selection the media tryptic soy agar (TSA; Sigma-Aldrich®) and sea salts with glucose as carbon source. The strains used in the present study were already isolated and deposited in a bacterial library at IBB-IST.

The cells were identified using the Sherlock® Microbial ID System (MIS) from MIDI (Newark, USA). Each isolated colony grown on TSA plate was incubated in a Memmert incubator at 30ºC for 24 ± 1 h. After this time, cells were harvested to a 1.5 mL Eppendorf tubes (Eppendorf, Hamburg, Germany) and the fatty acids (FAs) of the cells were extracted and simultaneously methylated to fatty acid methyl esters (FAMEs) using the Instant FAME method from MIDI. The FAMEs were analysed on a 6890N gas chromatograph from Agilent Technologies (Palo Alto CA), with a flame ionisation detector and a 7683 B series injector, equipped with a 25 m long Agilent J&W Ultra 2 capillary column. The Sherlock® software package (version 6.2) allowed bacterial identification based on the FAME profile of each bacterium using the ITSA1 method. Principal component analysis of the FAMEs profiles using the 2D plot function of the software was used to study relationships between the large number of isolated strains [9].

2.2. Screening for compounds of industrial interest

2.2.1. Production of biosurfactants

The cells identified of collection AZ1-C16 were harvested from agar plates to 1.5 mL Eppendorf tubes and resuspended in 1 mL of mineral medium (MM). The composition of MM used is described by de Cortes *et al.* (2015) [10].

To each well of a 96 deep-well microplate (Thermo Fisher Scientific, USA), 500 µL of MM, 50 µL of cell suspension and a given amount of hexadecane were added. On rows A, B and C, *n*-hexadecane (Merck-Schuchardt, Germany) was added to reach a concentration of 0.25 %(v/v), while on rows D, E and F, the amount was added to reach 0.50% (v/v). The last two rows, G and H, contained *n*-hexadecane at a concentration of 1% (v/v). The microplate was incubated at 30ºC and 200 rpm in an Agitorb 200 incubator (Aralab, Portugal).

To assess the production of biosurfactants, 100 µL of supernatant of each culture was removed after 3 days of cell growth. The cell-free supernatant was obtained by centrifugation of 96 deep-well microplate at 3,500 rpm for 5 min (Eppendorf centrifuge 5810 R). The supernatant was transferred to a new 96-well microtiter plate (Sarstedt, UK) and optical distortion was evaluated as described by de Walter *et al.* (2010) [11]. To help this evaluation, millimetric paper was placed under the microtiter plate.

2.2.2. Production of lipases/esterases

To assess the production of lipases and/or esterases, the cells were grown on 12 cm x 12 cm squared agar plate using as principal carbon and energy source tributyrin (Carl Roth, Germany). The composition of the agar medium was the following (per litre): 20 g of agar, 5 g of NaCl (Carl Roth, Germany), 3.5 g of yeast extract, 10 mL of 100x concentrated mineral medium and 10 mL of 100x concentrated phosphate buffer (to reach the concentrations mentioned in 2.2.1) and 10 mL of tributyrin. The plate was inoculated using sterile toothpicks and incubated at 30ºC during seven days. At least 28 isolates were tested. Lipase/esterase activities were determined by visualization of “halos” surrounding colonies with active enzymes.

2.3. Exploring the potential of *Bacillus pumilus*

2.3.1. Bacterial strain

One of the isolates from Ponta Delgada’s harbour was identified by the Sherlock® MIS as *Bacillus pumilus* (strain 3 AZ1-C16). Since this species produce biosurfactants [12] and presented enzymatic activities [13], it was selected for further studies.

2.3.2. Screening for biosurfactant production

Cells grown in two 100 mL Erlenmeyer flasks, containing 20 mL of MM with 35 g/L NaCl and 0.25% (v/v) of *n*-hexadecane. In one of the flasks, MTBE (Fluka) was added. The flasks were inoculated with cells previously grown under the same conditions but with MM without salt. After 24 h of growth, 15 mL from each Erlenmeyer were transferred to two 15 mL Falcon tubes (Thermo Fisher Scientific, USA), and the tubes were centrifuged at 4,500 rpm for 10 min in a Sigma Laborzentrifugen 2-15 centrifuge (B. Braun) to harvest the supernatant.

To assess the presence of biosurfactants, the superficial tension of ca. 15 mL of cell-free culture supernatants were measured in a K8 tensiometer from Krüss GmbH (Hamburg, Germany), using the ring method and superficial tension of distilled water was used as reference value.

2.3.3. Degradation of *n*-alkanes

2.3.3.1. Growth conditions

To evaluate the growth of *Bacillus pumilus* on *n*-alkane, dry weight of the cells was determined and the cell adaptations at the lipid level were assessed by FAME analysis.

Pre-inocula were prepared by growing cells in 20 mL of marine broth (MB) at 30ºC and 200 rpm in 100 mL Erlenmeyer flasks. Assays were carried out at least in duplicate. After ca. 24 h of growth, optical density of pre-inocula was measured, and all volume of one Erlenmeyer was collected and divided to two 15 mL Falcon tubes. The tubes were centrifuged at 1,500 rpm for 10 min (Sigma Laborzentrifugen 2-15 centrifuge) and supernatant was discharged. The pellet was resuspended in 3 mL of MM with 35 g/L NaCl, to wash the cells and remove the nutrients present in MB and centrifuged again under the same conditions. The supernatant was removed, and the pellet was resuspended in 3 mL of MM with

35 g/L NaCl. Growth on C5 to C17 *n*-alkanes was carried out in twenty-six 15 mL Falcon tubes (thirteen tubes for dry weight and another thirteen tubes for FAMEs analysis) containing 3 mL of MM with salt and 10 µL of one of the *n*-alkanes tested. The tubes were inoculated with an initial optical density of ca. 0.13 (100 µL of pre-inocula) and incubated at 30ºC and 200 rpm.

The *n*-alkanes used were the following: *n*-pentane (99%; Merck), *n*-hexane (>99%, Fisher Scientific, UK), *n*-heptane (99%, Panreac), *n*-octane (>99%; Merck-Schuchardt), *n*-nonane (99%; Acrós Organics), *n*-dodecane (99%; Acrós Organics), *n*-decane (>99%; Merck-Schuchardt), *n*-undecane, *n*-tridecane, *n*-tetradecane, *n*-hexadecane and *n*-heptadecane (99%; Sigma-Aldrich®) and *n*-pentadecane (≥98%; Sigma-Aldrich®).

2.3.3.2. Fatty Acid analysis

Cells were harvested after seven days of growth on each alkane by centrifugation at 5,000 rpm for 5 min (Eppendorf centrifuge 5810 R). The supernatant was discharged, the pellet was resuspended in 1 mL of distilled water, to wash the cells, and centrifuged again under the same conditions. The supernatant was removed. The cell lipids were extracted, and the FAs were simultaneously methylated to FAMEs using the Instant FAME method from MIDI as described in section 2.1, but identified by the PLFAD1 method of Sherlock® software v. 6.2. The degree of saturation of FAs of the cell membrane was defined by the ratio between the saturated straight chain FAs and mono-unsaturated FAs.

2.3.3.3. Dry weight cell measurements

At the end of the growth, 3.30 mL of culture were transferred to 15 mL Falcon tubes previously weighted. The tubes were centrifuged for 5 min at 5,000 rpm (Eppendorf centrifuge 5810 R). The supernatant was removed and the falcon tubes were dried at 65ºC for 24 h. After cooling to room temperature, the Falcon tubes containing the cells were weighted to determine the weight of the dried cell biomass.

2.3.4. Degradation of paraffin

2.3.4.1. Preparation of paraffin solution

In a 100 mL Erlenmeyer flask a suspension of paraffin was prepared, containing 0.5016 g of paraffin in 65 mL of MM. The suspension was autoclaved during 20 min at 121ºC. After cooling, and in sterile environment, 194.6 mg of surfactant produced by *Rhodococcus erythropolis* DCL14 were added, and the suspension was homogenized with the help of agitation. Afterwards, the suspension was sonicated (Bandelin, Sonopuls, Labometer) with the following conditions: power = 50 W, working 9 s on and 3 s off during 15 min. To help the homogenization of paraffin in the suspension, the suspension was heated in a microwave.

To precipitate paraffin particles with the form of spheres, the solution was stirred in an ice bath for 15 min. The morphology of the particles were observed in an optical microscope (Olympus CX40) so it and their particle size was measured by dynamic light scattering (Zetasizer Nano ZS, version 7.12, Malvern Instruments Ltd.) using a plastic cuvette with 2 mL of Milli-Q water and 20 µL of suspension.

2.3.4.2. Growth conditions

Pre-inocula were prepared by growing cells in 20 mL of MB at 30ºC and 200 rpm. Once the cells reached the exponential phase, which was determined by monitoring the optical density at 600 nm in spectrophotometer, 5 mL of each pre-inoculum were added to two 15 mL Falcon Tubes under sterile condition to recover the cells. The tubes were centrifuged at 6,000 rpm for 10 min (Eppendorf centrifuge 5810 R). The supernatant was discharged, the pellet was resuspended in MM to wash the cells and remove the nutrients present in MB and the cell suspension was centrifuged again at 6,000 rpm for 5 min. This procedure was repeated one more time under the same conditions. After the last removal of supernatant, the pellet was resuspended in MM and the optical density of cell suspension was measured at 600 nm.

To each well of 6-well plates (Sarstedt, UK), MM, paraffin to reach a concentration of 5%, 10% and 20% (v/v), and cell suspension to reach an initial optical density of ca. 0.2 were added. The total volume for each well was 4 mL and in each 6-well plates there were an assay for control and another for cells for each concentration of paraffin. Four 6-well plates were prepared: one to collect samples at 0 h (only controls) and another three plates for collection at 24, 48 and 120 h. The 6-well plates were incubated at 30ºC and 200 rpm.

2.3.4.3. Determination of paraffin consumption

To determine the concentration of paraffin consumed, paraffin was extracted from suicidal samples with 2 mL of *n*-hexane and the paraffin consumption over time was evaluated by gas chromatography-mass spectrometry.

Peak identification of paraffin hydrocarbons was confirmed by injecting both standard and selected samples on a gas chromatograph Agilent 7820A equipped with an autosampler and an Agilent 5977E quadrupole mass spectrometer. The capillary column was an Ultra-2 (25 m x 200 µm x 0.33 µm) from Agilent which worked at a constant flow of 1.5 mL/min. The mass detector was at 200ºC and the mass transfer line was set at 280ºC. The GC injector was set at 1:20 split mode and 250ºC.

2.3.4.4. Fatty acid analysis

The extraction of bacterial lipids of pre-inoculum was made according to the steps described in 2.3.3.2. For the remaining samples, after 24 h, 48 h and 120 h of growth, the Eppendorfs were centrifuged during 5 min at 10,000 rpm, the supernatant was removed, and the pellet was resuspended in 1 mL of Milli-Q water to wash the cells. The Eppendorfs were centrifuged again under the same conditions, the supernatant was discharged and the extraction of FAs from the cells was performed as described in section 2.3.3.2.

2.3.5. Production of EPS using sugars as carbon sources

2.3.5.1. Growth conditions

The cells of pre-inocula were grown in 100 Erlenmeyer flasks containing 20 mL Mueller-Hinto broth (MH) at 30ºC and 200 rpm in an Agitorb 200 incubator. The growth on inocula was carried out in cylindrical 200 mL closed flasks containing 40 mL of MH broth and 10 g/L of sugar. The initial optical

density was ca. 0.2. The extent of growth was monitored by measuring of the optical density at 600 nm using a spectrophotometer until 120 h of growth.

The sugars used in this work were glucose and sucrose from Fisher Chemical and galactose, arabinose, raffinose, mannose and xylose from Sigma-Aldrich®.

2.3.5.2. Extraction of EPS

To extract the EPS from the different cultures, 5 mL of medium culture were collected to 5 mL Eppendorf tubes and they were centrifuged at 8,000 rpm for 10 min (Eppendorf centrifuge 5810 R). The supernatant was placed on pre-weighted 15 mL Falcon tubes. The falcon tubes were placed on ice vessel, and 5 mL of absolute ethanol was added in order to precipitate the EPS. The tubes stayed overnight at 4ºC. To recover the EPS, the tubes were centrifuged at 6,000 rpm for 10 min and the supernatant was discharged. To determine the dry weight of EPS, the tubes were incubated at 65ºC. This procedure was repeated until 96 h of growth.

2.3.5.3. Dry weight cell measurements

At the same time as the samples of point 2.3.5.2 were collected, 1 mL of medium culture was harvested for dry cell weight to pre-weighted 1.5 mL Eppendorf tubes. The sample was centrifuged at 10,000 rpm for 5 min (Sigma 1-15 P sartorius). The supernatant was then removed, the pellet was resuspended in 1 mL of Milli-Q water to wash the cells, and centrifuged under same conditions. The supernatant was discharged and the eppendorfs were dried at 65ºC for 24 h. After cooling to room temperature, the eppendorf containing the cells were weighted to determine the weight of the dried cell biomass.

2.3.5.4. Superficial tension measurements

The superficial tension of cell-free supernatants (samples of 120 h) was measured in a K8 tensiometer from Krüss GmbH. The superficial tension of distilled water was used as reference value.

2.4. Exploring the potential of *Micrococcus luteus*

2.4.1. Bacterial strain

One of the isolates from Ponta Delgada's harbour identified by the Sherlock® MIS as *Micrococcus luteus* (12 AZ1-C16*)*. Since this species usually present interesting enzymatic activities [14,15], the strain was selected for further studies.

2.4.2. Impact of media composition on the production of extracellular lipase/ esterase activity

Pre-inocula was carried out in 100 mL Erlenmeyer flasks containing 20 mL of MH broth. The flasks were inoculated with 1 mL of exponentially growing cells and incubated at 30ºC and 200 rpm. The media used were MH, MB, Thioglycollate broth with resazurine (Thio) and Tryptic Soy Broth (TSB). Growth was monitored by measuring the optical density at 600 nm in a spectrophotomer. All assays were carried out in duplicate. After about 24 h, all volume of each flask was collected into a 50 mL Falcon tube (Sarstedt, UK), and they were centrifuged at 8,600 rpm for 10 min (Eppendorf centrifuge 5810 R). 10 mL of supernatant were transferred to 15 mL Falcon Tubes and analysed to assess the extracellular enzymatic activity using 4-nitrophenyl butyrate (*p*-NPB) as substrate. The activity of free lipases/esterases was determined by their capability to hydrolyse *p*-NPB into *p*-nitrophenol (*p*-NP) and butyric acid. In a cuvette of 3 mL, 200 µL of supernatant, 15 µL of substrate and 1,285 µL of 20 mM Tris HCl buffer, pH 8, were added to reach a final volume of 1.5 mL [16]. The enzymatic activity was measured at 410 nm in an HITACHI U-200 spectrophotometer. A mixture of 20 mM Tris HCl buffer (1,485 µL) and *p*-NPB (15 µL) was used as negative control to assess the existence of a chemical reaction. One enzyme unit (U) was defined as the amount of enzyme that converts 1 µmol of *p*-NP per min.

2.4.3. Inducing extracellular lipase/esterase activity by addition of tributyrin

To assess the influence of tributyrin in extracellular lipase/esterase activity, bacterial growth was promoted on 100 mL Erlenmeyer flasks, containing 20 mL of MH broth with tributyrin and without tributyrin. The flasks were inoculated with an exponential growing culture to an initial optical density of ca. 0.2 and incubated at 30ºC and 200 rpm. All assays were carried out at least in duplicate. After 24 h of growth, the supernatant of both cultures was collected to determine the extracellular enzymatic activity as described in 2.4.2.

2.5. Tolerance of marine bacteria from Aveiros-Algarve to salt

2.5.1. Bacterial strain

A strain from AV-SALT, identified by Sherlock® MIS, was used in this test, that was *Bacillus licheniformis* (32 AV-SALT).

2.5.2. Growth conditions

Cell growth was carried out in 100 mL Erlenmeyer flask containing 20 mL of TSB. The flasks containing TSB at different concentrations of salt (5, 35, 50, 100, and 150 g/L) which were inoculated to reach an initial optical density of ca. 0.2 at 30ºC and 200 rpm for 120 h. Growth was monitored by measuring the optical density at 600 nm in a spectrophotomer.

2.5.3. Fatty acid analysis

To assess the effects of different concentrations of salt in cell membrane, 1 mL was taken from medium culture to do FA analysis. The steps of this procedure were described in section 2.1. The unsaturation index (UI) of cell membrane was defined as the sum of the percentage of each unsaturated fatty acid multiplied by the number of double bonds in the molecule [17].

3. Results and Discussion

3.1. Screening for biosurfactant production

To assess if bacteria from the harbour of Ponta Delgada produced biosurfactants, a microplate assay was applied, where the optical distortion of the grid caused by altered superficial tension of culture supernatants was evaluated. The

bacteria used in this screening test were the bacteria identified by Sherlock® MIS from MIDI. Therefore, strains from AZ1-C16 grown in MM with 0.25, 0.50 and 1.00% (v/v) of *n*-hexadecane as carbon source. The MM with *n*-hexadecane (0.25, 0.50 and 1.00% (v/v)) and without *n*-hexadecane were used as control.

The results suggested that the strain 3 AZ1-C16, that was previously identified as *Bacillus pumilus* produced biosurfactants due to the distortion presented in the grid assay. Furthermore, in the literature was reported that the marine strain *B. pumilus* KMM 1364, isolated from the surface of ascidian *Halocynthia aurantium*, produced a mixture of lipopeptide surfactin in the presence of medium with salts, yeast extract, $FeSO_4$, Tris buffer, artificial seawater and distilled water [18]. Another study with the strain *B. pumilus* AAS3 isolated from the Mediterranean sponge *Acanthella acuta*, synthesized a diglucosyl-glycerolipid when grown on artificial seawater medium supplemented with yeast extract, glucose and nitrogen or phosphate sources [19].

3.2. Exploring the potential of *Bacillus pumilus*

3.2.1. Biosurfactant production

B. pumilus was identified in previous works as promising biosurfactant producer [18,19]. To confirm this ability, *B. pumilus* was grown in two different media (*vide* 2.3.2.) and after 24 h of growth, superficial tension of supernatant was measured.

Table 1 – Superficial tension for different cultures of *B. pumilus* after 24 h of growth and water as control.

Supernatant	Superficial tension (mN/m)
Water	77.0
MM with 35 g/L NaCl + 0.25% (v/v) C16	21.3
MM with 35 g/L NaCl + 0.25% (v/v) C16 + 0.25 % (v/v) MTBE	55.8

Table 1 shows that *B. pumilus* produced biosurfactants because the superficial tension of the supernatants was lower than the control (water). Besides that, effect of MTBE on biosurfactant production was also evaluated, and it was verified that MTBE did not favour extracellular production of the active surface compound. In another investigation with *B. pumilus* (CCT 2487) from the Tropical Foundation of Research and Technology, André, was reported that this bacterium could produce biosurfactants in the presence of 0.01 g/L of medium rich in mineral salts with 0.1 g/L yeast extract and 1% (v/v) of diesel. This medium was also supplemented with 1% to 5% total solids of vinasse and waste frying oil [12]. The lowest superficial tension for both substrates reached the value of 45 mN/m [12]. Production of biosurfactants by another *Bacillus* sp. was also observed. *B. megaterium* and *B. subtilis* from Tuticorin harbour, India, were isolated from sea water samples and after 7 days of growth in mineral salt medium with 1.0% (w/v) of crude oil, cell free culture broth of both bacteria presented superficial tensions of 30.8 mN/m and 38.75 mN/m, respectively [20].

3.2.2. Degradation of *n*-alkanes

To assess if *B. pumilus* could use alkanes as carbon and energy sources, this strain was grown in MM with 35 g/L NaCl and in the presence of *n*-alkanes, from *n*-pentane (C5) to *n*-heptadecane (C17), during seven days. The cell growth in each *n*-alkane was evaluated through biomass dry weight and the adaptations in cell membrane were verified by FA analysis.

In the dry weight of biomass analysis (data not shown), it was possible to verify that *B. pumilus* grew better in the presence of alkanes with long chain, especially in C16. The reason of this growth in C16 may due to the fact that *B. pumilus* (3 AZ1-C16) has been isolated in *n*-hexadecane.

With the aim of understanding the differences observed in the lipid composition of the cells in presence of *n*-alkanes, the FA were analysed (figure 1). FA were grouped into the different classes according to their chemical structure: saturated straight chain (SSFAs), mono-unsaturated (MUFAs), poly-unsaturated (PUFAs), saturated *iso*- and *anteiso*-methyl branched (BCFAs), 10-metyl branched (10MBFAs), saturated cyclopropyl-branched (SCBFAs) and dimethylacetals (DMAFAs).

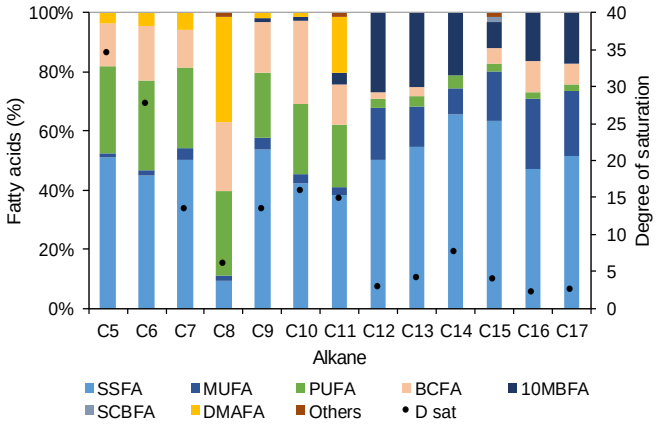

Figure 1 – Fatty acid composition of *B. pumilus* cells in the presence of C5-C17 n-alkanes after seven days of growth.

The composition of the cell membrane changed significantly from alkanes with small carbon chain (C5-C11) to long chain alkanes (C12-C17). The group of SSFAs constituted between 41.1% (C10) and 59.9% (C14) of the cell membrane, except for *n*-octane that reached the value of 9.26%. On other hand, MUFAs increased for alkanes with long chain (ca. 2.5% to 15.6%), which leads to a decrease in the degree of saturation. The degree of saturation decreased considerably from alkanes with small carbon chain to long chain alkanes, except in case of C8 alkane. In the C8 alkane, the percentage of SSFAs in cell membrane decreased significantly and then the degree of saturation also decreased.

The PUFAs decreased from ca. 25.9% in *n*-alkanes with small carbon chain to ca. 2.7% in long chain alkanes. The presence of PUFAs in greater amounts in C5-C11 alkanes, may be a form of the cells to maintain a suitable membrane fluidity [17]. Furthermore, the presence of BCFAs on cell membrane was higher in *n*-alkanes with small carbon chain (ca. 18.0%) than in long chain alkanes (ca. 4.4%), while the amount of 10MBFAs, presented an increase from ca. 0.9% in small carbon chains to ca. 17.6% for in long chain alkanes. These results suggest that BCFAs and 10MBFAs promote the fluidity of cell membrane when cells grow in *n*-alkanes with small chain and long chain, respectively [21]. Other reason to verify these differences between the groups of alkanes C5-C11 to C12-C17 is that some bacteria are able to accumulate

saturated and unsaturated straight long-chain FAs into cellular lipids during the cultivation under nitrogen-limiting conditions and, as well to other FAs derived from β-oxidation pathway [22]. In this case, when the cells grew on C12 up to C17 *n*-alkanes incorporated C14:0, C15:0 and C16:0 into their membrane phospholipids with a high percentage compared to other FAs. The results showed that these FAs had 45.3% in *n*-tetradecane, 45.6% in *n*-pentadecane and 33.2% in *n*-hexadecane of the cell membrane, respectively. It was reported in a study with *Rhodococcus erythropolis* that these cells grew on C13-C16 *n*-alkanes and then usually incorporate saturated FAs into their cell membrane, corresponding to the chain length of the substrate used for cell growth [23]. Other study with *Alcanivorax borkumesis* SK2 showed that generally FAs with a chain length of C14 to C18 are incorporated into the membrane, specially the C16:0 (palmitic acid) [24].

The results presented in figure 1 also suggest that the bacterium has changed its lipid composition as response to *n*-alkane, being visible the difference between C5-C11 to C12-C17 *n*-alkanes. In presence of *n*-octane, cell membrane changed significantly when comparing with other *n*-alkanes with small chain.) Chen, Janssen and Witholt (1995) reported that during growth on *n*-octane (C8), the accumulation of 1-octanol, a metabolite of the alkane monooxygenase, can be toxic to the cells [25], which means that C8 could have caused a toxic effect in the cells and to contrast this effect, cell membrane triggered some mechanisms of adaptation [23].

3.2.3. Degradation of paraffin

The microbial degradation of paraffin wax has been studied under the scope of the problems associated to oil industry and as an alternative to mechanical and chemical methods of paraffin degradation that are environmentally problematic [26].

In the present study, the efficiency of *B. pumilus* for degrading paraffin wax in different concentrations (5, 10 and 20%) was evaluated in terms of alkane degradation because this bacterium showed to produce biosurfactants that possibly solubilize hydrocarbons of alkanes. The adaptation of the cell membrane to paraffin was also assessed. The particle size of paraffin used in this work was measured by dynamic light scattering with at an angle 173º, having obtained an average size of 289 nm after sonication.

The analysis of consumption of paraffin wax (data not shown) was performed by gas chromatography, where the peak area of each *n*-alkane was determined for the samples collected at 0 h (control) and also after 24, 48 and 120 h (cells). The results showed that the paraffin consumption was higher when the concentration that was placed on medium culture was higher, except on the concentration of 10%, where it was verified more paraffin in 48 h than 24 h or 120 h. The reason for this was that suspension of paraffin is not totally well homogenised and the concentration present in medium culture could be more than expected. Therefore, experimental errors may have occurred when the paraffin suspension was placed in the culture media.

At 5% paraffin, almost the same quantity of paraffin was observed after 48 h and 120 h of growth, which can suggests mass transfer limitation between cells and particles of paraffin. Velankar *et al*. (1975) reported that when the cell surface is adjacent to the hydrocarbons, the transport of hydrocarbons to the cell surface is more quickly [27]. This concentration of paraffin can be considered relatively low and hydrocarbon surface area can be limiting, especially because *B. pumilus* grows in suspension and forms aggregates in MM. At a concentration of 10% paraffin, the same situation also happened.The consumption of paraffin at a concentration of 20% was ca. 1.4 times faster than at 10%, if the analysis is done until 120 h, because at the end of this time the peak area was similar for these two concentrations. This result suggests that the cell growth might be limited by the amount of substrate available. However, the monitoring time at 10% and 20% paraffin, should had been extended to verify if the degradation of paraffin could be complete. In conclusion, *B. pumilus* can degrade paraffin, so it could be a potential microorganism to do the biodegradation of long chain like alkanes.

To assess the influence of paraffin on cell membrane during growth (figure 2), it was analysed the FA composition at 0 h (control) and 24 h, 48 h and 120 h (cells).

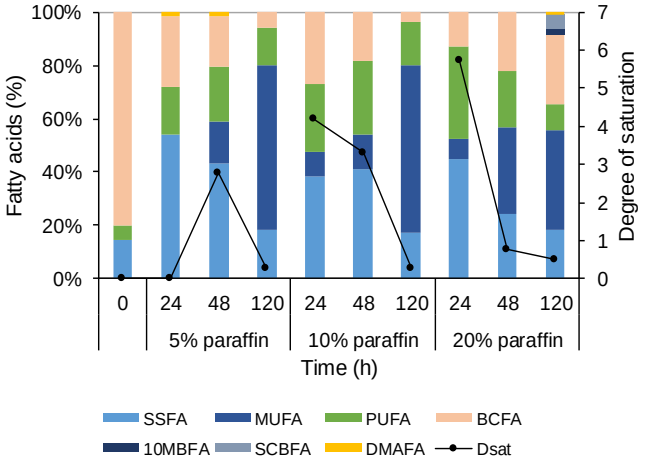

Figure 2 – Fatty acid composition of *B. pumilus* cells in the presence of different concentrations of paraffin after 24 h, 48 h and 120 h.

The FAs of cell membrane changed along time of growth. After 24 h of growth, the cells produced more ca. 32.3% of SSFAs and ca. 28.0% of PUFAs for all concentrations of paraffin tested. Relatively to MUFAs, cells produced ca. 9.1% and 7.8% for the 10% and 20% concentrations of paraffin when comparing to the control (0 h).

From 24 h to 120 h, SSFAs decreased ca. 36.3, 21.5 and 18.0% for the paraffin concentrations of 5, 10 and 20%, respectively, and increased the MUFAs in ca. 59.5, 51.8 and 46.0% for the paraffin concentrations of 5, 10 and 20%, respectively. These results led to a decrease of degree of saturation, which means that the cell membrane became more flexible with time [28].

Furthermore, the PUFAs decreased ca. 4.3, 10.0 and 20.3% from 24 h to 120 h of growth for the paraffin concentrations of 5, 10 and 20%, respectively.

Regarding BCFAs, this class of FAs decreased ca. 21.3 and 23.2% until 120 h of growth for 5% and 10% paraffin concentrations and increased ca. 24.7% from 24 h until 120 h in the paraffin concentration of 20%. The FAs that triggered these results was C15:0 iso and C15:0 anteiso. BCFAs affect the membrane fluidity due to the disruptive effect of the methyl group on acyl-chain packing, being that anteiso FAs promote a more fluid membrane [29]. After 120 h of growth in paraffin concentration of 20%, 10MBFAs constituted ca. 3.1% of cell membrane, and SCBFA and DMAFA represented 8.1% and 1.5% of cell membrane, respectively.

3.2.4. Production of EPS using sugars as carbon source

To assess the production of EPS by *B. pumilus*, this bacterium grew in different carbon sources: glucose, galactose, arabinose, sucrose, raffinose, manose and xylose. The effect of carbon source in the production biomass and EPS along time was evaluated and the potential of EPS to reduce the superficial tension of the supernatant was analysed (table 2).

The growth of *B. pumilus* was monitored by measuring the absorbance for each carbon source. The growth curves (data not shown) showed that the exponential phase of each growth ended after ca. 20 h for all carbon sources, except for xylose, and after this time, the growth curves tended to stationary phase. In case of xylose, the end of stationary phase was verified after ca. 48 h of growth.

The production of EPS occurred along time in all carbon sources tested. These compounds were quantified by dry weight. The maximum production of EPS was registered between 48 h and 72 h of growth, depending on the carbon source. This result showed that the production of EPS occurred essentially in stationary phase. After 96 h of growth, the production of EPS was lower than 0.1 g/L, which may suggest that in the absence of substrate, the cells consume the EPS produced by them. This time coincided with the stationary phase of growth or with the final of stationary phase depending on the carbon source used. The highest production of EPS (9.52 g/L) was noted when raffinose was used as carbon source and after 48 h of growth. This value represented almost 5.6 more times higher than the lowest production registered that was for glucose (1.70 g/L).

On the literature, marine bacterium *Saccharophagus degradans* was reported to have EPS productions of 1.5 g/L for glucose, 0.75 g/L for galactose, 0.9 g/L for sucrose and 0.8 g/L for xylose after 48 h of growth and using a concentration of 200 g/L of carbon source solution [30]. On the other hand, deep-sea bacterium *Zunongwangia profunda* SM-A8 showed the EPS production in presence of 30 g/L of carbon source, like glucose and sucrose. The results obtained for EPS production was 1.3 g/L for glucose and 1.6 g/L for sucrose after 144 h of growth [31]. Comparing these values with the obtained in this study, *B. pumilus* produced higher amounts of EPS with lower concentration of carbon source.

Table 2 – The effect of different substrates on superficial tension of EPS produced by *B. pumilus*.

Supernatant	Superficial tension (mN/m)
Water	77.0
Glucose	48.9
Galactose	27.8
Arabinose	14.5
Sucrose	33.3
Raffinose	12.4
Mannose	24.7
Xylose	23.9

In this study, a decrease in superficial tension of cell free culture was observed for all carbon sources tested after 120 h of culture. When comparing with the control, there are reductions in superficial tension of supernatant in ca. of 36.5% and 83.9% when cells used glucose and raffinose as carbon sources, respectively. Results also revealed that superficial tension reduction was greater with arabinose and raffinose. So, all carbon sources favoured the extracellular production of active surface agent by *B. pumilus*.

In the literature, it was reported a study with *Bacillus subtilis* HOB2 that produced EPS with the ability to reduce the superficial tension to 28 mN/m and 27 mN/m, using as carbon sources, glucose (10 g/L) and sucrose (10 g/L), respectively [32]. These results was registered after 48 hours of growth [32]. Other study with isolates from terrestrial and marine samples collected in areas contaminated with crude oil or its byproducts, presented cell free-cultures with a superficial tension between 37 and 42 mN/m after growth in 20 g/L of glucose for 120 h [33]. With these results, it can be concluded that EPS produced by *B. pumilus* did not reduce the superficial tension as other EPS produced by other microorganisms and when compared with the same carbon sources. However, *B. pumilus* was able to reduce the superficial tension below the values described using another substrate.

3.3. Screening for lipases/ esterases activities

In the present study, lipase/ esterase producing bacteria were screened from Harbour of Ponta Delgada, Azores. This screening was done using agar plates with medium containing as principal carbon and energy source the substrate, tributyrin or tween 80. The formation of opaque zones around the colonies, denominated by halos, is an indicator of the hydrolytic activity of the of the enzymes present in the cells. The diameter of colony formed after 24 hours and after seven days was considered, since diameter is indicative of the activity of the enzymes present in the cells. Higher enzymatic activities should lead to bigger halos surrounding colony of bacteria.

After 24 h of growth, visible halos surrounding colony of bacteria were observed in the tributyrin agar plate. For further studies, the strain 12 AZ1-C16 was selected because of the growth that this bacterium presented in tributyrin agar plates and the halo surrounding this colony. Besides that, this isolate was identified by Sherlock® MIS as *Micrococcus luteus*.

3.4. Exploring the potential of *Micrococcus luteus*

3.4.1. Analysis of enzymatic activity of supernatant in different culture media

Micrococcus luteus from Ponta Delgada harbour, Azores, was cultivated in different media cultures in order to do an evaluation of the presence of lipases/esterases in the supernatant and to assess which medium induced the production of larger amounts of extracellular lipases/esterases. The activity of free lipases/esterases was determined spectrophotometrically using *p*-NPB as substrate [16].

Table 3 – Enzymatic activity of extracellular lipase/esterase of *M. luteus* grown on different culture media after 24 h of growth.

Medium	Lipase/esterase activity (U/mL)
MH	0.06 ± 0.004
Thio	0.03 ± 0.005
MB	0.03 ± 0.006
TSB	0.04 ± 0.001

In this study, the supernatants of all culture medium of *M. luteus* presented extracellular lipase/esterase activity. The highest enzymatic activity was verified in MH culture medium, with 0.06 U/mL. A study carried out with strains of *Halobacteria* from an Algerian culture displayed diverse lipolytic activities with *p*-NPB, being that the highest lipolytic activity obtained was 0.02 U/mL and the lowest was 0.0018 U/mL when this strain grew in Gibbons medium [34]. In other study, halophilic archaeal isolates that grew in SG medium presented lipase activities between 0.02 and 0.09 U/mL depending on isolate and pH of medium [35]. Other study reported that lipases found in strain LAMA 582 (*Bacillus aerophilus*) from deep-sea have extracellular activities of ca. 0.03 and 0.04 U/mL when the strain grew in marine broth 2216 [36]. So, the extracellular activity achieved with *M. luteus* is in the same range than other microorganism studied.

3.4.2. Production of lipases/ esterases in presence and absence of tributyrin

To assess if the extracellular lipases/esterases activity could be induced by tributyrin, *M. luteus* was cultivated in MH with and without tributyrin. MH medium was chosen to this study, because in the previous point (3.4.1.), the extracellular lipase activity was higher in this medium.

Table 4 – Enzymatic activity of extracellular lipase/esterase of *M. luteus* grown on MH media with and without tributyrin after 24 h of growth.

Medium	Lipase/esterase activity (U/mL)
MH with tributyrin	0.09 ± 0.01
MH without tributyrin	0.06 ± 0.01

In the presence of tributyrin, *M. luteus* increase the extracellular lipase/esterase activity. In literature, it was reported that the extracellular lipase from *Pseudomonas aeruginosa* KM110 from wastewater of an oil processing plant located in Tehran, presented an enzymatic activity in presence of tributyrin of ca. 0.06 U/mL [37]. However, this strain displayed greater results when using other carbons sources, like olive oil (0.46 U/mL) [37]. These results suggest that extracellular lipase/esterase activity of *M. luteus* may be improved using other substrates.

3.5. Tolerance of 32 AV-SALT to different salinities

The bacterium 32 AV-SALT was cultivated under different salinity (5, 35, 50, 100 and 150 g/L) in TSB medium, to assess the influence of different NaCl concentrations on bacterial growth and cell membrane. The growth was followed by optical density measurements and the adaptation of cell membrane was evaluated by analysis of fatty acid profile.

The effect of salinity on the cell growth and growth rate is shown in figure 3.

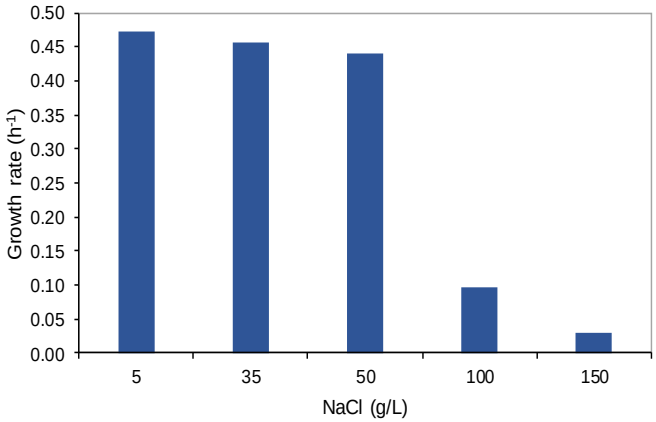

Figure 3 – Effect of different NaCl concentrations on growth rates of 32 AV-SALT.

Figure 3 shows that the maximum growth rate (0.47 h^{-1}) was verified for the lowest concentration of NaCl. This result was verified because the pre-inoculum of this bacterium has grown in the presence of 5 g/L of this salt. Then the adaptation to the new medium was easier for this concentration when compared with others. Furthermore, the growth rate just decreased when the salt concentration reached 100 g/L, which means that this bacterium is halotolerant to higher concentrations of salt.

As bacterium 32 AV-SALT grew better in 5 g/L than the higher concentrations, and the average concentration of salt in sea water is ca. 35 g/L, this result suggest that the bacteria present in this study may not be strictly halophilic.

To investigate the adaptative response of the cells to different concentrations of sodium chloride, the FA compositions were analysed, and UI was calculated (figure 4).

The concentrations of NaCl that allowed the highest growth rates of 32 AV-SALT cells (5, 35 and 50 g/L), induced a lower extent of changes in cell membrane than higher concentrations (100 and 150 g/L). The higher the salt concentration, the lower the percentage of BCFAs produced by 32 AV-SALT cells. This percentage decreased from ca. 85.3% in 5 g/L to ca.64.3% in 150 g/L of NaCl.

The sodium chloride concentrations tested induced modifications in the fatty acid profile after 6 h of growth, where in the lowest concentrations of salt, cell membrane stabilized at the end of this time. For the highest concentrations, fatty acid profile changed until 24 h of growth in case of 100 g/L, but in case of concentration of 150 g/L, cell membrane presented changes until 120 h. In the presence of 100 g/L NaCl, an increase of SSFAs and MUFAs were observed.

When compared with the lowest concentrations of NaCl (5, 35 and 50 g/L of NaCl), SSFAs increased from ca. 11.5% to 17.2% and MUFAs decreased from ca. 0% to 0.7%, respectively. An increase of SSFAs and decrease of BCFAs, from 50 g/L to 150 g/L, means a reduction of membrane fluidity and permeability [29]. These changes occur because cell membrane has the ability to prevent the release of solutes in the presence of high salinity in order to control the osmotic

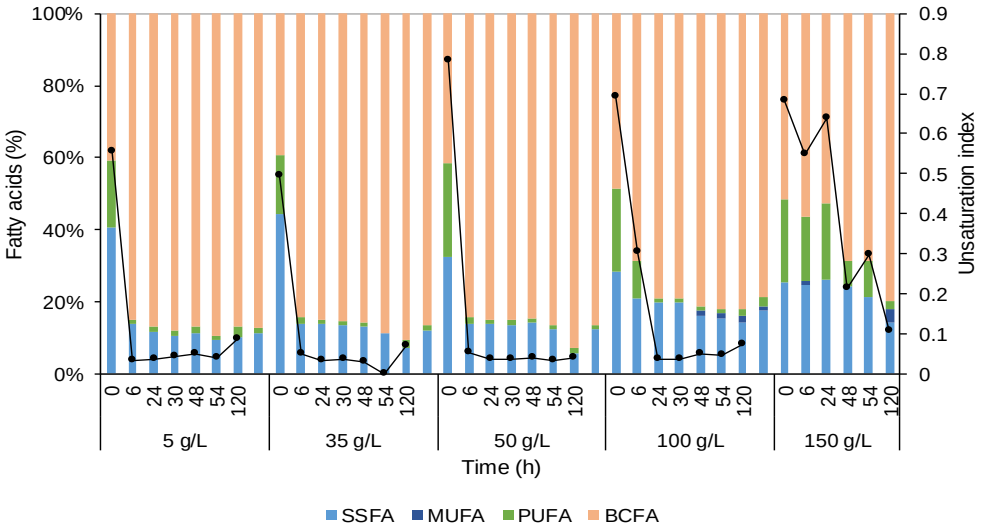

Figure 4 – Fatty acid composition of 32 AV-SALT cells exposed to different concentrations of NaCl (bars) and corresponding calculated unsaturation index (line).

pressure [38] and they were observed until the end of exponential phase. Other group of FAs that changed along time was PUFAs. These FAs increased in cell membrane from ca. 17.5% at 0 h for the salt concentrations of 5 and 35 g/L to ca. 24% for the remaining concentrations and for the same time. However, for the concentrations 100 and 150 g/L of salt, PUFAs did not decrease as in the lowest concentrations of NaCl. The presence of PUFAs in great amounts at higher salinities probably allowed the maintenance of cytoplasmatic membrane fluidity [17] and represented one of the most important adaptations under salt stress. Furthermore, UI decreased after 6 h of growth in presence of 5, 35 and 50 g/L of salt to ca. 0.3 and maintained this value in the remaining time of study. For the higher concentrations of salt, the stabilization of UI was observed after 24 h of growth for 100 g/L, but in the concentration of 150 g/L, UI decreased until 120 h. These results also showed a slower reduction of UI for higher concentrations of salt, which indicates that cell membrane had more difficulties to adapt and to overcome the problem, increased unsaturated FAs [17].

In the literature, there are some reported cases of bacteria that adapted their cell membrane with the increase of salinity, such as genus *Micrococcus* from Antarctica, which increased the amount of long FAs while decreased the iso chain and increased straight chain fatty acids [29] and *Rhodococcus erythropolis* which synthetized PUFAs, being this response is very uncommon for mesophilic bacteria [17].

4. Conclusions

The screening methods allowed to select two marine strains from harbour of Ponta Delgada, Azores, with suitable properties to produce compounds to be used in biotechnological processes: the strains #3 AZ1-C16 and #12 AZ1-C16, previously identified as *B. pumilus* as *M. luteus*, respectively.

In the presence of mineral medium with 35 g/L of NaCl and 0.25% of *n*-hexadecane, *B. pumilus* produced a biosurfactant with the ability to reduce the superficial tension until to 21.3 mN/m. *B. pumilus* also produced EPS using sugars as carbon sources. These EPS presented the ability to reduce the superficial tension (14.5-48.9 mN/m), being that could be applied in biotechnological applications of a day-to-day life, such as, cosmetic and food industry.

The biodegradation of *n*-alkanes and paraffin wax could be also performed by *B. pumilus*. The results showed that this marine microorganism can degrade hydrocarbon compounds, especially *n*-alkanes with long carbon chain (C12-C17) and paraffin at concentrations between 5 and 20% (v/v) in mineral medium. Furthermore, *B. pumilus* adapted its cell membrane to the composition of the medium, by changing the FAs, especially the MUFAs, PUFAs and BCFAs. So, as *B. pumilus* could degrade hydrocarbons, this strain can be applied in processes of bioremediation, such as, of crude oil.

Relatively to *M. luteus*, this microorganism showed extracellular lipase activity in different media, but in larger quantities in MH broth. This lipase activity can be enhanced by the addition of tributyrin to the medium culture that induced the enzymatic activity of lipases from 0.06 U/mL to 0.09 U/mL.

In the study with bacterium 32 AV-SALT, from Albufeira – Algarve, this strain showed to be halotolerant until 150 g/L. This bacterium could grow in high concentrations of salt due to adaptations of their cell membrane. Moreover, this bacterium may not be strictly halophilic because the cells grew better in the lowest concentrations of salt, especially in the concentration of 5 g/L.

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