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Exopolysaccharide production by the marine bacterium *Alteromonas macleodii* Mo169 using fruit pulp waste as the sole carbon source

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ABSTRACT

A sugar-rich apple pulp waste generated from fruit processing for juice production was used as the sole carbon source for the cultivation of *Alteromonas macleodii* Mo169, a marine bacterium known for its EPS-secreting ability. The strain efficiently utilized the glucose and fructose present in the apple pulp waste, reaching biomass and EPS production of 9.20 ± 0.61 and 3.51 ± 0.08 g L^{-1}, respectively, in 24-hour bioreactor cultivation. Two high molecular weight (Mw) fractions (1.7 ± 0.0 and 0.74 ± 0.0 MDa) were detected in the sample recovered from the cell-free supernatant by dialysis. The compositional analysis revealed the presence of glucose (31.1 ± 0.2 mol%), arabinose (23.9 ± 0.1 mol%), mannose (17.3 ± 0.1 mol%), glucosamine (10.3 ± 0.5 mol%), galactose (8.7 ± 0.0 mol%) and galacturonic acid (8.7 ± 0.0 mol%), as well as a high content in sulphate (6.0 ± 0.5 wt%). Given the presence of a high Mw polysaccharide in the apple pulp waste, probably pectin, a fraction of the detected sugar monomers might be attributed to that polymer, which was recovered together with *A. macleodii* Mo169 EPS. Concomitant with EPS synthesis, there was a viscosity build-up in the cultivation broth, which developed a shear-thinning fluid behaviour not observed in the initial medium. Therefore, this study demonstrates that apple pulp waste can be efficiently converted into a novel polysaccharide by *A. macleodii* Mo169 in a sustainable bioprocess. Moreover, the EPS sugar and acyl composition, together with its good thickening capacity, render the biopolymer of interest for use in several applications.

1. Introduction

Owing to their wide ecological and environmental characteristics, marine habitats are the widest source of biological and chemical diversity, including not only unexplored and/or unknown microorganisms, but also new biomolecules

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with unique features and properties that might find extensive biotechnological applications (Casillo et al., 2018; Finore et al., 2014). Among those biomolecules, marine bacterial polymers, such as exopolysaccharides (EPS), have emerged as innovative biomaterials with significant industrial importance (Sahana and Rekha, 2019). Marine EPS exhibit high diversity in terms of structure and composition and often possess biological activity, exploitable by the pharmaceutical and medical industries (Casillo et al., 2018; Concórdio-Reis et al., 2021). Moreover, their improved thickening, stabilizing, gelling, and/or emulsifying capacities support the application of marine EPS in a variety of industries, such as the food, pharmaceutical, cosmetics, or petroleum industries (Casillo et al., 2018; Roca et al., 2016).

Despite the potential of marine EPS, these polysaccharides represent only a small fraction of the current polymer's market, mainly due to the low productivity and high production costs (Casillo et al., 2018; Roca et al., 2015). EPS yield is greatly influenced by the fermentation conditions, including the carbon source (Casillo et al., 2018; Finore et al., 2014; Poli et al., 2010). Besides, the commonly used carbon sources, such as glucose, fructose, or sucrose, also represent a high fraction of the overall production expenses (Freitas et al., 2009; Pereira et al., 2021; Roca et al., 2015). Therefore, exploring novel and less expensive carbon sources for the cultivation of EPS-producing marine bacteria becomes crucial. In this perspective, agro-industrial wastes and by-products are relevant alternative feedstocks to produce these value-added macromolecules (Joulak et al., 2022). Agro-industrial wastes such as cheese whey, grape pomace, glycerol by-product from the biodiesel industry, pretreated sugar beet molasses, and starch molasses were already explored as feedstocks for the cultivation of marine bacteria belonging to the genus *Halomonas* (Küçükaşik et al., 2011; Joulak et al., 2022). The use of these wastes as substrates resulted in an overall higher EPS production in comparison with the reference carbon source (Küçükaşik et al., 2011; Joulak et al., 2022). Nonetheless, the knowledge of EPS synthesis by marine bacteria using alternative carbon sources and wastes is still quite limited (Finore et al., 2014).

Over the past few years, the generation of wastes and byproducts from the fruit juice industry has been increasing, representing, nowadays, 20%–60% (w/w) of processed fruit (Pereira et al., 2021). Upon apple juice manufacturing, 25%–35% (w/w) of the apple mass is not used (Pereira et al., 2021). The fruit pomace, mainly composed of peels, pulp, and seeds, has a content of simple sugars of 69–93 g L^{-1} that includes glucose (17–23 g L^{-1}) and fructose (29–53 g L^{-1}) (Pereira et al., 2021; Rebocho et al., 2019). Given their high sugar content, the wastes generated by the apple juice industry (which are discarded with associated costs) might be a valuable feedstock for marine bacteria to produce different microbial products, including EPS.

Alteromonas macleodii Mo169 was isolated from a giant clam collected in Moorea Island lagoon, French Polynesia (Concórdio-Reis et al., 2021). When cultivated in a glucose-enriched medium, this strain produced an EPS with the potential for utilization in different industrial fields, particularly in the food, pharmaceutical, biomedical, and cosmetic industries (Concórdio-Reis et al., 2021). The EPS had a high content of sulphate (2.8 wt%) and was mainly composed of (1→4)-linked glucuronic acid (39.3 mol%), mannose (12.8 mol%), glucose (11.2 mol%), and galactose (4.0 mol%) (Concórdio-Reis et al., 2021). *A. macleodii* Mo169 EPS had interesting rheological properties, namely thickening and weak gel-forming capacities that were rapidly recovered after exposure to either high shear rates or temperatures up to 95 °C (Concórdio-Reis et al., 2023). Additionally, the EPS could form hydrogels in the presence of copper (II) and iron (III) (Concórdio-Reis et al., 2021).

Envisaging a more cost-effective EPS production process, in this study, *A. macleodii* Mo169 was cultivated using apple pulp waste, a low-cost carbon source. To the best of our knowledge, this is the first time such feedstock is used for the cultivation of an *Alteromonas* strain. The study aimed at evaluating the strain's ability to utilize this feedstock as a substrate for cell growth and EPS synthesis. First, shake flask experiments were performed for cultivation medium selection, which was then used for bioreactor cultivation using apple pulp waste as a carbon source. Cell growth and EPS production were evaluated, and the produced polymer was characterized in terms of chemical composition and molecular mass distribution.

2. Materials and methods

2.1. Apple pulp processing

Apple pulp waste was supplied by SUMOL+COMPAL, S.A. (Portugal). For the cultivation experiments, the apple pulp waste was processed as described by Pereira et al. (2021). The waste was diluted with deionized water (3:1, v/v) for viscosity reduction, centrifuged (13 000 × g, 15 min, 4 °C) for the removal of insoluble solids, and autoclaved (121 °C, 1 bar, 30 min) for sterilization.

2.2. Characterization of the sugar-rich apple pulp supernatant

The diluted apple pulp supernatant was characterized in terms of viscosity, density, pH, conductivity, total dry mass, elemental composition, and the content of salts, ammonium, phosphate, sugars, acyls, and high molecular weight soluble compounds. The sample's apparent viscosity was determined at room temperature using a viscometer (Fungi Lab S.A., Alpha series, Spain). The density was determined gravimetrically by weighing three different volumes (10, 20, and 30 mL) of the sample. The total dry mass was determined gravimetrically by freeze-drying the sample (∼5 g) and weighing the obtained dry material. For the moisture content determination, the sample (∼5 g) was subjected to a temperature

of 100 °C and weighed once a constant weight was attained. The moisture content was calculated as the difference in mass before and after the treatment (Antunes et al., 2017). Afterward, the dried sample was placed at a temperature of 550 °C for 24 h, and the inorganic salt content was determined gravimetrically by weighing the resulting ashes. Elemental analysis was performed on the freeze-dried sample using an elemental analyser (Thermo Finnigan-CE Instruments, Flash EA 1112 CHNS series, Italy). Ammonium and phosphate concentrations were determined by colorimetry using a flow segmented analyser (Skalar 5100, Skalar Analytical, The Netherlands). Standard solutions of phosphorus (KH_2PO_4) and ammonium (NH_4Cl) were prepared at concentrations between 4 and 20 mg L^{-1}.

Sugars, organic and inorganic acyls were quantified by liquid chromatography (HPLC) after controlled hydrolysis of the EPS, as previously reported by Concórdio-Reis et al. (2020). Briefly, the lyophilized samples were dissolved in deionized water (1 g L^{-1}) and hydrolysed with 2% (v/v) trifluoroacetic acid (TFA, 99%), at 120 °C, for 2 h. The hydrolysate was used for the identification and quantification of the constituent monosaccharides by HPLC using a Carbopac PA10 column (Thermo Scientific™ Dionex™, Sunnyvale, CA, USA) equipped with an amperometric detector. Glucose (99%, Fluka), galactose (99%, Fluka), arabinose (99%, Sigma), fructose (99%, Scharlau), and sucrose (99%, Fluka), were used as standards (1 to 100 ppm). The acid hydrolysates were also used for the quantification of acyl groups by HPLC with an Aminex HPX-87H 300 × 7.8 mm column (Biorad, Hercules, CA, USA) coupled to a UV detector (210 nm). Acetate, pyruvate, and succinate (Sigma-Aldrich) were used as standards at concentrations between 0.015 and 1.0 g L^{-1}. Sulphate concentration in the hydrolysates was determined by HPLC using a Thermo Ionpac AS9-HC 250 × 4 mm column and a Thermo Ionpac AG11HC column (Thermo Scientific™ Dionex™, Sunnyvale, CA, USA), equipped with a conductivity detector. The analysis was performed at 25 °C, using sodium acetate (8 mM) at a flow rate of 1 mL min^{-1}.

For determination of the sample's high molecular weight (Mw) compounds content, a sample (5 mL) of the apple pulp supernatant was dialysed with a 12 kDa MWCO (molecular weight cut-off) membrane (ZelluTrans/Roth) against deionized water at room temperature, under constant stirring. The dialysis water was changed frequently and the efficiency of the process (i.e., removal of low molecular weight compounds) was monitored by measuring the conductivity of the dialysis water until it reached a value below 10 μS cm^{-1} (48 h). When this conductivity was reached, the sample was lyophilized and weighed for the determination of the content in high molecular weight compounds. The lyophilized sample was analysed in terms of chemical composition and molecular mass distribution. The monosaccharide and acyl composition were determined as described above. The number and average molecular weight (Mn and Mw, respectively) of the sample, as well as the polydispersity index (PDI, Mn/Mw), were determined by Size Exclusion-High Performance Liquid Chromatography (SE-HPLC). The analysis was performed at 25 °C on a KNAUER Smartline HPLC equipped with a Phenomenex Phenogel Linear LC Column 300 × 7.8 mm (USA), using 0.1 M $LiNO_3$ as eluent, at a flow rate of 0.6 mL min^{-1}. The sample (50 μL) diluted in the eluent was injected, and a Water 2414 Refractive Index Detector was used for detection. The values of Mw and Mn were calculated using a calibration curve generated with pullulan standards (P50 to P80) (Paz-Samaniego et al., 2015).

2.3. Microbial cultivation

2.3.1. Microorganism

Alteromonas macleodii Mo169 (CNCM I-5374), isolated from Moorea Island lagoon, French Polynesia (Concórdio-Reis et al., 2021) was used in all assays. For inoculum preparation, an isolated colony was inoculated into a 250 mL baffled shake flask containing 100 mL of marine 2216 medium (Millipore, Merck KGaA) (Oppenheimer and Zobell, 1952; Zobell, 1941) supplemented with glucose (30 g L^{-1}, 99%, Fluka). After incubation for 24 h at 30 °C in an orbital shaker (200 rpm), the culture (10%, v/v) was used as inoculum for the shake flask and bioreactor experiments.

2.3.2. Shake flask assays: fructose as the sole substrate

The experiments were performed in 500 mL baffled shake flasks containing 200 mL of marine 2216 medium (Oppenheimer and Zobell, 1952; Zobell, 1941) supplemented with 30 g L^{-1} of fructose (99%, Scharlau). Glucose (30 g L^{-1}, 99%, Fluka) was also used as the standard reference carbon source. The flasks were incubated at 30 °C in an orbital shaker (200 rpm) for 24 h. Samples were collected at times 0 and 24 h for cell dry weight (CDW) and EPS quantification.

2.3.3. Shake flask assays: cultivation media

The marine 2216 medium (Oppenheimer and Zobell, 1952; Zobell, 1941), previously used for the cultivation of this bacterium (Concórdio-Reis et al., 2021), was not compatible with the cultivation using the sugar-rich apple pulp (precipitation occurred). Therefore, this medium was used as a standard reference in the shake flask experiments conducted to select a medium suitable for supplementation with apple pulp waste. The following four media, supplemented with glucose (30 g L^{-1}), were tested:

(1) modified Schatz medium containing (g L^{-1}): NaCl, 30; KH_2PO_4, 1; NH_4NO_3, 1; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2 \cdot 2H_2O$, 0.01 (Fondi et al., 2015).

(2) modified semi-complex medium (SCM) composed of (g L^{-1}): NaCl, 30; $MgCl_2 \cdot 6H_2O$, 13; $MgSO_4 \cdot 7H_2O$, 9; KCl, 1.3; $CaCl_2 \cdot 2H_2O$, 0.2; NaBr, 0.15; $NaHCO_3$, 0.05; yeast extract, 0.3; and peptone, 0.2 (Joulak et al., 2022).

(3) salted Medium E* with the following composition (per litre): NaCl, 30 g; $(NH_4)_2HPO_4$, 3.3 g; K_2HPO_4, 5.8 g; KH_2PO_4, 3.7 g; 10 mL of a 100 mM $MgSO_4$ solution and 1 mL of a micronutrients' solution. The micronutrients solution was

composed of (per litre of 1 N HCl): $FeSO_4{\cdot}7H_2O$, 2.78 g; $MnCl_2{\cdot}4H_2O$, 1.98 g; $CoSO_4{\cdot}7H_2O$, 2.81 g; $CaCl_2{\cdot}2H_2O$, 1.67 g; $CuCl_2{\cdot}2H_2O$, 0.17 g; and $ZnSO_4{\cdot}7H_2O$, 0.29 g (Concórdio-Reis et al., 2018).

(4) liquid A1 medium containing per litre (750 mL natural seawater (Naturitas, Spain) and 250 mL deionized water): yeast extract, 4 g; peptone, 2 g (Roca et al., 2016).

The pH value of all media was adjusted to 7.6 with 1 M NaOH before autoclaving. The experiments were performed in 500 mL baffled shake flasks containing 200 mL of the appropriate medium and were performed in an orbital shaker, as described above. Samples were collected at times 0 and 24 h for CDW and EPS quantification.

2.3.4. Bioreactor cultivation

A. macleodii Mo169 was cultivated using salted Medium E* supplemented with the sugar-rich apple pulp supernatant, obtained as described above. The bioreactor cultivation was initiated by inoculating 200 mL of culture grown in salted medium E* supplemented with glucose (30 g L^{-1}). The cultivation was conducted in a BioStat B-Plus bioreactor (Sartorius, Germany) with a working volume of 2 L. A constant aeration rate (2 standard litres per minute) was kept during the cultivation, and the dissolved oxygen concentration was controlled at 40% of the air saturation by the automatic variation of the stirring speed (200–1200 rpm). The bioreactor was operated at 30 ± 0.2 °C, and the pH was maintained at 7.6 ± 0.1 by the automatic addition of NaOH 5 M. Foam formation was controlled by the automatic addition of Antifoam A (Sigma-Aldrich). Samples (15 mL) were withdrawn during the cultivation runs for the culture's broth apparent viscosity measurement, and for the quantification of the CDW, carbon sources, ammonium, phosphate, and EPS.

2.3.5. Analytical techniques

The samples' viscosity was determined at 25 °C using a MCR92 modular compact rheometer (Anton Paar, Graz, Austria) equipped with a cone-plate geometry (angle 2°, diameter 35 mm, 0.145 mm gap). Culture broth samples were centrifuged (13 000 × g, 15 min, 4 °C) for cell separation. Samples with high viscosity were diluted in deionized water (1:2 or 1:3, v/v) before centrifugation for viscosity reduction. The cell-free supernatant was used for the quantification of the carbon source, ammonium, and EPS. For CDW determination, the bacterial pellets obtained by centrifugation were washed twice with a 30 g L^{-1} NaCl solution (resuspension in the solution followed by centrifugation at 13 000 × g and 4 °C, for 15 min), lyophilized, and weighed. Glucose and fructose concentrations in the cell-free supernatant were determined by HPLC with a VARIAN Metacarb column (BioRad) coupled to an infrared (IR) detector. The analysis was performed at 50 °C, using H_2SO_4 (0.01 N) as eluent at a flow rate of 0.6 mL min^{-1}. Glucose and fructose were used as standards at concentrations between 0.016 and 1.0 g L^{-1}. Ammonium concentration was determined as described above. The EPS was quantified by dialysis of the cell-free supernatant, as described above for the determination of high molecular weight compounds in the apple pulp supernatant.

2.3.6. Kinetic parameters

The maximum specific cell growth rate μ_{max} (h^{-1}) was calculated using the following Eq. (1):

$$\ln\left(\frac{x}{x_0}\right) = \mu_{max} \times t \tag{1}$$

where x_0 is the initial biomass concentration (g L^{-1}) and x is the biomass concentration at time t (h). The EPS volumetric productivity (r_p, g L^{-1} h^{-1}) and the biomass volumetric productivity (r_x, g L^{-1} h^{-1}) were determined according to Eqs. (2) and (3), respectively:

$$r_p = \frac{\Delta p}{\Delta t} \tag{2}$$

$$r_x = \frac{\Delta x}{\Delta t} \tag{3}$$

where Δp and Δx correspond to the EPS (g L^{-1}) and biomass (g L^{-1}) produced during the cultivation time Δt (h).

2.4. EPS characterization

The EPS was characterized in terms of its monosaccharide and acyl substituents (organic and inorganic) contents, as described above for the apple pulp waste characterization. Average Mw and PDI determination were also performed as described above.

2.5. Statistical analysis

All experiments were performed in duplicate or triplicate, and their values were expressed as the mean ± standard deviation. Student's t-test was used to determine statistical differences ($p < 0.05$) among averages at a confidence level of 95%.

3. Results and discussion

3.1. Characterization of the sugar-rich apple pulp supernatant

The apple pulp supernatant was rich in glucose and fructose, with contents of 29.1 ± 0.8 and 11.5 ± 0.4 g L^{-1}, respectively. In comparison with the apple pulp supernatant used by Pereira et al. (2021), it had approximately twice the sugar content, which demonstrates the feedstock's variability. Traces (below 1.5 g L^{-1}) of sucrose, rhamnose, and arabinose were also noticed. It was characterized by a pH of 3.59, which is within the values (3.59–4.16) reported for different apple varieties (Pereira et al., 2021; Rebocho et al., 2019; Wu et al., 2007). The low viscosity (0.004 Pa s) and density (0.95 g cm^{-3}) of the apple pulp supernatant, similar to that of water, indicated that the processing method was adequate for its utilization as feedstock for the cultivation experiments. The inorganic salt content (0.09 ± 0.02 wt%) was lower than that previously described in the literature (1.65 wt%) (Pereira et al., 2021). The elemental analysis revealed that the apple pulp supernatant was mainly composed of carbon (36.8 ± 0.6 wt%), with only traces of sulphur (0.09 ± 0.00 wt%). No nitrogen was detected, contrary to Pereira et al. (2021) who reported traces of this element in the apple pulp. This difference might be due to variations between apple pulp batches and/or the methodology employed for nitrogen determination. Additionally, a minor phosphate content (38.9 ± 0.0 mg L^{-1}) was detected.

A high molecular weight fraction (1.60 ± 0.04 g L^{-1}), with a Mw of 5.73 ± 0.16 × 10^5 Da and a PDI of 4.22 ± 0.94, was detected in the apple pulp supernatant. It was mainly composed of galacturonic acid (42.9 ± 1.3 mol%), arabinose (33.1 ± 2.0 mol%) and galactose (13.7 ± 0.6 mol%), with minor contents of glucose (3.75 ± 0.47 mol%), xylose (3.68 ± 0.41 mol%) and rhamnose (2.42 ± 0.05 mol%). This high molecular weight soluble fraction was probably composed of different polysaccharides, including pectins that are high molecular weight (∼2 ×10^5 Da) polymers mainly composed of galacturonic acid (up to 81 wt%), with minor contents of arabinose, galactose, glucose, xylose and/or rhamnose (Antunes et al., 2017; Dranca et al., 2020).

3.2. Fructose as the sole substrate

Although *A. macleodii* Mo169 was not previously cultivated with fructose, literature indicated that this specie metabolizes glucose and fructose through an inducible Entner–Doudoroff pathway, suggesting that fructose can be used as the main carbon and energy source (Mikhailov et al., 2006). Shake flask experiments were conducted to evaluate the impact of this substrate on *A. macleodii* Mo169 growth and EPS production. Glucose was used as the standard reference substrate for *A. macleodii* Mo169 cultivation. Cultivation with fructose resulted in a biomass production of 1.94 ± 0.05 g L^{-1}, a value statistically comparable to that obtained with glucose (2.23 ± 0.13 g L^{-1}) ($p > 0.05$). Interestingly, EPS synthesis by *A. macleodii* Mo169 was enhanced with fructose as a carbon source (Table 1). On the opposite, marine isolates *Halomonas* strain CRSS and *Pseudoalteromonas* sp. AM preferred glucose to fructose as a substrate for EPS production (Al-Nahas et al., 2011; Poli et al., 2004). In Gram-negative bacteria, such as *A. macleodii* and the examples above, EPS synthesis and secretion follow one of two mechanisms: Wzx–Wzy-dependent pathway, in which the polymer repeat unit is assembled at the inner face of the cytoplasmic membrane and polymerized at the periplasm, and the ABC transporter-dependent pathway, in which polymerization occurs at the cytoplasmic face of the inner membrane (Freitas et al., 2011b). The variation in EPS production depending on the substrate is possibly due to the regulatory mechanisms of the bacterium, which need to be understood for the future optimization of EPS production.

Nevertheless, with fructose as a carbon source, *A. macleodii* Mo169 reached an EPS productivity of 0.089 ± 0.001 g L^{-1} h^{-1}. With the exception of *Microbacterium aurantiacum* FSW-25, *Rhodobacter johrii* CDR-SL 7Cii and *Virgibacillus dokdonensis*, this value was much higher than that reported for other marine bacteria in shake flask experiments (0.008–0.06 g L^{-1} h^{-1}, Table 1), thus, supporting the utilization of the glucose- and fructose-rich apple pulp waste as substrate.

3.3. Cultivation media

Four different media, namely the Schatz medium (Fondi et al., 2015), the SCM (Joulak et al., 2022), salted Medium E* (Concórdio-Reis et al., 2018), and the A1 medium (Roca et al., 2016), were evaluated for the cultivation of *A. macleodii* Mo169, and compared with the marine 2216 medium as a control (Fig. 1). The strain was able to grow in all the tested media, although some differences were noticed in terms of cell growth and EPS synthesis. In comparison to the control medium, which presented an overall biomass production of 2.23 ± 0.13 g L^{-1}, the Schatz and the SCM media resulted in significantly lower biomass production (0.55 ± 0.05 and 1.38 ± 0.10 g L^{-1}, respectively) (Fig. 1). On the contrary, no statistically significant difference was observed in the CDW between the control medium and the salted Medium E* ($p > 0.05$), in which a CDW of 2.33 ± 0.07 g L^{-1} was reached. The use of A1 medium resulted in the highest CDW production (5.75 ± 0.75 g L^{-1}). However, no EPS production was observed with this medium (Fig. 1). The highest EPS production was obtained with the control medium (1.30 ± 0.05 g L^{-1}). Nonetheless, the presence of a significant amount of high molecular weight compounds was noticed in this medium, probably associated with its content in yeast extract and peptone (Roca et al., 2016), resulting in overestimating EPS production. Out of the other four media, the salted Medium E* presented the highest EPS production value (0.72 ± 0.22 g L^{-1}) (Fig. 1). Additionally, no precipitation occurred when this medium was supplemented with the apple pulp waste supernatant, thus, it was chosen for conducting the subsequent bioreactor experiments.

Table 1
EPS production and volumetric productivity obtained in the cultivation of different marine bacteria (n.a., not available).

Bacterium	Carbon source	Cultivation method	EPS ($g\ L^{-1}$)	Productivity ($g\ L^{-1}\ h^{-1}$)	References
Alteromonas macleodii Mo169	Glucose	Shake flask	1.30	0.054	This study
	Fructose	Shake flask	2.12	0.089	
	Apple pulp waste	Bioreactor	3.42	0.190	
Alteromonas macleodii subsp. *fijiensis*	Glucose	Bioreactor	6.00	0.1	Raguénès et al. (1996)
Alteromonas macleodii MS907	Glucose	Bioreactor	9.00	0.125	Raguénès et al. (2003)
Alteromonas sp. strain 1644	Fructose	Bioreactor	7.50	0.107	Samain et al. (1997)
Alteromonas gracilis sp.	Glucose	Shake flask	1.82	0.019	Matsuyama et al. (2015)
Alteromonas infernus GY785	Glucose	Bioreactor	5.5	0.102	Raguénès et al. (1997b)
Brevibacillus borstelensis M42	Glucose	Shake flask	1.88	0.031	Srivastava et al. (2022)
Halomonas strain CRSS	Glucose	Shake flask	0.1	n.a.	Poli et al. (2004)
	Fructose	Shake flask	0.0001	n.a.	
Halomonas almeriensis	Glucose	Bioreactor	1.7	0.014	Llamas et al. (2012)
Halomonas anticariensis	Glucose	Shake flask	2.9–4.9	0.024–0.041	Mata et al. (2006)
Halomonas caseinilytica K1	Glucose	Shake flask	0.044	0.011	Joulak et al. (2019)
Halomonas elongata K4	Glucose	Shake flask	0.061	0.015	
Halomonas smyrnensis S3	Glucose	Shake flask	0.100	0.033	
Halomonas halophila S4	Glucose	Shake flask	0.030	0.010	
Halomonas maura S-30	Glucose	Shake flask	4.28	0.036	Arias et al. (2003)
Halomonas ventosae	Glucose	Shake flask	2.89	0.024	Mata et al. (2006)
Microbacterium aurantiacum FSW-25	Glucose	Shake flask	7.81	0.108	Sran et al. (2019b)
Neorhizobium urealyticum	Glucose	Shake flask	3.38	0.047	Roychowdhury et al. (2021)
Polaribacter sp. SM1127	Glucose	Shake flask	2.11	0.018	Sun et al. (2016)
Pseudoalteromonas sp. MD12-642	Glucose	Bioreactor	2.50–4.40	0.17–0.25	Roca et al. (2016)
Pseudoalteromonas ruthenica	Glucose	Shake flask	1.80	0.025	Saravanan and Jayachandran (2007)
Pseudoalteromonas sp. AM	Glucose	Shake flask	9.50	0.06	Al-Nahas et al. (2011)
	Fructose	Shake flask	3	0.018	
Pseudoalteromonas SM20310	Glucose	Shake flask	0.567	0.008	Liu et al. (2013)
Rhodobacter johrii CDR-SL 7Cii	Glucose	Shake flask	6.2	0.103	Sran et al. (2019a)
Vibrio alginolyticus	Glucose	Bioreactor	1	0.014	Drouillard et al. (2018)
Vibrio diabolicus	Glucose	Bioreactor	2.5	0.052	Raguénès et al. (1997a)
Vibrio diabolicus strain CNCM I-1629	Glucose	Bioreactor	0.563	0.012	Delbarre-Ladrat et al. (2022)
Virgibacillus dokdonensis	Glucose	Shake flask	23.2	0.241	Andrew and Jayaraman (2022)

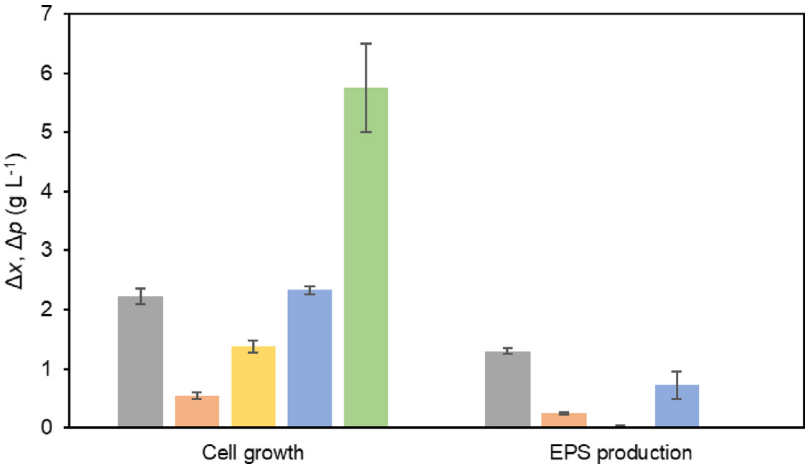

Fig. 1. *A. macleodii* Mo169 growth (Δx) and EPS production (Δp) in different media: control (2216 medium,), Schatz ( ), SCM ( ), salted Medium E* ( ), and A1 ( ) media.

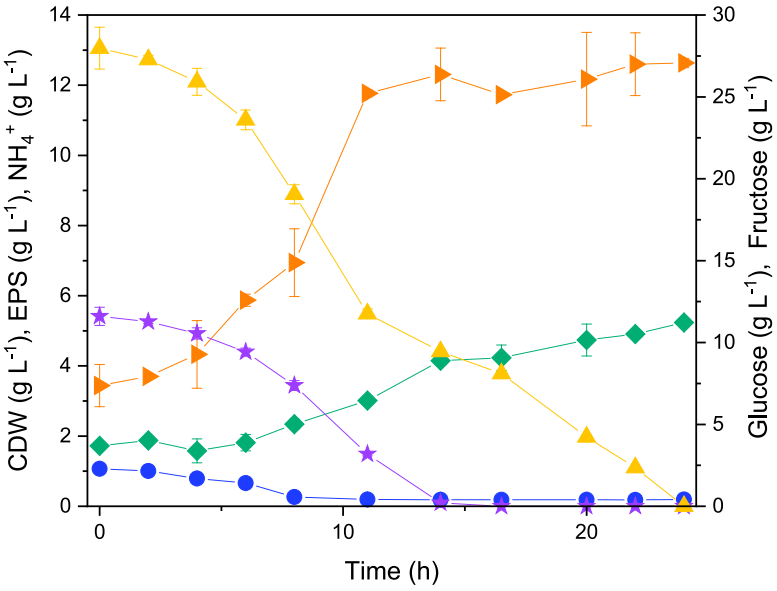

Fig. 2. Cultivation profile of *A. macleodii* Mo169 bioreactor cultivation with salted Medium E* supplemented with apple pulp waste (CDW (▶), EPS (◆), glucose (★), fructose (▲), and ammonium (●) concentrations).

3.4. Bioreactor cultivation

The glucose- and fructose-rich supernatant obtained from waste apple pulp was used as the sole feedstock for the cultivation of *A. macleodii* Mo169 and EPS production under controlled bioreactor conditions (Fig. 2). After inoculation, the culture grew at a maximum specific growth rate of 0.139 ± 0.012 h^{-1}, with no noticeable lag phase. The available ammonium (1.0 ± 0.0 g L^{-1}) was consumed within 11 h of cultivation, and the culture reached the stationary growth phase with a CDW of 11.77 ± 0.09 g L^{-1}. An initial CDW of 3.23 ± 0.60 g L^{-1} was noticed, which was associated with the presence of particles from the pulp waste in suspension. Therefore, there was an effective CDW production of 8.53 ± 0.61 g L^{-1}, which corresponded to a biomass volumetric productivity of 0.776 ± 0.055 g L^{-1} h^{-1}. As depicted in Fig. 2, the two sugars present in the apple pulp, glucose and fructose, were efficiently used by the culture. Both sugars were consumed simultaneously after inoculation, with glucose being exhausted first within 14 h, and fructose at the end of the cultivation (24 h) (Fig. 2).

A high molecular weight fraction, accounting for 1.72 ± 0.08 g L^{-1}, was detected in the cultivation medium at the beginning of the run, which was associated with the presence of the soluble apple pulp polysaccharide, as described above. Nonetheless, a significant increase in EPS concentration was observed concomitant with cell growth (Fig. 2). Interestingly, EPS synthesis continued after the culture entered the stationary cell growth phase (Fig. 2), reaching an EPS concentration of 5.24 ± 0.01 g L^{-1} at the end of the assay. Similarly, for *Halomonas elongata* K4, *Halomonas halophila* S4, *Pseudoalteromonas* sp. MD12-642, and *Pseudoalteromonas* sp. AM, EPS synthesis was partially growth-associated (Al-Nahas et al., 2011; Joulak et al., 2022; Roca et al., 2016), while for other strains, such as *Halomonas caseinilytica* K1 and *Salipiger mucosus* A3T, it only occurred during the exponential growth phase (Joulak et al., 2022; Llamas et al., 2010).

Considering the time between 6 and 24 h of cultivation, 3.42 ± 0.23 g L^{-1} of EPS were produced, corresponding to a maximum EPS volumetric productivity of 0.190 ± 0.013 g L^{-1} h^{-1}. Although higher EPS concentrations were obtained for several marine bacteria (5.5–9.5 g L^{-1}), their productivity was lower than that presented by *A. macleodii* Mo 169 (Table 1). Examples include *Alteromonas macleodii* subsp. *Fijiensis* (Raguénès et al., 1996), *Alteromonas macleodii* MS907 (Raguénès et al., 2003), *Alteromonas* sp. strain 1644 (Samain et al., 1997), *Alteromonas infernus* GY785 (Raguénès et al., 1997b), *Microbacterium aurantiacum* FSW-25 (Sran et al., 2019b), *Pseudoalteromonas* sp. AM (Al-Nahas et al., 2011), and *Rhodobacter johrii* CDR-SL 7Cii (Sran et al., 2019a), whose reported EPS volumetric productivity ranged between 0.06 and 0.125 g L^{-1} h^{-1} (Table 1). *Pseudoalteromonas* sp. MD12-642 presented a higher value when cultivated under fed-batch mode (0.25 g L^{-1} h^{-1}), but a lower value was reported for batch experiments (0.17 g L^{-1} h^{-1}) (Roca et al., 2016). Among marine bacteria, a higher EPS concentration and productivity (23.2 g L^{-1} and 0.241 g L^{-1} h^{-1}, respectively) was only found for *Virgibacillus dokdonensis* (Andrew and Jayaraman, 2022).

Concomitant with EPS synthesis, the apparent viscosity of the cultivation broth increased dramatically, and a shear-thinning fluid behaviour was developed (Fig. 3). This viscosity build-up is a common characteristic during EPS production by several microorganisms, and it usually dictates the end of the cultivation run due to the loss of bulk homogeneity (i.e., mixing, mass and oxygen) (Alves et al., 2010; Freitas et al., 2011a; Poli et al., 2010). This broth fluid behaviour is correlated with the presence of the EPS and indicates that the biopolymer can be used as a rheology modifier in aqueous media.

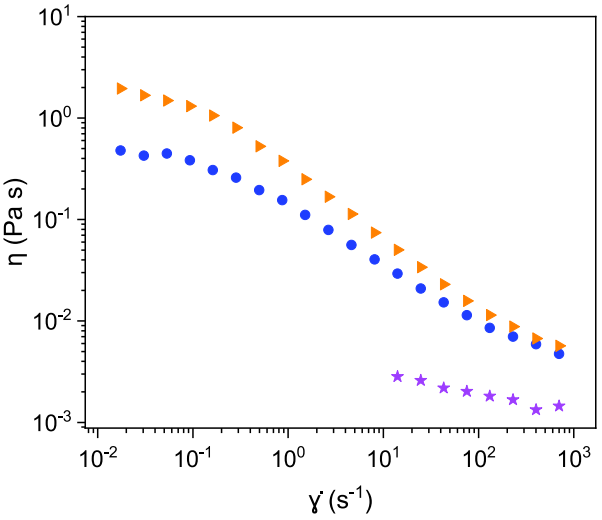

Fig. 3. Flow curves of the cultivation medium (★) and of the broth at the end of the cultivation runs with glucose (●) and apple pulp waste (▶) as carbon sources (measured at 25 °C).

3.5. EPS characterization

3.5.1. Chemical composition

The composition of the EPS produced using the salted Medium E* supplemented with apple pulp waste is presented in Table 2. Contrary to the EPS obtained using marine 2216 medium supplemented with glucose (Concórdio-Reis et al., 2021, 2023), no glucuronic acid was detected in the EPS synthesized with salted Medium E* supplemented with apple pulp waste, thus resulting in a polymer with lower anionic character. Also, the EPS obtained from apple pulp waste had an arabinose content of 23.9 ± 0.1 mol%, a sugar not detected in previous studies. However, galacturonic acid and arabinose were the main monosaccharides in the high molecular weight fraction of apple pulp waste, accounting for 42.9 ± 1.3 and 33.1 ± 2.0 mol%, respectively, of the carbohydrate fraction. Therefore, it should be considered that the content of these monosaccharides in the sample might be related to the presence of such high molecular weight contaminants (e.g., pectins), and further analysis (e.g., determination of the EPS chemical linkages and structure) and purification steps are required to obtain the individual polymers. If not degraded by the bacteria during the cultivation run, pectin would account for up to 30% of the high molecular weight polysaccharide obtained at the end of the assay. Nevertheless, pectin is a biocompatible polysaccharide with potential as a therapeutic agent (Kedir et al., 2022), thus, the EPS/pectin blend might have interesting properties for high-value applications.

Except for arabinose, both EPS had the same neutral monosaccharides (mannose, galactose, glucose, and glucosamine) but at different molar proportions. Although the contents of all the neutral sugars increased in the apple pulp waste EPS, the most significant difference was the higher glucosamine content (10.3 ± 0.5 mol%), which was higher than galactose's content (8.7 ± 0.1 mol%), and the high content in glucose (31.1 ± 0.2 mol%). Bacteria belonging to the *Alteromonas* genus are known to produce EPS composed of glucose, mannose, galactose, and their uronic acid derivatives (Delbarre-Ladrat et al., 2017; Sahana and Rekha, 2019), whereas amino-sugars are not commonly found within this group (Delbarre-Ladrat et al., 2017). Nonetheless, *Alteromonas* sp. PRIM-28 produced an EPS composed of glucose, mannuronate and N-acetyl glucosamine (1:3.67:0.93), which was biocompatible and capable of modulating various cellular events during *in vitro* wound healing, suggesting its potential as a multifunctional bioactive material for wound care (Sahana and Rekha, 2019). Due to the EPS' high content in glucosamine, *A. macleodii* Mo169 might be a potential source of novel and improved biomaterials for health applications.

It should be noted that the marine 2216 medium used by Concórdio-Reis et al. (2021, 2023) had a high content of high molecular weight soluble compounds arising from the presence of yeast extract and peptone in the medium. Such compounds, if not totally degraded during the bacterial cultivation, might be present in the broth at the end of the run. Due to their high molecular weight, such compounds were probably extracted together with the EPS secreted by *A. macleodii* Mo169 when it was grown in 2216 medium. Consequently, their presence in the extracted sample impacted the analysed sugar composition, increasing some monomers content, namely glucose, mannose, and galactose, which are commonly present in yeast extract (Roca et al., 2016) thus increasing their contents in the sample recovered from such cultivations.

Considering the acyl content, lactate and pyruvate were within the ranges previously described for the EPS produced by *A. macleodii* Mo169 (Table 2). Such acyl groups have been reported as components of the EPS produced by marine bacteria (Casillo et al., 2018). On the other hand, higher contents of acetate and sulphate were detected (1.2 ± 0.0 wt% and 6.0 ± 0.5 wt%, compared to 0–0.5 wt%, and 2.8–5.3 wt%, respectively). Although sulphated EPS are usually isolated

Table 2
Sugar composition, acyl content, and molecular mass distribution of the EPS produced by *A. macleodii* Mo169 in bioreactor cultivation experiments using different carbon sources and medium compositions, namely 2216 E medium supplemented with glucose (reported in the literature) and using salted Medium E* supplemented with apple pulp waste (Ara, arabinose, Gal, galactose; GalA, galacturonic acid; Glc, glucose; GlcA, glucuronic acid; GlcN, glucosamine; Man, mannose; Mw, average molecular weight; and PDI, polydispersity index).

	Reported in literature Concórdio-Reis et al. (2021, 2023)		This study	
Sugar monomers (mol%)				
Man	12.8		17.3 ± 0.1	
Gal	4		8.7 ± 0.1	
Glc	11.2		31.1 ± 0.2	
GlcN	2.4		10.3 ± 0.5	
Ara	n.d.		23.9 ± 0.1	
GlcA	39.3		n.d.	
GalA	10.4		8.7 ± 0.0	
Acyl content (wt.%)				
Sulphate	2.8–5.3		6.0 ± 0.5	
Pyruvate	0.063–4.9		0.5 ± 0.1	
Lactate	0–0.63		n.d.	
Acetate	0–0.5		1.2 ± 0.0	
Molecular mass distribution				
Mw (MDa)	1.6	4.6	1.7 ± 0.0	0.74 ± 0.01
PDI	1.3	1.3	1.0 ± 0.0	1.1 ± 0.0

from algae and are quite uncommon among prokaryotic, some marine bacteria belonging to the *Alteromonas, Halomonas and Pseudoalteromonas* genera are reported as producers of sulphate-containing EPS. The presence of sulphate renders the polysaccharide increased interest, due to its higher charge density which can be useful for several applications, such as the removal of toxic metals from contaminated samples. Additionally, similarly to glycosaminoglycans, this type of biopolymers has been reported to display anticoagulant, antiviral, and immuno-inflammatory activities. As a result of these features, sulphate-containing EPS might find relevant use in bioremediation, cosmetic, pharmaceutical, and nutraceutical food applications (Casillo et al., 2018).

3.5.2. Molecular mass distribution

The polymer's average molecular weight (Mw) and polydispersity index (PDI) are presented in Table 2. Independently of the cultivation conditions, two high molecular weight fractions were obtained. For the EPS produced from the apple pulp waste, this might partly be attributed to the presence of high molecular weight compounds of the apple pulp waste supernatant that were not completely degraded by the bacteria. One of the fractions had a Mw (0.74 ± 0.01 MDa) similar to that described above for the high molecular weight polysaccharide found in the apple pulp waste supernatant (0.57 ± 0.00 MDa). Nonetheless, the Mw values obtained in this study were within the range reported for other *Alteromonas* EPS (0.17–2 MDa) (Le Costaouëc et al., 2012; Raguénès et al., 2003; Sahana and Rekha, 2019; Zhang et al., 2017; Zykwinska et al., 2018), and lower than those previously reported with marine 2216 medium (1.6 and 4.6 MDa) (Table 2). Additionally, both EPS fractions were found to be homogeneous polymers, as shown by their low PDI values (1.0–1.1) (Table 2).

4. Conclusions

This work demonstrated for the first time the ability of a marine bacterium, *A. macleodii* Mo169, to grow on apple pulp waste, a low-cost feedstock, as sole substrate, and secrete high amounts of a novel EPS. Higher cell growth and EPS production were achieved from the glucose- and fructose-rich apple pulp waste, compared to the use of glucose alone. The EPS volumetric productivity of 0.190 ± 0.013 g L^{-1} h^{-1} suggests the potential of this biotechnological process as a perfect illustration of a circular economy, where waste is converted into a value-added product. Also, alterations in the EPS composition were noticed, which opens the possibility for the discovery of new EPS with unique compositions and properties by implementing different cultivation conditions. The produced EPS comprised two high Mw fractions, which were composed of glucose, arabinose, mannose, glucosamine and galacturonic acid as major sugar monomers, and a higher content of sulphate. The presence of glucosamine, galacturonic acid, and sulphate in the EPS composition is often associated with biological activities, thus, biotechnological value. Moreover, the secreted polysaccharide conferred a shear-thinning fluid behaviour to the cultivation broth with a high apparent viscosity. Given these features, the EPS is worth exploiting for a variety of applications, including in the pharmaceutical, cosmetic, food, or petroleum sectors. Thus, not only does this study contribute to the valorization of wastes and the reduction of EPS production costs, but also provides evidence of the high potential of marine resources as producers of EPS with unusual compositions with high-value applications.

CRediT authorship contribution statement

Patrícia Concórdio-Reis: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Bruno Serafim:** Methodology, Investigation, Formal analysis. **João R. Pereira:** Methodology, Investigation, Formal analysis. **Xavier Moppert:** Writing –review and editing. **Jean Guézennec:** Writing – review and editing. **Maria A.M. Reis:** Funding acquisition, Supervision, Writing – review and editing. **Filomena Freitas:** Conceptualization, Funding acquisition, Supervision, Writing – review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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