Extremophiles (2023) 27:30
https://doi.org/10.1007/s00792-023-01313-3

ORIGINAL PAPER

Production of poly (3-hydroxybutyrate) and extracellular polymeric substances from glycerol by the acidophile *Acidiphilium cryptum*

Ernesto González[1,2] · Camila Zuleta[1] · Guiselle Zamora[1] · Nataly Maturana[1] · Belén Ponce[1] · María Virginia Rivero[3,4] · Alberto Rodríguez[3,4] · Juan Pablo Soto[5] · Felipe Scott[6] · Álvaro Díaz-Barrera[1]

Received: 20 April 2023 / Accepted: 26 September 2023

Abstract

Acidiphilium cryptum is an acidophilic, heterotrophic, and metallotolerant bacteria able to use dissolved oxygen or Fe(III) as an electron sink. The ability of this extremophile to accumulate poly(3-hydroxybutyrate) (PHB) and secrete extracellular polymeric substances (EPS) has also been reported. Hence, the aim of this work is to characterize the production of PHB and EPS by the wild strain DSM2389 using glycerol in shaken flasks and bioreactor. Results showed that maximum PHB accumulation (37–42% *w*/*w*) was obtained using glycerol concentrations of 9 and 15 g L^{-1}, where maximum dry cell weight titers reached 3.6 and 3.9 g L^{-1}, respectively. The culture in the bioreactor showed that PHB accumulation takes place under oxygen limitation, while the redox potential of the culture medium could be used for online monitoring of the PHB production. Recovered EPS was analyzed by Fourier-transform infrared spectroscopy and subjected to gas chromatography—mass spectrometry after cleavage and derivatization steps. These analyses showed the presence of sugars which were identified as mannose, rhamnose and glucose, in a proportion near to 3.2:2.3:1, respectively. Since glycerol had not been used in previous works, these findings suggest the potential of *A. cryptum* to produce biopolymers from this compound at a large scale with a low risk of microbial contamination due to the low pH of the fermentation process.

Keywords *Acidiphilium cryptum* · Acidophile · Extracellular polymeric substance · Extremophile · Glycerol · poly(3-hydroxybutyrate)

Communicated by Atomi.

✉ Ernesto González
ernesto.gonzalez@pucv.cl; ernest02@ucm.es

1 Escuela de Ingeniería Bioquímica, Pontificia Universidad Católica de Valparaíso, Av. Brasil, 2085 Valparaíso, Chile

2 Department of Chemical and Materials Engineering, Faculty of Chemistry, Universidad Complutense de Madrid, Avda. Complutense s/n, 28040 Madrid, Spain

3 Polymer Biotechnology Lab, Centro de Investigaciones Biológicas Margarita Salas (CIB-CSIC), Ramiro de Maeztu 9, 28040 Madrid, Spain

4 Interdisciplinary Platform for Sustainable Plastics Towards a Circular Economy-Spanish National Research Council (SusPlast-CSIC), Madrid, Spain

5 Instituto de Química, Pontificia Universidad Católica de Valparaíso, Av. Universidad 330, Curauma, Valparaíso, Chile

6 Green Technologies Research Group, Facultad de Ingeniería y Ciencias Aplicadas, Universidad de los Andes, Av. Mons. Álvaro del Portillo, Las Condes, 12455 Santiago, Chile

Abbreviations

BDM	Basal defined medium
BEM	Basal E medium
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
DTGS	Deuterated triglycine sulfate
DSMZ	German Collection of Microorganisms and Cell Cultures
EPS	Extracellular polymeric substances
FTIR	Fourier-transform infrared spectroscopy
GC	Gas chromatography
GMP	Good manufacturing practice
HPLC	High-performance liquid chromatography
MEM	Minimum essential medium
MS	Mass spectrometry
OD_{600}	Optical density at 600 nm
pE	Negative logarithm of the electron activity
PHA	Polyhydroxyalkanoate
PHB	Poly(3-hydroxybutyrate)
RI	Refractive index
rpm	Revolutions per minute
TIC	Total ion current

Published online: 17 October 2023

TMCS	Trimethylchlorosilane
TMS	Trimethylsilylation
UDP	Uridine diphosphate
UV–Vis	Ultraviolet–visible
vvm	Volume of gas (under standard conditions) per volume of liquid per minute
v/v	Volume by volume
w/w	Weight by weight

Introduction

Prokaryotic life, through a long evolutionary history, has become ubiquitous in all environmental niches pushing the boundary of habitable conditions. Microorganisms living in these environments, collectively known as extremophiles, are categorized according to the conditions under which they grow, being possible to identify thermophiles/hyperthermophiles, psychrophiles, barophiles, acidophiles, alkalophiles, halophiles, xerophiles and metallophiles, among others (Chettri et al. 2021). The application of extremophiles has been focused on the isolation of extremozymes, industrial catalysis of processes under extreme conditions (e.g. biomining), and the production of valuable molecules or nanoparticles (Beeler and Singh 2016; Coker 2016). However, some microorganisms, such as acidophiles, alkalophiles and halophiles, are interesting subjects of basic and applied research for the production of compounds in fermentation processes due to the self-protected nature of the fermentations. The advantages of these processes include savings in media sterilization, simpler equipment design, and reduced chances of losing a batch of product due to culture contamination (Chen and Jiang 2018; Koller 2017).

Microorganisms belonging to the genus *Acidiphilium*, mainly *A. cryptum* strains, stand out among acidophiles for their ability to carry out reductive biomining operations (González et al. 2015, 2018) but they can also accumulate the biopolymer poly(3-hydroxybutyrate) (PHB), the most common type of polyhydroxyalkanoate (PHA) (Xu et al. 2013, 2010). *Acidiphilium cryptum* is a heterotrophic bacterium able to use several substrates, although some authors have reported a meager growth in chemically defined medium and inhibition by organic acids and high concentrations of organic compounds (Harrison 1981; Kishimoto et al. 1990). Since its bacterial activity is maintained in the presence of heavy metals such as chromium, zinc, aluminum, and manganese (Cummings et al. 2006; Fischer et al. 2002Zhou et al. 2014), it can also be classified as a metallotolerant bacteria.

Biopolymers such as PHA/PHB have been widely studied because they are biodegradable polymers that can be obtained from renewable sources (Palmeiro-Sánchez et al. 2022). Nevertheless, their production has not reached a large scale due to the lack of cost-effectiveness, where the carbon source contributes to approximately 50% of the total cost (Sirohi et al. 2020). Glycerol has been explored as an alternative carbon source, instead of sucrose or glucose, for PHB production in neutrophilic bacteria (Sriyapai et al. 2022; Wen et al. 2020) because crude glycerol (80% purity) can be obtained at low cost from the biodiesel industry (0.09–0.20 USD kg^{-1}), while the price of pure glycerol is almost five-folds higher (0.60–0.91 USD kg^{-1}) (Monteiro et al. 2018). Despite *A. cryptum* can use of glycerol as a carbon and energy source (Harrison 1981; Küsel et al. 1999), and the affordable price of this feedstock, there are no reports about the utilization of this compound to produce PHB.

On the other hand, a price comparable to petroleum-based plastics is not a goal for medical applications of bioplastics where other characteristics, such as biocompatibility, good mechanical properties and high purity, are desirable too (Luef et al. 2015). In particular, the production of biopolymers for medical purposes must accomplish good manufacturing practice (GMP) standards, where chemically defined media are usually preferred. These media are typically employed in research to increase the reproducibility and to study the effect of individual compounds (Link and Weuster-Botz 2011), although Zhang and Greasham (1999) and Posch et al. (2012) have also encouraged their employment in industrial processes. The reported advantages include (1) enhanced process consistency, (2) better control and monitoring, (3) improved process scale-up and (4) simplified product recovery, having (5) equal or higher fermentation productivities.

In addition to the ability of certain microorganisms to accumulate PHA/PHB, others also secrete extracellular polymeric substances (EPS), mixtures of polysaccharides, proteins and DNA (Costa et al. 2018). In bacteria with geomicrobiological relevance, the production of EPS has been related to biosorption, biomineralization, particle trapping/aggregation, redox cycling, metals complexation, mineral dissolution, bioleaching and corrosion (Tourney and Ngwenya 2014). Despite several subfractions of EPS can be isolated, two major fractions involve bound and soluble EPS (Shi et al. 2017). As far as we know there are no reports about production of soluble EPS for strains belonging to the genus *Acidiphilium*, while Tapia et al. (2009) reported the protein and carbohydrate content found in bound EPS obtained from *Acidiphilium* 3.2Sup(5) by different methods. The bound EPS of this strain was then subsequently used to study the adsorption of Fe(III) and Fe(II) from a mechanistic point of view (Tapia et al. 2016, 2011).

Hence, this work focuses on characterizing the production of PHB and EPS by the acidophilic and metallotolerant wild strain *Acidiphilium cryptum* DSM2389 using glycerol. The cultures were performed in shaken flasks and bioreactor

using complex and defined culture media supplemented with this compound as carbon and energy source. This study shows an opportunity to exploit the metabolic potential of extremophiles to improve the fermentation process for addressing the current needs of biodegradable polymers in the context of bioeconomy (Antranikian and Streit 2022).

Materials and methods

Microbial strain and culture media

The strain *A. cryptum* DSM2389 was maintained in flasks at 35 °C and 150 rpm using basal E medium (BEM) supplemented with glucose or glycerol. The BEM, formulated based on E medium from González et al. (2015), contained: $(NH_4)_2SO_4$ 2.0 g L^{-1}, KCl 0.1 g L^{-1}, K_2HPO_4 0.5 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.5 g L^{-1} and yeast extract 0.3 g L^{-1}. A basal defined medium (BDM) was formulated as follows: $(NH_4)_2SO_4$ 4.0 g L^{-1}, KCl 0.4 g L^{-1}, K_2HPO_4 0,5 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.2 g L^{-1}, $CaCl_2{\cdot}2H_2O$ 0.1 g L^{-1}, ferric citrate (CAS: 3522–50-7) 0.1 g L^{-1}, trace element solution 10 mL L^{-1} and vitamin solution 5 mL L^{-1}. The trace element solution had the composition shown by the DSMZ (German Collection of Microorganisms and Cell Cultures) for the medium 141 (methanogenium medium), while the MEM (Minimum Essential Medium) vitamin solution 100X (Gibco, 11,120,052) was used to supply required vitamins. The BDM was also supplemented with glycerol.

Experimental conditions

Experiments in flasks were performed to evaluate (1) the microbial growth using unbuffered and buffered ($NaH_2PO_4{\cdot}2H_2O$ 7.5 g L^{-1} and H_3PO_4 0.23 g L^{-1}) defined medium at glycerol or glucose concentrations of 3 g L^{-1}, and (2) the effect of glycerol as carbon and energy source at different levels (3, 9 and 15 g L^{-1}) in buffered medium. The medium used for pre-cultures was the same employed for actual cultures but using 3 g L^{-1} of glycerol or glucose, depending on the carbon source used in the experiment. Cultures were carried out in 1000 mL flasks with 300 mL of culture medium inoculated with 30 mL of a pre-grown broth. The cultures were performed in an orbital incubator (New Brunswick Scientific, Innova 4000) at 35 °C and 150 rpm.

The culture using the basal defined medium amended with 15 g L^{-1} glycerol was scaled from flasks to a 3 L bioreactor (Applikon Ez-Control). The bioreactor was equipped with a polarographic oxygen electrode, combined redox electrode and a pH control system. The culture was performed with 2 L of broth at 35 °C, 340 rpm and 0.1 vvm, controlling the pH above 3.0 by the addition of NaOH. Samples were periodically withdrawn to measure biomass, glycerol and the PHB content (as a percentage of the total dry cell weight). At the end of the culture, the biomass was removed by centrifugation and the spent medium was mixed (1:3 *v*/*v*, 30 min) with cold ethanol 96% (*v*/*v*) to precipitate soluble EPS. All cultures were performed in duplicate.

Analytical methods

Fresh samples were separated into two aliquots. The first aliquot was used to measure the optical density at 600 nm (OD_{600}), which was employed to obtain the dry cell weight using a previously made calibration curve. The second aliquot was centrifuged (Thermo Scientific, SL16R) at 10,000 rpm and 4 °C for 10 min to pellet the biomass. The supernatant was stored at − 20 °C to measure the concentration of glycerol or glucose while the biomass pellet was washed twice and dried at 105 °C. The dried biomass pellet was used to measure the PHB content or to obtain a direct quantification of dry biomass weight.

Glycerol and glucose were measured by HPLC (high-performance liquid chromatography) using the column Bio-Rad Aminex HPX-87H (300 mm × 7.8 mm) at 50 °C, H_2SO_4 5 mmol L^{-1} as eluent (0.6 mL min^{-1}) and a refractive index (RI) detector. The PHB in dried biomass was measured by means of the crotonic acid method coupled with HPLC (Karr et al. 1983). Hence, 2.5–3.5 mg of dried pellet was weighted into a 2 mL microtube and treated with 1 mL of H_2SO_4 95–98% at 90 °C and 700 rpm during 1 h (Thermo-Shaker TS-100, SC-20N, 20 × 2.0 mL) for transforming PHB intro crotonic acid. The reaction mix was cold-down, diluted with milliQ water, filtered (polyvinylidene fluoride membrane and 0.22 µm pore size) and measured in HPLC under similar conditions as those described previously for glycerol using an ultraviolet–visible (UV–Vis) detector at 210 nm (Díaz-Barrera et al. 2016).

Samples of EPS were analyzed by Fourier-transform infrared spectroscopy (FTIR) using a Thermo Nicolet iS5 FTIR spectrometer (Thermo Electronic Inc. USA) having a resolution of 4 cm^{-1} with DTGS (deuterated triglycine sulfate) detector and with the OMNIC 9.2 software in the 400–4000 cm^{-1} region. For this analysis, the sample was ground with KBr powder (spectroscopic grade) and pressed into a 1 mm pellet. Samples were also subjected to pyrolysis by heating at 900 °C using purified nitrogen as a carrier gas to remove gaseous and condensable products while providing an inert atmosphere.

The EPS was assayed to determine the monosaccharide composition. Hence, samples were treated to cleave the oligosaccharide into their components, while two derivatization methods were used after the glycosidic bond cleavage to detect the highest number of polysaccharides: formation of alditol acetates and oxime-trimethylsilylates. For analysis of sugars, the polysaccharides (between 1–2 mg) were

hydrolyzed with 3 mol L^{-1} trifluoroacetic acid (121 °C, 90 min). On the one hand, the neutral monosaccharides were converted into their corresponding alditol acetates by reduction with $NaBH_4$ and subsequent acetylation (Notarangelo et al. 2013). The second method applied to derivatize the samples after the hydrolysis step involved the formation of oxime-trimethylsilylation (TMS) derivatives. Firstly, the formation of the oxime derivative was carried out with a solution of hydroxylamine hydrochloride 2.5% in pyridine (70 °C, 30 min). Then, TMS reagent [N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA): trimethylchlorosilane (TMCS)] was added to the solution and the mixture was heated at 80 °C for 20 min. In both cases, the identification and quantification were performed by gas chromatography (GC) mass spectrometry (MS) on a gas chromatograph coupled to a quadrupole mass detector 7980A-5975C (Agilent Technologies, Santa Clara, CA, USA) equipped with a DB-5HT ((5%-Phenyl)-methylpolysiloxane) column (Agilent, Santa Clara, CA, USA; 30 m × 0.25 mm internal diameter; 0.10 µm film thickness). The detector condition was 70 eV, while the temperatures used were 150 °C for the MS Quad, 230 °C for the MS Source, and 280 °C for the transfer line. All sugars were quantified in TIC (total ion current) mode using calibration curves of each monosaccharide with inositol as the internal standard. For alditol acetates the oven program started at 175 °C for 1 min, increasing at 2.5 °C min^{-1} until 204 °C, and the ion source of the mass spectrometer (electronic impact) was set at 250 °C. For oxime-trimethylsilylates the oven program started at 160 °C for 2 min, increasing at 2 °C min^{-1} until 185 °C and the ion source of the mass spectrometer (electronic impact) was set at 275 °C. The carrier gas was helium (1 mL min^{-1}) in both cases.

Results

Figure 1 shows the dry cell weight and carbon sources titers, pH and PHB accumulation quantified along the batch cultures of *A. cryptum* in basal E medium (BEM) and basal defined medium (BDM) supplemented with glucose and glycerol. In unbuffered media, the dry cell weight achieved similar maximum values in the range of 1.07–1.34 g L^{-1} while glucose and glycerol decreased to reach low or negligible values and the pH decreased 1.1–1.4 units achieving a value near 2.4. The incorporation of phosphate was effective in limiting the pH change to 0.6 units without affecting microbial growth. The maximum value for PHB content was recorded at 24 h, which was followed by a decrease in this parameter.

Figure 2 shows the profiles for dry cell weight, glycerol, PHB accumulation and pH obtained for the culture of *A. cryptum* in buffered BDM supplemented with 3, 9 and 15 g L^{-1} of glycerol. The maximum dry cell weight titer

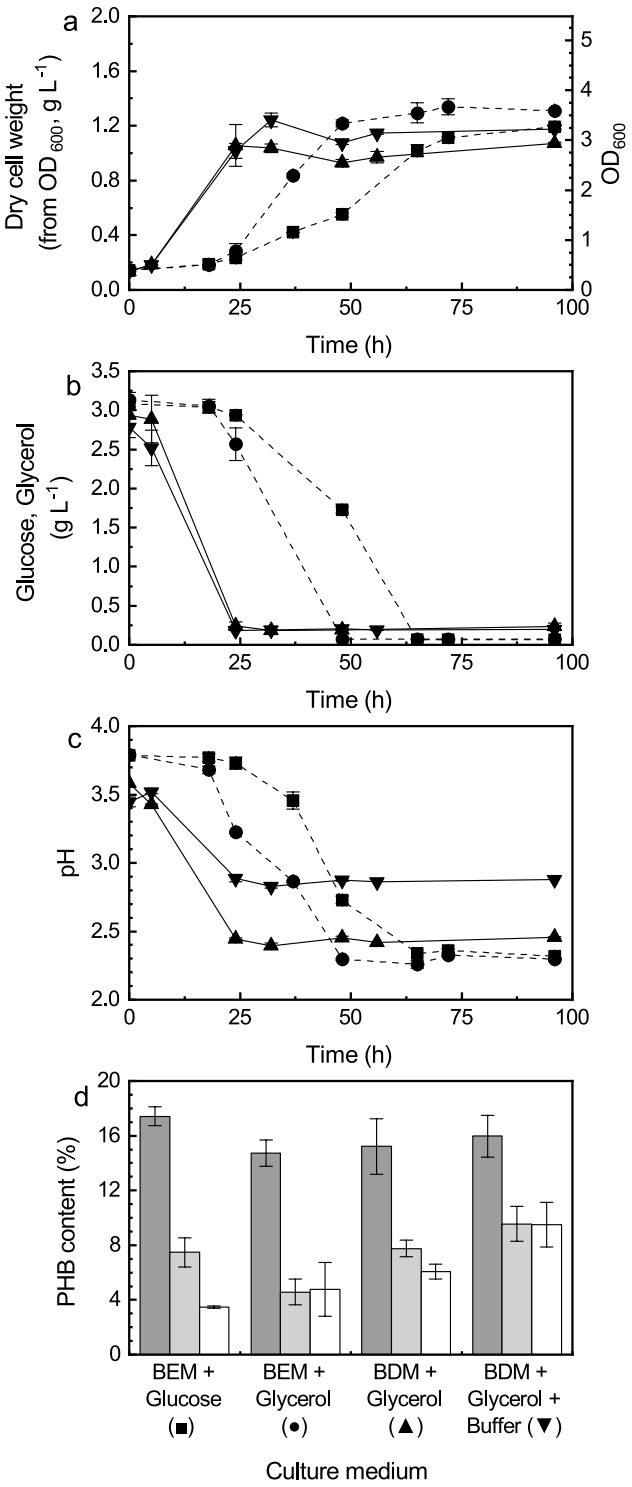

Fig. 1 Profiles for dry cell weight **a**, substrate **b** and pH **c** recorded during the culture of *Acidiphilium cryptum* in flasks at 35 °C and 150 rpm using basal E medium (BEM, dashed line) supplemented with 3 g L^{-1} of glucose (■) and glycerol (●), and basal defined medium (BDM, solid line) supplemented with 3 g L^{-1} of glycerol without (▲) and with (▼) phosphate buffer. Panel d shows the PHB content at different times: 24 h (dark grey), 48 h (light grey) and 96 h (white). Mean ± standard deviation

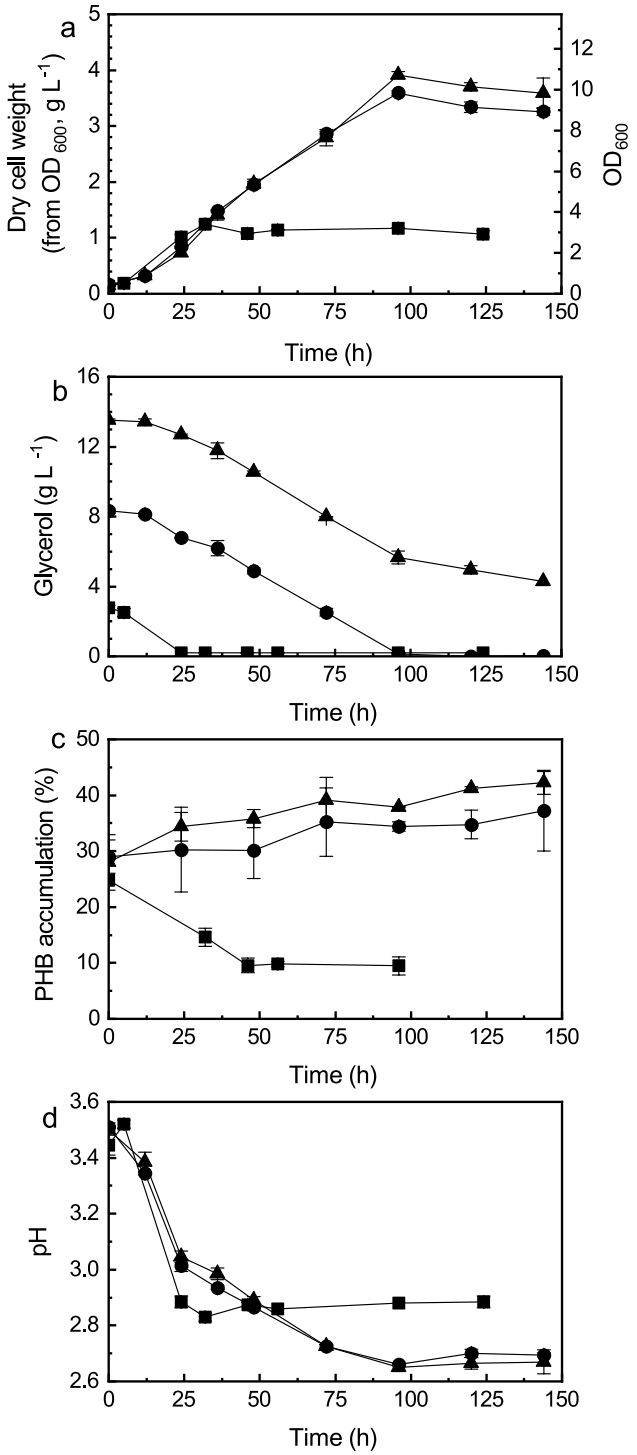

Fig. 2 Profiles for dry cell weight **a**, glycerol **b**, PHB accumulation **c** and pH **d** recorded during the culture of *Acidiphilium cryptum* in flasks at 35 °C and 150 rpm using the buffered basal defined medium (BDM) supplemented with 3 g L^{-1} (■), 9 g L^{-1} (●) and 15 g L^{-1} (▲) of glycerol. Mean ± standard deviation

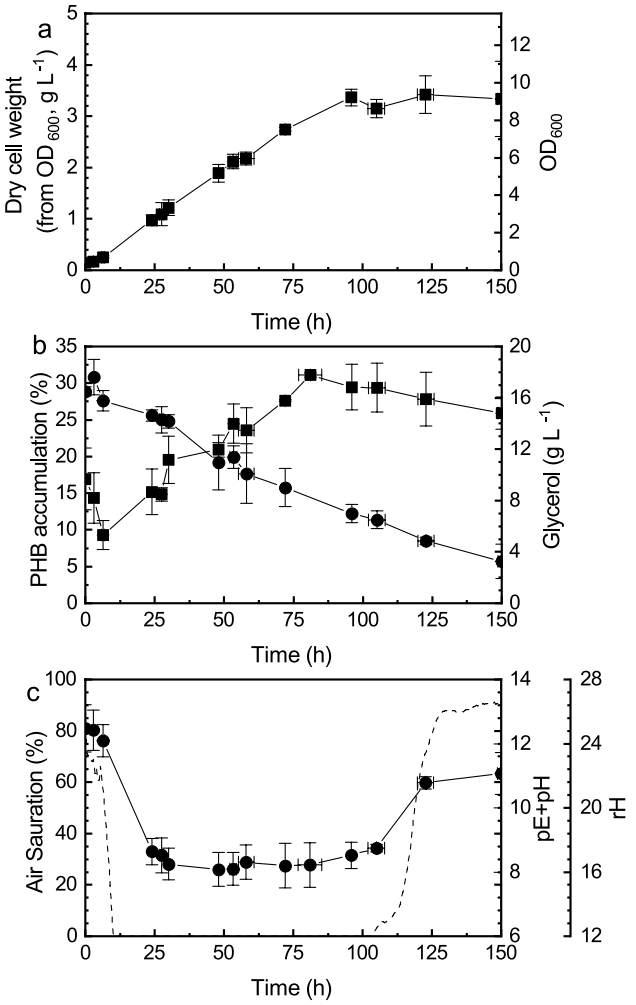

Fig. 3 Profiles for dry cell weight **a**, PHB accumulation (■) and glycerol (●) **b**, dissolved oxygen (dashed line, as air saturation) and redox potential (●, as rH and pE + pH) **c** recorded during the culture of *Acidiphilium cryptum* in a bioreactor at 35 °C, 340 rpm and 0.1 vvm using the basal defined medium (BDM) supplemented with 15 g L^{-1} of glycerol. Mean ± standard deviation while the profile for dissolved oxygen is presented for one culture

increased threefold when the concentration of glycerol in the medium was raised from 3 to 9 g L^{-1}, but a further increase in glycerol concentration did not yield significantly more biomass. PHB accumulation profiles recorded using 9 and 15 g L^{-1} of glycerol showed a different trend to that obtained using 3 g L^{-1} of glycerol because the PHB content decreased to achieve a 9.5% in the culture performed in a medium supplemented with 3 g L^{-1} of glycerol while this parameter reached 37–42% in the cultures with higher initial concentrations of glycerol.

Profiles for dry cell weight, PHB accumulation, glycerol, redox potential and dissolved oxygen recorded for the culture of *A. cryptum* in a bioreactor using BDM medium supplemented with glycerol are presented in Fig. 3. The

dry cell weight and PHB accumulation achieved in this fermentation process were slightly lower than those obtained in shaken flasks using the same medium. The microbial growth finished at 96 h obtaining a yield equal to 0.35 ± 0.03 g of dry cells per gram of consumed glycerol. After that time the dry cell weight remained constant while glycerol concentration decreased. The culture was then ceased, and the soluble EPS was freeze-dried after the recovery step with cold ethanol obtaining 0.71 g of EPS per liter of spent medium. A similar mass of soluble EPS was also obtained from the fermentation of *A. cryptum* using BEM supplemented with glycerol 15 g L^{-1}, although the culture time was 30% longer (Online Resource Fig. S1). The comparison between measured dry biomass weight and dry cell weight calculated from OD_{600} showed a good correspondence, although calculated values were $13 \pm 2\%$ higher than measured ones (Online Resource Fig. S2).

The pyrolysis test showed that the inorganic fraction in EPS recovered from cultures in the bioreactor was near 80% and 30% (w/w) for those samples obtained using glycerol-supplemented BDM and BEM, respectively. Fig. 4 (Table 1, and Online Resource Fig. S3 and Table S1) shows the FTIR spectrum for EPS samples while Fig. 5 (Table 2, and Online Resource Fig. S4) shows that mannose, rhamnose and glucose are the main constituents of the exopolysaccharide while traces of mannitol, sorbitol, fructose, galactose and galacturonic acid were also detected.

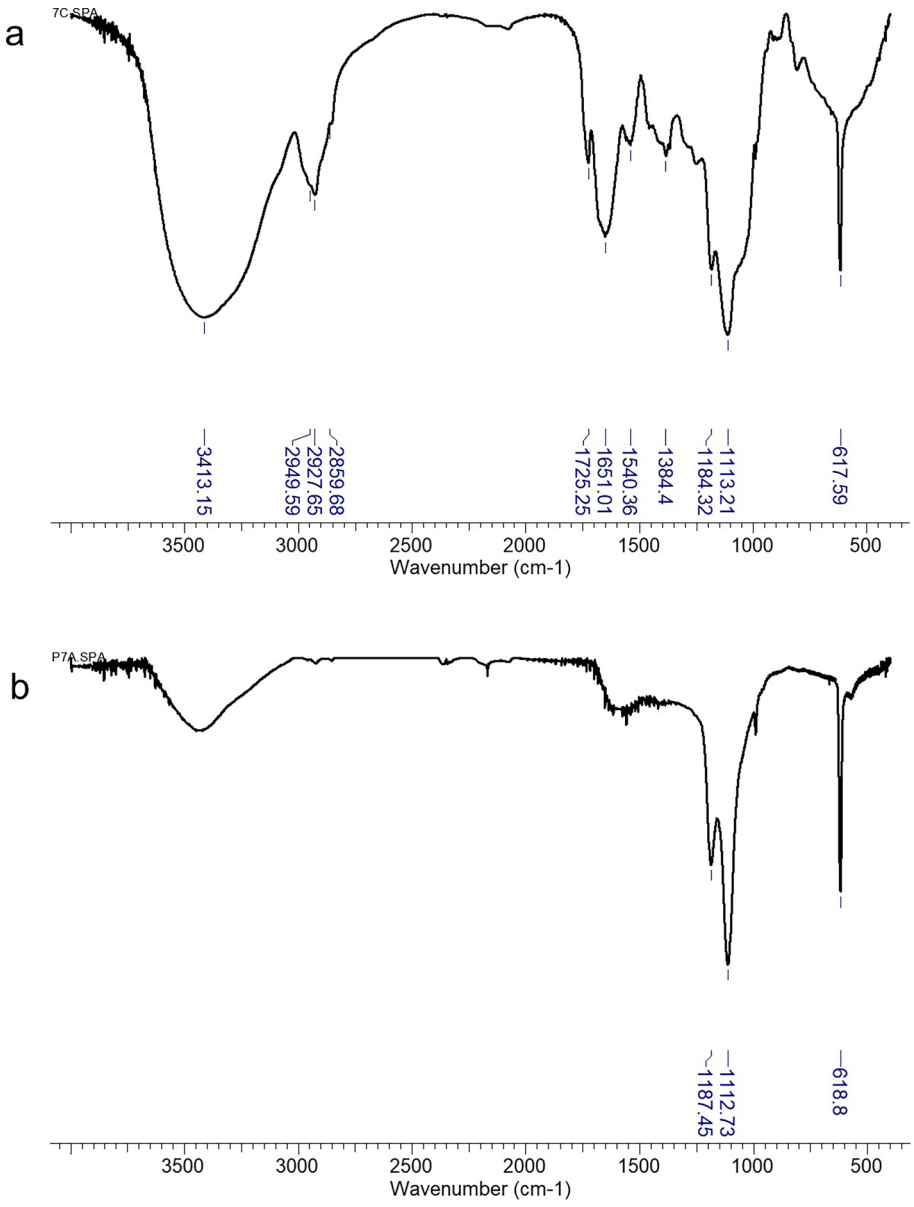

Fig. 4 FTIR spectra of EPS recovered from the culture of *Acidiphilium cryptum* in bioreactor using basal E medium (BEM) supplemented with glycerol 15 g L^{-1} **a** and its inorganic residue after pyrolysis **b**

Table 1 Table of peaks in FTIR spectrum shown in Fig. 4a

N°	Wavenumber (cm^{-1})	%T	Intensity
1	617.59	35.280	S
2	1113.21	18.926	VS
3	1184.32	35.412	S
4	1384.40	64.106	M
5	1540.36	66.836	M
6	1651.01	43.625	S
7	1725.25	62.329	M
8	2859.68	72.380	M
9	2927.65	54.287	M
10	2949.59	56.696	M
11	3413.15	23.349	VS

Discussion

Growth using a defined culture medium with glycerol

Different fermentation times were registered in Fig. 1 for cultures in unbuffered media: 65 h for BEM + Glucose, 48 h for BEM + Glycerol and 24 h for BDM + Glycerol. Hence, replacing glucose by glycerol was effective in reducing the length of microbial culture, also showing that *A. cryptum* grows well in a chemically defined medium. Bhattacharyya et al. (1991) reported that glycerol would be a better substrate than glucose to grow *A. cryptum*, a fact here observed in a lower fermentation time. Harrison (1981) described a scant growth of *A. cryptum* in chemically defined medium but the change of BEM to BDM was positive in this research. The tendency to decrease the pH was opposite to that previously observed for *A. cryptum* JF-5 grown anoxically in the presence of magnetite and jarosite (González et al. 2015), which was explained by protons consumed for the dissolution of ferric compounds. Since the final pH were near the lower limit reported for *A. cryptum* type strain (Harrison 1981) and JF-5 strain (Küsel et al. 1999), a buffer was included to avoid restrictions caused by this variable in subsequent cultures aimed at reaching a higher biomass. The buffer did not affect the fermentation time and caused a slight increase in the PHB accumulation measured at 48 h and 96 h. This slight positive effect may be related to the mechanisms used by acidophiles for maintaining a near-neutral intracellular pH. Since the flow of electrons through the electron transport chain is usually associated with proton translocation from the cytoplasm to the periplasmic space (Bird et al. 2011), the accumulated PHB can be degraded to maintain the electron transport chain operating when glycerol has been depleted. Thus, the energy generated in this process may be used for cell maintenance and to drive potassium pumps required to maintain a reverse membrane potential which inhibits the entry of protons into the cell (Sharma et al. 2016).

Effect of glycerol concentration

Figure 2 shows that the culture performed in BDM supplemented with 15 g L^{-1} of glycerol did not reach glycerol depletion, which agrees with the fact that the maximum dry cell weight titer did not increase when glycerol was raised from 9 to 15 g L^{-1}. Since phosphate buffer remained effective in limiting the decrease in pH caused by the improved growth, this culture was probably limited by another nutrient. The dry cell weight followed a linear growth from 24 h onwards for those cultures performed in a medium supplemented with 9 and 15 g L^{-1} of glycerol. This behavior, similar to that obtained by Carvalho et al. (2010) for *Bacillus subtilis*, is typical for a culture kinetically controlled by oxygen. Production of PHB may take place under nutrient limitation or continuously throughout microbial growth. *Azotobacter vinelandii* accumulate this biopolymer under oxygen limitation (Díaz-Barrera et al. 2019) and *Cupriavidus necator* accumulates PHB when the culture is limited by oxygen, nitrogen or phosphorous (Batt 2014), although PHB is a growth-associated product in *Azohydromonas lata* (Scott et al. 2021). Thus, data in Fig. 2 suggest that *A. cyptum* can grow to accumulate PHB when the culture is limited by oxygen. The maximum values for PHB content in Fig. 2c are in the range of 28% to 64% shown by Naranjo et al. (2013) for PHB accumulation using agro-industrial wastes and are significantly higher than those depicted in Fig. 1d.

Culture in bioreactor

Figure 3 proves the feasibility of translating the process one order of magnitude from 300 mL in flasks to 2 L in a bioreactor. The profile for PHB accumulation in Fig. 3b allowed us to identify three phases. The first one was characterized by a decrease in the PHB accumulation during the early 10 h, which was followed by a significant increase until the 80^{th} hour of culture, followed by a slight decrease until the end of the fermentation. Interestingly, the decrease in PHB content in the first phase coincided with exponential biomass growth and a decrease in the dissolved oxygen content. These observations are consistent with a reduced or nil PHB accumulation during this period, producing a dilution of the already accumulated PHB in the newly formed biomass. This is characteristic of the so-called non-growth associated PHB producers, such as *Cupriavidus necator*, where the fraction of PHB accumulated and the growth rate are inversely correlated (Henderson and Jones 1997).

Figure 3c shows that the second phase takes place when dissolved oxygen is low or negligible, supporting

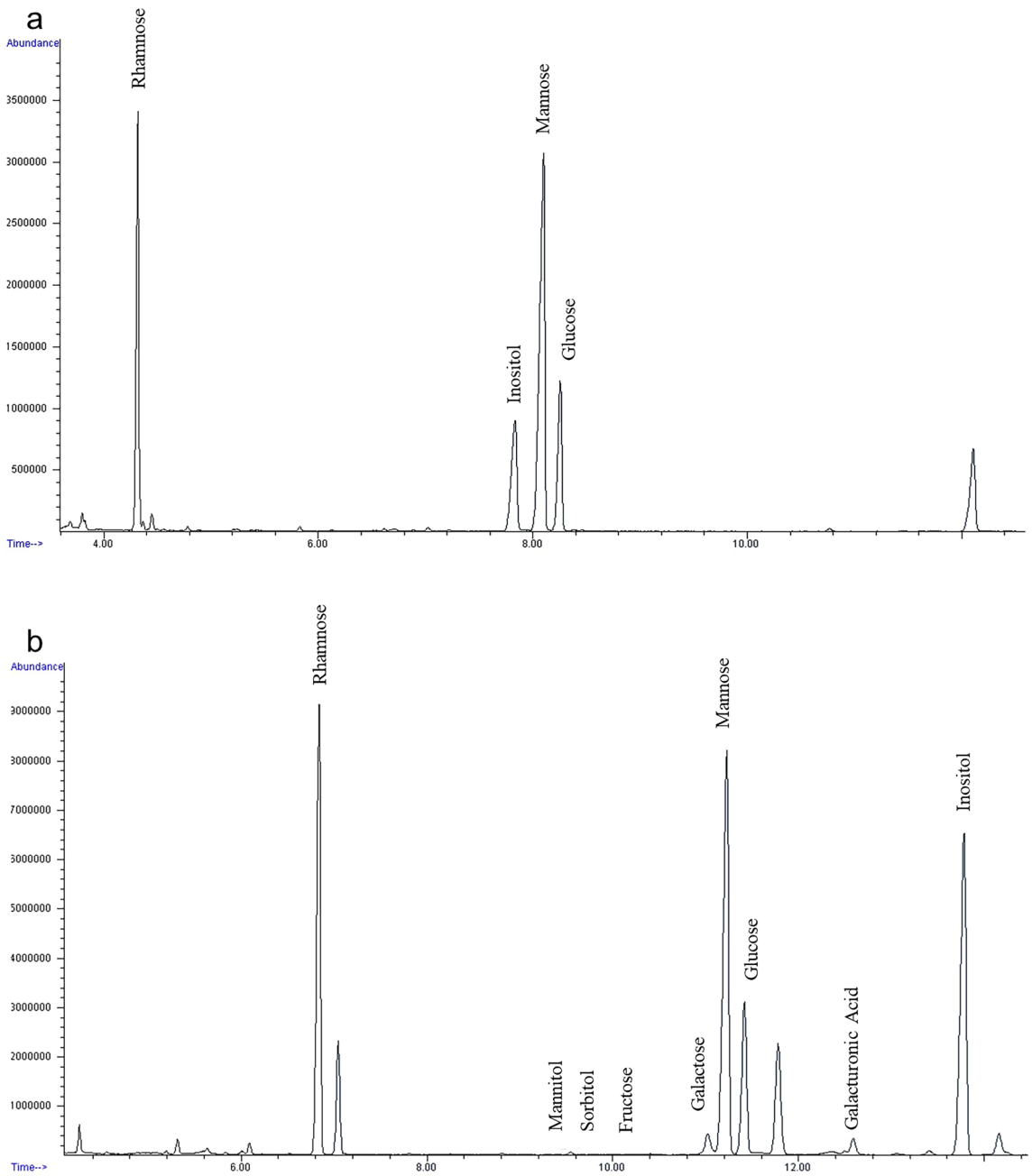

Fig. 5 GC–MS chromatograms obtained for the treatments of EPS recovered from the culture of *Acidiphilium cryptum* in a bioreactor using basal E medium (BEM) supplemented with glycerol 15 g L^{-1}. Derivatization to alditol acetates **a** and oxime-trimethylsilylates **b**

the premise that PHB accumulation in *A. cryptum* occurs under oxygen limitation in microaerobic conditions. As expected from Liu et al. (2017), the profile recorded for redox potential showed a similar pattern to that obtained for the dissolved oxygen. The average minimum value (8.19 ± 0.09 for pE + pH) was maintained until 80 h, coinciding this time with the maximum accumulation of PHB. From that point on, the redox potential tended to increase while the accumulation of PHB gradually decreased. Hence, this variable could be used for online monitoring the PHB production under microaerobic conditions (Liu et al. 2013). Recent reports have shown the convenience of using the redox potential to monitor the xylose fermentation with *Spathaspora passalidarum* (Bonan et al. 2020) and to guide the expression and harvesting of a recombinant protein in *Escherichia coli* (Guo et al. 2021). The discrepancy between measured dry biomass weight and calculated dry cell weight may be attributed to the loss

Table 2 Composition (mass percentage) of EPS recovered from the culture of *Acidiphilium cryptum* in bioreactor using basal E medium (BEM) and basal defined medium (BDM) supplemented with glycerol 15 g L^{-1}. The range for two measurements is shown in brackets

Sugar	BEM		BDM
	Alditol acetates method	Oxime-trimethylsilylates method	Alditol acetates method
Mannose	13.90 [1.48]	7.33 [0.54]	2.48 [0.14]
Rhamnose	10.02 [0.08]	7.23 [0.93]	1.72 [0.27]
Glucose	4.01 [0.76]	2.60 [0.41]	0.83 [0.01]
Xylose	ND	< 0.1	ND
Mannitol	ND	< 0.1	ND
Sorbitol	ND	< 0.1	ND
Fructose	ND	< 0.1	ND
Galactose	ND	< 0.1	ND
Galacturonic acid	ND	< 0.1	ND

ND Non-detected

of bound EPS during biomass washing before the drying process.

EPS analysis

The strong absorption band from 3600 to 3100 cm^{-1} in Fig. 4a represents hydroxyl (OH) stretching, and a medium band at 2949 cm^{-1} corresponds to C-H stretching from the aliphatic methylene group. Further, stretching at 1725 cm^{-1} corresponds to the carbonyl group of aldehydes while the intensive band at around 1651 cm^{-1} was attributed to the anion carbohydrate group (Vinoth et al. 2021). The band characteristic of polysaccharides around 1384–113 cm^{-1} corresponds to the vibration of sugar skeletal (Kavita et al. 2011). The FTIR spectrum for the EPS obtained using BDM showed similar peaks (Online Resource Fig. S3), while the spectrum of the inorganic residue (Fig. 4b) also showed the presence of sulphate ion.

The identification of glycosyl residues composing oligosaccharides and polysaccharides has been previously addressed by means of gas chromatography (e.g. coupled to detectors for mass spectrometry) after derivatization to their volatile forms such as oxime-trimethylsilyl ether and alditol-acetate derivatives (Wang and Fang 2004). In this study, the oxime-trimethylsilyl and alditol-acetate methods were successfully used for the derivatization and identification of the monosaccharides that were released from the EPS by the hydrolysis method. The repeated application of both methods allowed us to quantify the abundance of the most abundant compounds as shown in Table 2 showing a proportion near to 3.2:2.3:1 for mannose:rhamnose:glucose (calculated from alditol acetates measurements).

The synthesis of EPS has been pointed to be a key factor in achieve an effective microbial adhesion to ores in natural environments (Costa et al. 2018) or electrodes in bioelectrochemical systems (Verma et al. 2023). Despite rhamnose being a rare sugar (Roca et al. 2015), *Acinetobacter calcoaceticus* (Bryan et al. 1986) and *Pseudomonas fluorescens* (Fett et al. 1989) also produce EPS composed of rhamnose, mannose and glucose residues; derived from UDP-glucose (Li et al. 2020). As far as we know, this is the first report where *A. cryptum* has been cultured in a bioreactor, the poly(3-hydroxybutyrate) content has been consistently measured and the exopolymer has been analyzed for identification and quantification of glycosyl residues. Additionally, glycerol had not been used to produce biopolymers with *Acidiphilium* strains. Obtained findings may be used to improve the fermentation process using this or more productive strains, such as the native *A. cryptum* DX1-1 (Xu et al. 2013) or engineered strains, to develop industrial processes performed at low pH with low risk of culture contamination. Hence, this fermentation process may take part in a glycerol-based biorefinery to generate biodegradable polymers, derived from PHB and EPS, for substitution of petroleum-based plastics, dust suppression, metal chelation and food industry, among other applications.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00792-023-01313-3.

Author contribution EG, AD conceived and designed research. CZ, GZ, NM performed microbial cultures in shaken flasks and bioreactor. JPS performed EPS analysis by FTIR. MVR and AR performed the identification and quantification of glycosyl residues. EG, MVR and BP analyzed data. EG, FS and AD wrote the manuscript. All authors reviewed the manuscript.

Funding This study was funded by the National Agency for Research and Development (ANID), Chile, grants Fondecyt Iniciación 11180688 and Fondecyt Regular 1211434.

Data availability All data generated or analyzed during this study are included in this published article and the supplementary information file.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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

References

Antranikian G, Streit WR (2022) Microorganisms harbor keys to a circular bioeconomy making them useful tools in fighting plastic pollution and rising CO2 levels. Extremophiles 26:10. https://doi.org/10.1007/s00792-022-01261-4

Batt CA (2014) Alcaligenes. In: Batt CA, Tortorello ML (eds) Encyclopedia of food microbiology, 2nd edn. Academic Press, Oxford, pp 38–41

Beeler E, Singh OV (2016) Extremophiles as sources of inorganic bionanoparticles. World J Microbiol Biotechnol 32:156. https://doi.org/10.1007/s11274-016-2111-7

Bhattacharyya S, Chakrabarty BK, Das A, Kundu PN, Banerjee PC (1991) *Acidiphilium symbioticum* sp.nov., an acidophilic heterotrophic bacterium from *Thiobacillus ferrooxidans* cultures isolated from Indian mines. Can J Microbiol 37:78–85. https://doi.org/10.1139/m91-012

Bird LJ, Bonnefoy V, Newman DK (2011) Bioenergetic challenges of microbial iron metabolisms. Trends Microbiol 19:330–340. https://doi.org/10.1016/j.tim.2011.05.001

Bonan CIDG, Biazi LE, Dionísio SR, Soares LB, Tramontina R, Sousa AS, de Oliveira Filho CA, Costa AC, Ienczak JL (2020) Redox potential as a key parameter for monitoring and optimization of xylose fermentation with yeast *Spathaspora passalidarum* under limited-oxygen conditions. Bioprocess Biosyst Eng 43:1509–1519. https://doi.org/10.1007/s00449-020-02344-2

Bryan BA, Linhardt RJ, Daniels L (1986) Variation in composition and yield of exopolysaccharides produced by *Klebsiella* sp. strain K32 and *Acinetobacter calcoaceticus* BD4. Appl Environ Microbiol 51:1304–1308. https://doi.org/10.1128/aem.51.6.1304-1308.1986

Carvalho AL, Oliveira FH, Mariano RR, Gouveia ER, Souto-Maior AM (2010) Growth, sporulation and production of bioactive compounds by *Bacillus subtilis* R14. Braz Arch Biol Technol 53:643–652. https://doi.org/10.1590/S1516-89132010000300020

Chen G-Q, Jiang X-R (2018) Next generation industrial biotechnology based on extremophilic bacteria. Curr Opin Biotechnol 50:94–100. https://doi.org/10.1016/j.copbio.2017.11.016

Chettri D, Verma AK, Sarkar L, Verma AK (2021) Role of extremophiles and their extremozymes in biorefinery process of lignocellulose degradation. Extremophiles 25:203–219. https://doi.org/10.1007/s00792-021-01225-0

Coker J (2016) Extremophiles and biotechnology current uses and prospects. F1000Research. https://doi.org/10.12688/f1000research.7432.1

Costa OYA, Raaijmakers JM, Kuramae EE (2018) Microbial extracellular polymeric substances: ecological function and impact on soil aggregation. Front Microbiol. https://doi.org/10.3389/fmicb.2018.01636

Cummings DE, Fendorf S, Singh N, Sani RK, Peyton BM, Magnuson TS (2006) Reduction of Cr(VI) under acidic conditions by the facultative Fe(III)-reducing bacterium *Acidiphilium cryptum*. Environ Sci Technol 41:146–152. https://doi.org/10.1021/es061333k

Díaz-Barrera AROM, Andler R, Martínez I, Peña C (2016) Poly-3-hydroxybutyrate production by *Azotobacter vinelandii* strains in batch cultures at different oxygen transfer rates. J Chem Technol Biotechnol 91(1063):1071. https://doi.org/10.1002/jctb.4684

Díaz-Barrera A, Urtuvia V, Padilla-Córdova C, Peña C (2019) Poly(3-hydroxybutyrate) accumulation by *Azotobacter vinelandii* under different oxygen transfer strategies. J Ind Microbiol Biotechnol 46:13–19. https://doi.org/10.1007/s10295-018-2090-9

Fett WF, Osman SF, Dunn MF (1989) Characterization of exopolysaccharides produced by plant-associated fluorescent Pseudomonads. Appl Environ Microbiol 55:579–583. https://doi.org/10.1128/aem.55.3.579-583.1989

Fischer J, Quentmeier A, Gansel S, Sabados V, Friedrich C (2002) Inducible aluminum resistance of *Acidiphilium cryptum* and aluminum tolerance of other acidophilic bacteria. Arch Microbiol 178:554–558. https://doi.org/10.1007/s00203-002-0482-7

González E, Espada A, Muñoz JÁ, Blázquez ML, González F, Ballester A (2015) Reductive dissolution of magnetite and jarosite by *Acidiphilium cryptum* JF-5. Hydrometallurgy 157:292–297. https://doi.org/10.1016/j.hydromet.2015.08.003

González E, Rodríguez JM, Muñoz JÁ, Blázquez ML, Ballester A, González F (2018) The contribution of *Acidiphilium cryptum* to the dissolution of low-grade manganese ores. Hydrometallurgy 175:312–318. https://doi.org/10.1016/j.hydromet.2017.12.008

Guo J, Wu Y, Tanaka T, Lin Y-H (2021) Development of redox potential-driven fermentation process for recombinant protein expression. Biotechnol Lett 43:99–103. https://doi.org/10.1007/s10529-020-03030-9

Harrison AP (1981) *Acidiphilium cryptum* gen. nov., sp. nov., heterotrophic bacterium from acidic mineral environments. Int J Syst Bacteriol 31:327–332. https://doi.org/10.1099/00207713-31-3-327

Henderson RA, Jones CW (1997) Physiology of poly-3-hydroxybutyrate (PHB) production by *Alcaligenes eutrophus* growing in continuous culture. Microbiology 143:2361–2371. https://doi.org/10.1099/00221287-143-7-2361

Karr DB, Waters JK, Emerich DW (1983) Analysis of Poly-b-Hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exclusion high-pressure liquid chromatography and UV detection. Appl Environ Microbiol 46:1339–1344. https://doi.org/10.1128/aem.46.6.1339-1344.1983

Kavita K, Mishra A, Jha B (2011) Isolation and physico-chemical characterisation of extracellular polymeric substances produced by the marine bacterium *Vibrio parahaemolyticus*. Biofouling 27:309–317. https://doi.org/10.1080/08927014.2011.562605

Kishimoto N, Inagaki K, Sugio T, Tano T (1990) Growth inhibition of *Acidiphilium* species by organic acids contained in yeast extract. J Ferment Bioeng 70:7–10. https://doi.org/10.1016/0922-338X(90)90021-N

Koller M (2017) Production of poly hydroxyalkanoate (PHA) biopolyesters by extremophiles? MOJ Poly Sci 1:69–85. https://doi.org/10.15406/mojps.2017.01.00011

Küsel K, Dorsch T, Acker G, Stackebrandt E (1999) Microbial reduction of Fe(III) in acidic sediments: isolation of *Acidiphilium cryptum* JF-5 capable of coupling the reduction of Fe(III) to the oxidation of glucose. Appl Environ Microbiol 65:3633–3640. https://doi.org/10.1128/AEM.65.8.3633-3640.1999

Li L, Liu Z, Zhang M, Meng D, Liu X, Wang P, Li X, Jiang Z, Zhong S, Jiang C, Yin H (2020) Insights into the Metabolism and Evolution of the genus *Acidiphilium*, a typical acidophile in acid mine drainage. mSystems 5:e00867-e920. https://doi.org/10.1128/mSystems.00867-20

Link H, Weuster-Botz D (2011) 2.11 - Medium formulation and development. In: Moo-Young M (ed) Comprehensive biotechnology (Second Edition). Academic Press, Burlington, pp 119–134

Liu C-G, Xue C, Lin Y-H, Bai F-W (2013) Redox potential control and applications in microaerobic and anaerobic fermentations. Biotechnol Adv 31:257–265. https://doi.org/10.1016/j.biotechadv.2012.11.005

Liu C-G, Qin J-C, Lin Y-H (2017) Fermentation and redox potential. In: Jozala A (ed) Fermentation Processes. IntechOpen, Londan

Luef KP, Stelzer F, Wiesbrockb F (2015) Poly(hydroxy alkanoate)s in medical applications. Chem Biochem Eng Q 29:287–297. https://doi.org/10.15255/CABEQ.2014.2261

Monteiro MR, Kugelmeier CL, Pinheiro RS, Batalha MO, da Silva CA (2018) Glycerol from biodiesel production: Technological paths for sustainability. Renew Sustain Energy Rev 88:109–122. https://doi.org/10.1016/j.rser.2018.02.019

Naranjo JM, Posada JA, Higuita JC, Cardona CA (2013) Valorization of glycerol through the production of biopolymers: The PHB case using *Bacillus megaterium*. Bioresour Technol 133:38–44. https://doi.org/10.1016/j.biortech.2013.01.129

Notararigo S, Nácher-Vázquez M, Ibarburu I, Werning ML, de Palencia PF, Dueñas MT, Aznar R, López P, Prieto A (2013) Comparative analysis of production and purification of homo- and

hetero-polysaccharides produced by lactic acid bacteria. Carbohydr Polym 93:57–64. https://doi.org/10.1016/j.carbpol.2012.05.016

Palmeiro-Sánchez T, O'Flaherty V, Lens PNL (2022) Polyhydroxyalkanoate bio-production and its rise as biomaterial of the future. J Biotechnol 348:10–25. https://doi.org/10.1016/j.jbiotec.2022.03.001

Posch AE, Spadiut O, Herwig C (2012) Switching industrial production processes from complex to defined media: method development and case study using the example of *Penicillium chrysogenum*. Microb Cell Fact 11:88. https://doi.org/10.1186/1475-2859-11-88

Roca C, Alves VD, Freitas F, Reis MAM (2015) Exopolysaccharides enriched in rare sugars: bacterial sources, production, and applications. Front Microbiol. https://doi.org/10.3389/fmicb.2015.00288

Scott F, Yañez L, Conejeros R, Araya B, Vergara-Fernández A (2021) Two internal bottlenecks cause the overflow metabolism leading to poly(3-hydroxybutyrate) production in *Azohydromonas lata* DSM1123. J Environ Chem Eng 9:105665. https://doi.org/10.1016/j.jece.2021.105665

Sharma A, Parashar D, Satyanarayana T (2016) Acidophilic Microbes: Biology and Applications. In: Rampelotto PH (ed) Biotechnology of extremophiles: advances and challenges. Springer International Publishing, Cham, pp 215–241

Shi Y, Huang J, Zeng G, Gu Y, Chen Y, Hu Y, Tang B, Zhou J, Yang Y, Shi L (2017) Exploiting extracellular polymeric substances (EPS) controlling strategies for performance enhancement of biological wastewater treatments: an overview. Chemosphere 180:396–411. https://doi.org/10.1016/j.chemosphere.2017.04.042

Sirohi R, Prakash Pandey J, Kumar Gaur V, Gnansounou E, Sindhu R (2020) Critical overview of biomass feedstocks as sustainable substrates for the production of polyhydroxybutyrate (PHB). Bioresour Technol 311:123536. https://doi.org/10.1016/j.biortech.2020.123536

Sriyapai T, Chuarung T, Kimbara K, Samosorn S, Sriyapai P (2022) Production and optimization of polyhydroxyalkanoates (PHAs) from *Paraburkholderia* sp. PFN 29 under submerged fermentation. Electron J Biotechnol 56:1–11. https://doi.org/10.1016/j.ejbt.2021.12.003

Tapia JM, Muñoz JA, González F, Blázquez ML, Malki M, Ballester A (2009) Extraction of extracellular polymeric substances from the acidophilic bacterium *Acidiphilium* 3.2Sup(5). Water Sci Technol 59:1959–1967. https://doi.org/10.2166/wst.2009.192

Tapia JM, Muñoz JA, González F, Blázquez ML, Ballester A (2011) Mechanism of adsorption of ferric iron by extracellular polymeric substances (EPS) from a bacterium *Acidiphilium* sp. Water Sci Technol 64:1716–1722. https://doi.org/10.2166/wst.2011.649

Tapia JM, Muñoz J, González F, Blázquez ML, Ballester A (2016) Sorption of ferrous iron by EPS from the acidophilic bacterium *Acidiphilium* Sp a mechanism proposal. Rev Metal 52:e076. https://doi.org/10.3989/revmetalm.076

Tourney J, Ngwenya BT (2014) The role of bacterial extracellular polymeric substances in geomicrobiology. Chem Geol 386:115–132. https://doi.org/10.1016/j.chemgeo.2014.08.011

Verma M, Singh V, Mishra V (2023) Moving towards the enhancement of extracellular electron transfer in electrogens. World J Microbiol Biotechnol 39:130. https://doi.org/10.1007/s11274-023-03582-8

Vinoth S, Gurusaravanan P, Arun M, Saradhadevi M, Senthilkumar N, Gowtham P, Sivakumar SR (2021) Biostimulant activity of sulfated polysaccharide extract from red seaweed *Halymenia dilatata* on yield of Mung bean in greenhouse conditions. J Appl Phycol 33:3309–3317. https://doi.org/10.1007/s10811-021-02507-y

Wang Q, Fang Y (2004) Analysis of sugars in traditional Chinese drugs. J Chromatogr B 812:309–324. https://doi.org/10.1016/j.jchromb.2004.09.027

Wen Q, Liu B, Li F, Chen Z (2020) Substrate strategy optimization for polyhydroxyalkanoates producing culture enrichment from crude glycerol. Bioresour Technol 311:123516. https://doi.org/10.1016/j.biortech.2020.123516

Xu A-L, Xia J, Liu K, Li L, Yang Y, Nie Z, Qiu G (2010) Real-time PCR Analysis of metabolic pathway of PHB in *Acidiphilium cryptum* DX1–1. J Microbiol Biotechnol. https://doi.org/10.4014/jmb.0906.06054

Xu A-l, Xia J-l, Song Z-w, Jiang P, Xia Y, Wan M-x, Zhang R-y, Yang Y, Liu K-k (2013) The effect of energy substrates on PHB accumulation of *Acidiphilium cryptum* DX1-1. Curr Microbiol 67:379–387. https://doi.org/10.1007/s00284-013-0373-y

Zhang J, Greasham R (1999) Chemically defined media for commercial fermentations. Appl Microbiol Biotechnol 51:407–421. https://doi.org/10.1007/s002530051411