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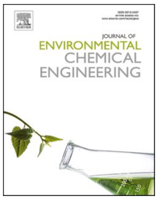

Dynamics in the profile of biopolymers produced by mixed microbial cultures from ethanol-rich feedstocks

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ABSTRACT

The use of agro-industrial wastes as substrates for polyhydroxyalkanoate (PHA) production by mixed microbial cultures is an alternative to lower the price of these biopolymers. However, when submitting these wastes to pre-fermentation, lactate and ethanol can be produced in addition to volatile fatty acids (VFAs). In this study, the impact of ethanol on PHA storage and in the carbon flux of the process were assessed. The co-production of extracellular polymeric substances (EPS) and its impact on PHA production was investigated. PHA production yield showed a decrease from 0.92 ± 0.004–0.63 ± 0.002 Cmol-PHA.Cmol-S^{-1} with the increase of ethanol content from 5 to 20 Cmmol.L^{-1}. EPS production was directly proportional to the ethanol's fed, reaching a maximum EPS total content of 6.70 Cmmol for the maximum ethanol supplied. EPS acted as a protection strategy, which benefited PHA specific storage rate for the highest ethanol content tested. These results indicate that ethanol-rich feedstocks can be valorised through the simultaneous production of two value-added biopolymers.

1. Introduction

The environmental concern regarding the build-up of petrochemical plastic residues across the globe is continuously growing. Researchers have been focusing on the development of fully biodegradable alternatives, such as polyhydroxyalkanoates (PHAs). PHAs are biodegradable, biocompatible and environmentally friendly thermoplastics. Similar to many petrochemical plastics currently in use, PHA vary in flexibility and hardness, according on their monomeric composition, and they can be used in various applications [1].

Researchers are presently working towards decreasing the cost of these biopolymers to make them more price-competitive comparing with the conventional plastics. The combination of using mixed microbial cultures (MMCs) and low-cost/zero value agro-industrial wastes as substrate can significantly lower PHA production costs, since the needs of maintaining sterile conditions and of using refined substrates are eliminated [2]. PHA production by MMCs using agro-industrial wastes is usually performed with a three-step process comprising: (1) the acidogenic fermentation stage, where the feedstock is fermented to obtain a volatile fatty acid (VFA) enriched stream that is used in the subsequent steps; (2) the culture selection stage, for the production of biomass with high PHA storage ability and (3) the PHA accumulation stage, operated in fed-batch mode to maximize PHA cell content [3].

VFAs are the predominant products in pre-fermented feedstocks [4], but other types of fermentation products (such as lactate, ethanol, succinate and glycerol) can be present at high concentrations in fermented wastewaters as well. Temudo et al. [5] observed that in an open mixed culture fermentation system, fermentation products distribution shifted from acetate, butyrate and hydrogen at lower pH (4.0 – 6.5) into ethanol, acetate and formate at higher pH (6.5 – 8.5). Moreover, Hofvendahl and Hahn-Hägerdal [6] also showed that operating factors such as temperature, pH, fermentation mode and type of carbon source can act as a trigger to lactate production in the acidogenic fermentation process. Zheng et al. [7] also demonstrated that, during fermentation of vegetable and fruit wastes, after the spontaneous pH decrease to 3.0 ~ 4.0, a stable ethanol-type fermentation was obtained. At such low pH

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values, the fermentation can diminish the variety of fermentation products since only a few microorganisms, namely ethanol-producing bacteria, would survive at these low pH conditions. Indeed, temporary shifts in the operation conditions of the acidogenic fermentation reactor can originate high amounts of compounds other than VFAs, which are the typical PHA precursors used for MMC PHA production. One advantage of working with MMCs is their continuous ability to adapt to changes in the feedstock composition [2]. PHA production from substrates other than VFAs is not the subject of frequent research but gains importance when acidogenic reactors can routinely yield high concentrations of non-VFA compounds. This is an important aspect to be investigated since the composition of fermented products will directly influence the monomeric composition of the polymers and consequently their properties and applications.

Presence of high ethanol concentrations in the fermented stream may also have impact on the PHA production process performance since this product is considered inhibitory for most of bacteria. The adverse effects of ethanol on bacterial growth, viability, and metabolism are caused primarily by ethanol-induced leakage of the plasma membrane [8]. Nevertheless, previous work [9] validated that it is possible to perform the selection of a robust PHA accumulating MMC using a complex feedstock rich in lactate and ethanol and still obtain significant PHA yields and productivities, although lower than the obtained for other VFAs. However, the impact of ethanol on the PHA production process, namely on the yield and productivity in the presence of high contents of ethanol is still to be explored. Additionally, it is important to investigate the carbon flux in these conditions, as ethanol is known to trigger various defence mechanisms in response to its potentially inhibitory effect. MMCs are known to produce various polymers other than PHA in response to stressors. Simultaneous PHA synthesis with other value-added products like microbial proteins, polysaccharides and carotenoids was already investigated. Depending on their characteristics, these co-products can be commercialized separately, therefore improving the economic viability of the PHA production process [10]. Concomitant production of PHA and extracellular polymeric substances (EPS) has been reported in numerous microorganisms such as *Azotobacter beijerinckii, Anabaena cylindrica, Azotobacter chroococcum, Sinorhizobium meliloti, Cupriavidus Necator, Pseudomonas* sp. and *Haloferax mediterranei* [11]. EPS are composed by a highly hydrated matrix, that originates a polymeric network where the cells are implanted. They are constituted mainly by polysaccharides and proteins, although lipids, humic acids and extracellular DNA can be also present [12]. EPS presents numerous physiological functions, including protection from environmental stress factors (e.g. temperature, osmotic stress, pH, heavy metals, damage by UV light, desiccation), and can provide cell adherence to surfaces and also carbon and water storage reserves. If bacteria produce EPS as a protection pathway against ethanol, this will reduce the PHA yield and productivity since part of the carbon substrate is deviated to EPS instead of PHA. However, there is no current knowledge about the effect of ethanol on the carbon flux in a MMC PHA production process.

The goal of this study was to investigate the impact of ethanol in driving the carbon flux in a MMC PHA production system away from PHA and into the production of an EPS protection matrix. The impact of different concentrations of ethanol on the yield and productivity of PHA in the presence of acetic, butyric and lactic acids was also assessed. This study is important to assess the impact of the presence of ethanol in the substrate on the metabolism of MMC PHA production systems, to be able to manage the process accordingly for an optimal performance.

2. Materials and methods

2.1. Culture selection setup

A 2 L sequencing batch reactor (SBR) was inoculated with activated sludge retrieved from the municipal wastewater treatment plant of Mutela, Almada, Portugal. The SBR was operated with a sludge retention time (SRT) of 4 d, hydraulic retention time (HRT) of 1 d and organic loading rate (OLR) of 100 Cmmol.L^{-1}.d^{-1}. Several timers were used to control the reactor aeration and stirring, feeding and withdrawing. Reactor operation was performed in a temperature-controlled room (20°C) using a Feast and Famine regime with 12 hours cycles. Reactor cycles comprised 11 hours of reaction, 40 minutes of settling, 10 minutes of withdrawing of 1 L of supernatant, which was then substituted by 1 L of renewed medium in the first 5 minutes of the next cycle. pH was maintained in the range of 6.50–8.20 using automatic dosing of 0.5 M NaOH and 0.5 M HCl.

The carbon feeding solution was prepared by dilution in a nutrient mineral solution with a composition described by Serafim et al. [13] This medium contained lactic acid (20 Cmmol.L^{-1}), acetic acid (10 Cmmol.L^{-1}), butyric acid (10 Cmmol.L^{-1}) and ethanol (10 Cmmol.L^{-1}), as carbon source with concentrations determined to attain the intended OLR. This profile of soluble fermentation products (SPFs) was selected in order to mimic the SFPs profile of the fermented wastewater used in a previous study [9]. A solution of NH_4Cl and KH_2PO_4 corresponding to a C/N/P ratio of 100:7:1 (mol basis) was supplied at the start of the famine phase [14].

Aeration was supplied by an air compressor, maintaining the dissolved oxygen (DO) above 2 mgO_2.L^{-1}. Analog sensors (Mettler Toledo, LLC, Columbus, OH, USA) were used for online measurement of DO, temperature and pH. Reactor stirring was performed at 250 rpm by a double impeller stainless steel shaft.

The reactor was monitored regularly by the collection of samples during the whole cycle length. Samples were analysed using the analytical methods described in the Section 2.3.

2.2. Fed-batch assays

Using the biomass selected in the SBR, several fed-batch tests were performed in duplicate with the objective of evaluating the PHA accumulation potential of the selected culture and to investigate the culture's response to different ethanol contents. The tests were carried out using an agitation of 250 rpm, airflow of 3.0 L.min^{-1}, pH of 8.50 and temperature of 20 ºC in a New Brunswick™ BioFlo®/CelliGen® 115 system (Eppendorf AG, Germany) with a 1 L working volume.

The composition of the feedstock used in each one of the tests is presented in Table 1. Four tests (A, B, C and D) were performed using only ethanol as carbon source at increasing concentrations (5, 10, 15 and 20 Cmmol.L^{-1}). For Test E, a feed with the same SFP profile as the one fed to the SBR was used, consisting of 20 Cmmol.L^{-1} lactic acid, 10 Cmmol.L^{-1} acetic acid, 10 Cmmol.L^{-1} butyric acid and 10 Cmmol.L^{-1} ethanol. For Test F, the concentration of ethanol was increased to 15 Cmmol.L^{-1}, maintaining the concentrations of all the other components, resulting in a slight increase of organic load when comparing to the culture selection SBR. Nitrogen and phosphorus limitation was applied in all the tests in order to maximize PHA accumulation [15]. In order to follow the carbon consumption and the intracellular PHA accumulation, samples were collected throughout the assays. By the end of each assay, the centrifugation of the cultivation broth was performed (10,000 rpm, 15 minutes) and the cell-free supernatant was used for EPS

Table 1
Carbon source compositions tested in fed-batch accumulation assays.

	Concentrations (Cmmol.L^{-1})				
Test	Ethanol	Lactic Acid	Acetic Acid	Butyric Acid	Total
A	5				5
B	10				10
C	15				15
D	20				20
E	10	20	10	10	50
F	15	20	10	10	55

quantification.

2.3. Bioreactor monitoring

The samples collected from the SBR and the fed-batch reactor were centrifuged (10,000 rpm, 3 min) for cell removal, the supernatant was filtered using 0.20 µm syringe membrane filters and the pellets were freeze-dried. The supernatant was used for quantification of SFPs and ammonia and the pellets were used for PHA quantification.

PHA was determined by gas chromatography (GC) with flame ionization detector as described by Lanham et al. [16] SFPs (lactate, ethanol, acetate and butyrate) were quantified by high-performance liquid chromatography (HPLC) and soluble ammonia concentrations were determined by a segmented continuous flow analyser, according to the method described by Oliveira et al.[15] Total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to the standard methods [17].

2.4. EPS characterization at the end of the fed-batch accumulation assays

EPS quantification at the end of all fed-batch accumulation assays was performed on cell-free supernatants as previously reported by Concórdio-Reis et al. [18] For each sample, dialysis of 30 mL of cell-free supernatant was performed using 12 kDa MWCO membranes (Zellu-Trans/Roth) using constant stirring at room temperature against deionized water. Conductivity of the water was monitored for 48 hours until it reached a value lower than 10 µS.cm^{-1}. To inhibit biological degradation of the EPS during the dialysis, sodium azide (10 mg.L^{-1}) was used. By the end of the dialysis, the content of each membrane was freeze-dried and its gravimetric quantification was performed.

As described by Antunes et al. [19], HPLC after hydrolysis was used to determine the sugar composition of the EPS samples. 5 mg of the freeze-dried samples were dissolved in 5 mL of deionized water and hydrolysis with trifluoroacetic acid (TFA) (0.1 mL TFA 99%) was performed at 120 °C for 2 h. The determination of the monosaccharides of these hydrolysates was performed by HPLC using a CarboPac PA10 column (Thermo Dionex).

Elemental analysis of the freeze-dried samples was performed by a Flash EA 1112 CHNS analyser (Thermo Scientific, Waltham, MA, USA). Eq. 1 was used to estimate the protein content:

$$\text{Protein} = 6.25 \times \text{N} \tag{1}$$

where 6.25 is the conversion factor of nitrogen-to-protein and N symbolises the total nitrogen [20].

Total organic carbon (TOC) determination for samples of a known EPS concentration allowed to obtain the total carbon concentration in g-C.L^{-1}, which was then converted to C-mmol.L^{-1}. The supernatant (filtered with a 0.45 µm syringe filter) was analysed using a Total Organic Carbon Analyser (TOC-V$_{CSH}$ Shimadzu). TOC quantification was performed to all EPS samples in order to convert EPS concentration in g.L^{-1} (obtained by gravimetric quantification) to C-mmol.L^{-1}.

2.5. Calculations

The active biomass concentration (X_A, g.L^{-1}) was estimated by deducting the PHA concentration (g.L^{-1}) from the VSS concentration (g.L^{-1}) according to Eq. 2. Generic chemical formula for the biomass $C_5H_7NO_2$ was applied to express the X_A in Cmol (22.6 g.Cmol^{-1}).

$$X_A \quad (g.L^{-1}) = VSS \quad (g.L^{-1}) - PHA \quad (g.L^{-1}) \tag{2}$$

The percentage of PHA content in the biomass (wt. basis) was determined in terms of percentage of TSS. PHA concentration (in g.L^{-1}) was estimated by multiplying the intracellular PHA content by the biomass concentration (VSS) according to Eq. 3.

$$PHA \quad (g.L^{-1}) = \%PHA \quad (wt.basis) \times VSS \quad (g.L^{-1}) \tag{3}$$

Feast to famine ratio (F/F) was determined by dividing the duration of the feast phase by the duration of the famine phase (Eq. 4). The moment of depletion of all the SFPs (end of feast) was related with the sudden increase in DO and was previously confirmed by the SFPs determination by HPLC.

$$F\Big/F = \frac{Feast \quad phase \quad duration(h)}{Famine \quad phase \quad duration(h)} \tag{4}$$

The total substrate concentration (S) corresponded to the sum of all constituents of the feedstock (lactate, acetate, ethanol and butyrate) in Cmmol.L^{-1}. Specific uptake rate (-q$_S$, Cmol-S.Cmol-X_A^{-1}.L^{-1}) and specific PHA storage rate (q$_{PHA}$, Cmol-PHA.Cmol-X_A^{-1}.L^{-1}) were determined using the linear regression of the SFPs and PHA specific concentrations, respectively, plotted over time.

The volumetric PHA production rate (r$_{PHA}$, g-PHA.L^{-1}.h^{-1}) was calculated by dividing the produced PHA concentration by the time elapsed according to Eq. 5.

$$r_{PHA} = \frac{PHA_{prod.}(g - PHA.L^{-1})}{Time(h)} \tag{5}$$

The PHA storage yield (Y$_{PHA/S}$, Cmol-PHA.Cmol-S^{-1}) was estimated by dividing the total amount of produced PHA by the total amount of consumed SFPs/ethanol according to Eq. 6.

$$Y_{PHA/S} = \frac{PHA_{prod}.(Cmol - PHA.L^{-1})}{SFP_{cons.}(Cmol - S.L^{-1})} \tag{6}$$

The EPS production yield (Y$_{EPS/S}$, Cmol-EPS.Cmol-S^{-1}) was calculated as per Eq. 7 by dividing the total amount of EPS produced by the total amount of SFPs/ethanol consumed.

$$Y_{EPS/S} = \frac{EPS_{prod.}(Cmol - EPS.L^{-1})}{SFPs_{cons.}(Cmol - S.L^{-1})} \tag{7}$$

3. Results and discussion

3.1. Effect of ethanol concentration on PHA production from mixed precursors

3.1.1. Culture selection

The selection SBR was fed with medium containing lactic acid (20 Cmmol.L^{-1}), acetic acid (10 Cmmol.L^{-1}), butyric acid (10 Cmmol.L^{-1}) and ethanol (10 Cmmol.L^{-1}), maintaining an OLR of 100 Cmmol.L^{-1}d^{-1}. The performance of the SBR was monitored regularly, and when a stabilization of the biomass concentration and F/F was observed, the reactor was considered to be in a stable state. Figure I available in Supplementary Material shows the evolution of the F/F and the active biomass (X_A) concentration during the SBR operation. The stabilization of these two parameters occurred remarkedly fast, around the 20th day of operation, so a stable period was assumed to be achieved at this moment. During the stable period, several cycles were monitored and kinetic and stoichiometric parameters were determined for these cycles.

Fig. 1 illustrates one SBR cycle during the stable period, where the profiles of SFPs, %PHA, X_A and ammonia are displayed. The cycle starts with the replenishment with fresh media, so in the beginning (t=0 h) the concentration of SFPs is at its maximum value, accounting a total of 50 Cmmol-SFP.L^{-1}. There is a clear preference of the culture for acetic, lactic and butyric acids, and ethanol is the last substrate to be consumed. Ethanol is barely consumed when acetic and butyric acids are available, and ethanol consumption only escalates after their depletion. The specific substrate uptake rates of the selected MMC during the stable period also demonstrated the preference for acetate consumption (0.066 ± 0.01 Cmol.Cmol-X_A^{-1}.h^{-1}), followed by lactate (0.055 ± 0.02 Cmol.Cmol-X_A^{-1}.h^{-1}) and butyrate (0.054 ± 0.01 Cmol.Cmol-X_A^{-1}.h^{-1}).

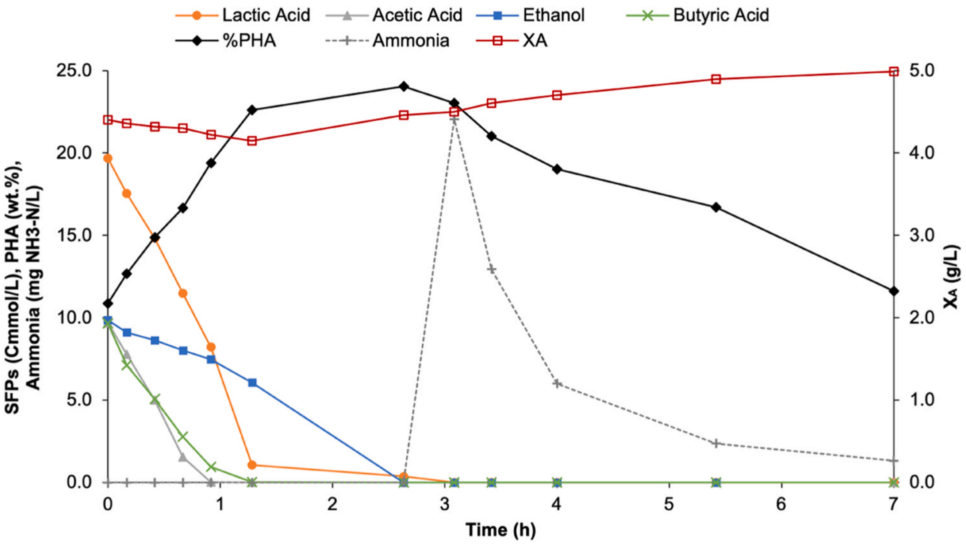

Fig. 1. Concentration profiles for %PHA, SFP, ammonia and X_A during a cycle in the stable operation stage of the SBR, corresponding to the 21th day of operation.

Ethanol presented a much lower specific uptake rate of 0.021 ± 0.002 Cmol.Cmol-X_A^{-1}.h^{-1}. The culture selected in previous work [9] presented a different trend of substrate preference. That culture presented a preference for lactate (0.16 ± 0.03 Cmol.Cmol-X_A^{-1}.h^{-1}), followed by ethanol (0.080 ± 0.04 Cmol.Cmol-X_A $^{-1}$.h^{-1}) and acetate (0.073 ± 0.03 Cmol.Cmol-X_A^{-1}.h^{-1}). However, it should be noted that in the previous work a complex feedstock was used containing higher concentrations of lactate and ethanol (26.7 and 20.4 Cmmol.L^{-1}, respectively). Moreover, despite the same operational conditions (SRT, HRT, OLR) being used in both works, a different microbial culture was selected with different substrate preferences which was likely dependent on the feedstock composition.

Throughout the stable period, the average F/F was of 0.19 ± 0.02 h.h^{-1} and the PHA yield was of 0.93 ± 0.02 Cmol-PHA.Cmol-S^{-1}. A low F/F (< 0.2) ensures the physiological adaptation of the microorganisms, favouring PHA storage in the feast phase [3]. At the end of the feast phase, the PHA content in the cells was 27.6 ± 3.6% (w/w). These values are higher than those obtained in our previous work using real ethanol-rich feedstock [9], where the PHA yield was of 0.80 ± 0.09 Cmol-PHA.Cmol-S^{-1} and maximum PHA cell content was of 17.7 ± 1.9% (w/w). The other closest comparison that we found in the literature was Jiang at al. [21], who selected PHA accumulating MMCs using lactate and a mixture of lactate and acetate. However, Jiang et al. [21] used a feedstock with no ethanol and the total carbon concentration supplied to the selection reactor was around 20 Cmmol.L^{-1}, while in the present study the carbon concentration in the SBR after feeding was 50 Cmmol.L^{-1}. Notwithstanding, Jiang et al. [21] obtained PHA storage yields between 0.60 and 0.67 Cmol-PHA.Cmol-S^{-1} for the feast phase of the SBR, which is lower than the obtained in the present study (0.93 ± 0.02 Cmol-PHA.Cmol-S^{-1}). The high PHA yields are a strong indicator that all the carbon sources were consumed and converted to PHA, even the ethanol. Beccari et al. [22] selected a PHA accumulating MMC using a fermented olive mill effluent containing 17% of the overall COD in ethanol and observed a substantial contribution to the PHA production from substrates other than VFAs, especially for ethanol. Poly (3-hydroxybutyrate) (P(3HB)) was the only polymer produced by the selected culture in the current study. Since the feedstock was constituted by lactate, ethanol, acetate and butyrate, these results are coherent with prior findings suggesting that these SFPs are metabolized directly to P(3HB).

3.1.2. *Accumulation assays*

After the SBR reached the stable state, accumulation tests were performed aiming at evaluating the influence of ethanol content on the PHA storage capacity and on trends of consumption of other carbon sources, at two different ethanol contents: 10 (test E) and 15 Cmmol.L^{-1} (test F).

For the Test E, four pulses of feeding were supplied, which took 7.1 ± 0.2 h to be consumed, while Test F with only three pulses of feeding, had a duration of 9.0 ± 0.04 h, as shown in Fig. 2. Replicates of tests E and F are presented in the Supplementary material section (Figure II). The fact that the culture took much longer to consume all the substrate in the second condition was already a preliminary indicator of the negative impact of ethanol concentration on the process performance, and for that reason the duration of the accumulation assays in test F was not further extended.

Increasing the ethanol concentration in the PHA accumulation step in 5 Cmmol.L^{-1}, compared to the content that the biomass was acclimatized to in the SBR (10 Cmmol.L^{-1}), had a very significant impact on the PHA production performance (Table 2). For the ethanol concentration of 15 Cmmol.L^{-1}, the maximum PHA cell content, PHA production yield, volumetric production rate and PHA storage rate were significantly lower than for 10 Cmmol.L^{-1}. The increase of ethanol concentration from 10 Cmmol.L^{-1} to 15 Cmmol.L^{-1} resulted in a decrease in the PHA production yield from 0.82 ± 0.03–0.61 ± 0.005 Cmol-PHA.Cmol-S^{-1}, and in the PHA production rate from 0.13 ± 0.01–0.059 ± 0.003 Cmol-PHA.Cmol-X_A^{-1}.h^{-1}. The decrease of these parameters with the increase of ethanol content is a clear indicator of the ethanol's inhibitory effect on PHA production. P(3HB) was the only polymer produced in these assays.

For both the conditions, the culture showed a preference for acetic, lactic and butyric acids, and ethanol showed the lowest specific uptake rate (Table 2), similarly to what had been observed in the culture selection SBR. As presented in Table 2, ethanol uptake rate for Test F was slightly lower than for the Test E, 0.031 ± 0.002 Cmol.Cmol-X_A^{-1}.h^{-1} and 0.035 ± 0.001, respectively.

Interestingly, a lower PHA yield was obtained for the accumulation assay E (0.82 ± 0.03 Cmol-PHA.Cmol-S^{-1}) than for the SBR (0.93 ± 0.02 Cmol-PHA.Cmol-S^{-1}) that was operated with the same feedstock composition and concentration. Since the goal of the PHA accumulation step is to maximize PHA storage, this result is noteworthy and suggests that a fraction of the carbon in the fed-batch tests was deviated to another metabolic product.

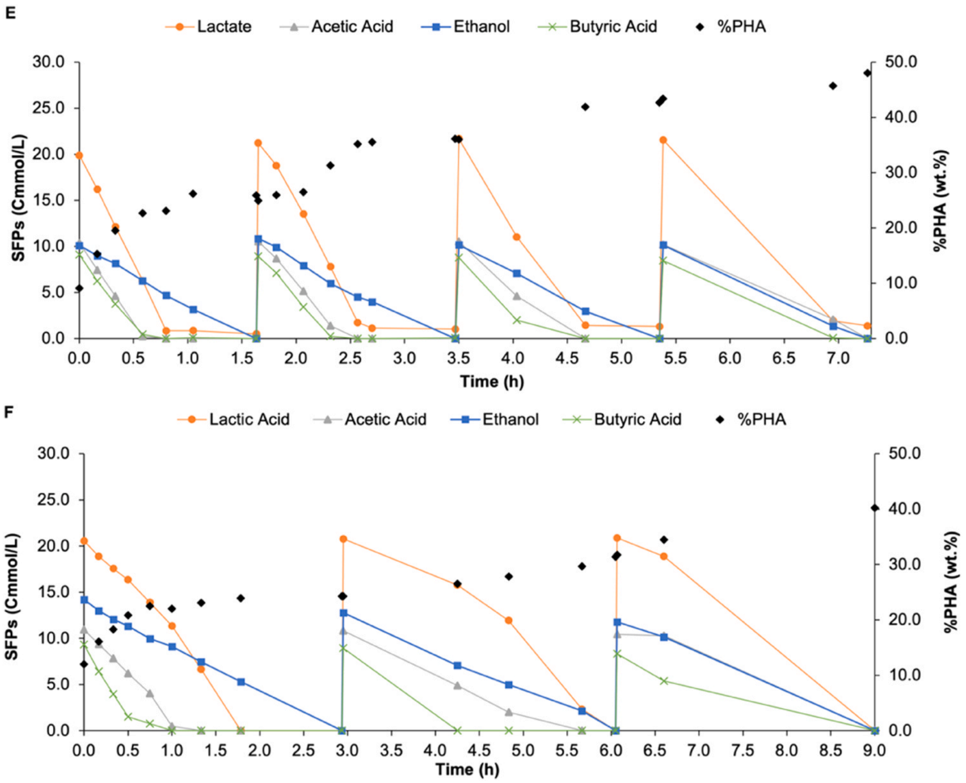

Fig. 2. Results obtained for the accumulation assays performed for ethanol concentrations of 10 Cmmol.L^{-1} (test E) and 15 Cmmol.L^{-1} (test F), with the selected biomass taken from the SBR. Two replicates were performed for each condition, only one is represented.

Table 2

Performance parameters from the accumulation assays E and F. Values presented are mean ± standard deviation. S refers to total SFPs supplied in the test.

	E	F
Ethanol concentration per pulse (Cmmol.L^{-1})	10	15
Overall accumulation performance		
PHA^{max} (wt%)	46.5 ± 1.5	40.7 ± 0.5
$Y_{PHA/S}$ (Cmol-PHA.Cmol-S^{-1})	0.82 ± 0.03	0.61 ± 0.005
r_{PHA} (g-PHA.L^{-1}.h^{-1})	0.53 ± 0.004	0.23 ± 0.002
q_{PHA} (Cmol-PHA.Cmol-X_A^{-1}.h^{-1})	0.13 ± 0.01	0.059 ± 0.003
Maximum substrate uptake rates (-q^{max})		
Lactic Acid (Cmol.Cmol-X_A^{-1}.h^{-1})	0.122 ± 0.001[a]	0.061 ± 0.009[a]
Acetic Acid (Cmol.Cmol-X_A^{-1}.h^{-1})	0.065 ± 0.004[a]	0.060 ± 0.005[a]
Ethanol (Cmol.Cmol-X_A^{-1}.h^{-1})	0.035 ± 0.001[a]	0.031 ± 0.002[a]
Butyric Acid (Cmol.Cmol-X_A^{-1}.h^{-1})	0.061 ± 0.001[a]	0.056 ± 0.001[a]

[a] maximum value was calculated for the first pulse of each test.

3.2. Effect of ethanol concentration on PHA production in the absence of other carbon sources

Though all ethanol and other available carbon sources were completely consumed in each pulse, the decrease in the PHA production yield from 0.82 ± 0.03–0.61 ± 0.005 Cmol-PHA.Cmol-S^{-1} when the ethanol concentration increased from 10 to 15 Cmmol.L^{-1} seems to indicate that in Test F a higher fraction of the carbon was used for production of other metabolic products or for cell maintenance rather than for PHA synthesis.

In order to better elucidate the impact of ethanol concentration on PHA production yield and productivity, fed batch tests were performed with ethanol as sole carbon source using the selected culture from the SBR. Four assays with increasing ethanol concentrations (A: 5 Cmmol.L^{-1}, B: 10 Cmmol.L^{-1}, C: 15 Cmmol.L^{-1} and D: 20 Cmmol.L^{-1}) were performed in duplicate (R1 and R2). In these tests, two feeding pulses of ethanol were supplied to biomass retrieved from the culture selection SBR at the end of the famine phase. The second pulse was supplied when an increase in the DO was detected, indicating that all the substrate was exhausted. All tests were performed in the absence of ammonia and phosphate in order to limit biomass growth and promote PHA production.

Fig. 3 represents the evolution of intracellular PHA content (wt%) and bulk ethanol concentration during assays A, B, C and D. Each condition was tested in duplicate (R1 and R2). Kinetic and stoichiometric parameters were also calculated for the four conditions and are presented in Table 3.

As seen in Fig. 3 and Table 3, the selected culture was able to consume all the ethanol available and to produce PHA in all the assays, even at the highest ethanol concentration tested (20 Cmmol.L^{-1}, equivalent to 0.46 g.L^{-1}). For all the assays, the only polymer produced was P(3HB). The highest PHA volumetric productivity and yield of 0.13 ± 0.002 g-PHA.L^{-1}.h^{-1} and 0.92 ± 0.004 Cmol-PHA.Cmol-S^{-1} respectively, were registered for the minimum ethanol concentration tested (Test A). For the highest ethanol concentration of ~20 Cmmol.L^{-1} (Test D), the minimum PHA yield of 0.63 ± 0.002 Cmol-PHA.Cmol-S^{-1} was registered. Given that all ethanol was consumed, and no ammonia was supplied, the missing carbon (almost 40% in Test D) cannot be assigned to cell growth, but likely to another metabolic product.

By plotting the PHA yield and specific storage rate as a function of total ethanol supplied (Fig. 4), the results show that: 1) the PHA yield decreased with the ethanol content, suggesting deviation of the carbon to another product; 2) the specific PHA storage rate first decreased between ethanol concentrations of 5–15 Cmmol.L^{-1} and then increased for the highest ethanol content tested, seeming to partially overcome the ethanol inhibitory effect on metabolism. These results led us to hypothesise that for higher ethanol contents the culture deviated its

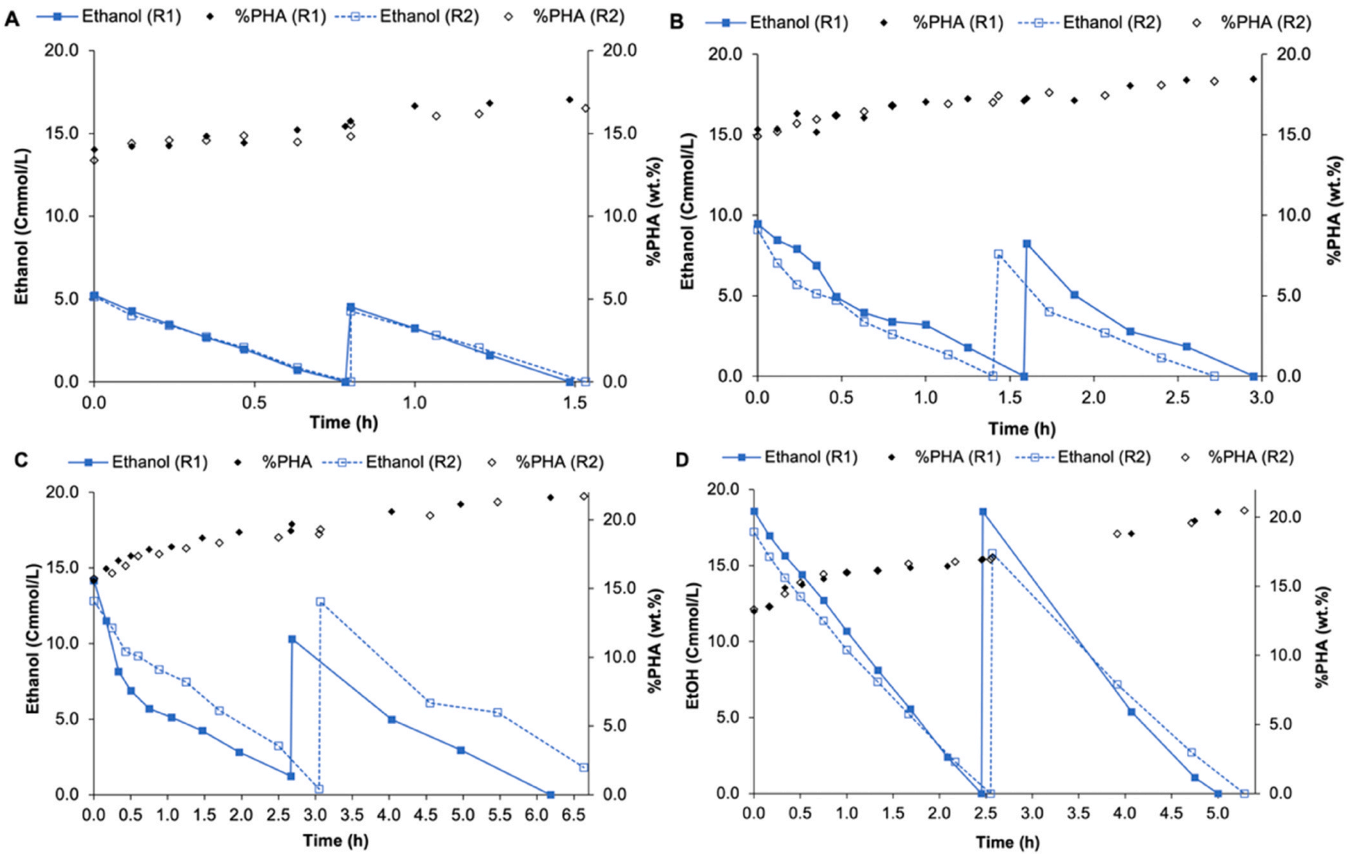

Fig. 3. Intracellular PHA content and bulk ethanol concentration profiles obtained for assays A, B, C and D where R1 and R2 represent the two replicates for each assay.

Table 3
Summary of performance parameters calculated for the fed-batch assays performed with ethanol concentrations of 5, 10, 15 and 20 Cmmol.L^{-1}. Values presented are mean ± standard deviation.

Test	A	B	C	D
Ethanol concentration (Cmmol.L^{-1})	5	10	15	20
Number of pulses	2	2	2	2
X_A (g.L^{-1})	3.9 ± 0.2	4.2 ± 0.2	4.0 ± 0.02	4.1 ± 0.1
Ethanol consumption				
Initial ethanol concentration on 1st pulse (Cmmol.L^{-1})	5.2 ± 0.04	9.3 ± 0.2	14.1 ± 0.03	18.8 ± 0.2
- $q_{ethanol}$ – 1st pulse (Cmol.Cmol-X_A^{-1}.h^{-1})	0.037 ± 0.001	0.031 ± 0.001	0.023 ± 0.001	0.040 ± 0.001
- $q_{ethanol}^{max}$ (Cmol.Cmol-X_A^{-1}.h^{-1})	0.043 ± 0.002	0.037 ± 0.001	0.035 ± 0.002	0.043 ± 0.001
Overall accumulation performance				
$Y_{PHA/S}$ (Cmol-PHA.Cmol-S^{-1})	0.92 ± 0.004	0.73 ± 0.01	0.70 ± 0.0003	0.63 ± 0.002
q_{PHA} (Cmol-PHA.Cmol-X_A^{-1}.h^{-1})	0.034 ± 0.0002	0.022 ± 0.001	0.017 ± 0.0007	0.024 ± 0.001
%PHA^{max} (wt%)	16.8 ± 0.3	18.4 ± 0.1	21.7 ± 0.05	20.4 ± 0.05
r_{PHA} (g-PHA.L^{-1}.h^{-1})	0.13 ± 0.002	0.091 ± 0.007	0.067 ± 0.002	0.097 ± 0.004

metabolism for the production of EPS, which is often produced in response to stress factors such as inhibitory substances, to provide a shielding effect.

3.3. EPS production as a protection strategy against ethanol

During fed-batch assays, visual inspection of the cultivation broth indicated that the broth viscosity increased by the end of the assay, especially for the highest ethanol concentrations tested. This led us to investigate the potential production of EPS by the culture, since in low cell density systems the accumulation of EPS in the cultivation broth increases its apparent viscosity, which can increase substantially and originate a transformation to shear thinning fluid behaviour [23]. EPS can be produced in reaction to stress factors and/or to adapt to extreme conditions [24]. The main purpose of EPS is the protection against environmental pressures, so it is possible that the increase of ethanol content, a known microbial growth inhibitor, triggered EPS production as a protection strategy against ethanol. In this context, EPS extraction and quantification was performed for all the fed-batch tests. The final EPS content in each test as a function of the total ethanol supplied is presented in Fig. 5.

Fig. 5 shows that the EPS content of the cultivation broth by the end of each test increased linearly with the increase of the ethanol content supplied. For the tests where only ethanol was supplied, the maximum EPS content of 6.70 Cmmol was registered for test D, where the total ethanol supplied was the highest (18.7 Cmmol). For tests E and F, where other carbon sources were available, EPS production follows the same linear trend with the ethanol. Indeed, the increase in ethanol content from assay E to F also resulted in an increase in EPS final content in the cultivation broth. The maximum EPS production was registered for these assays, since a higher carbon concentration was fed in each pulse and a higher number of carbon pulses occurred during these tests with the objective of maximizing PHA cell content.

Representation of PHA and EPS production yields for assays A, B, C and D (Fig. 6) indicates that the increase in yield of EPS is accompanied

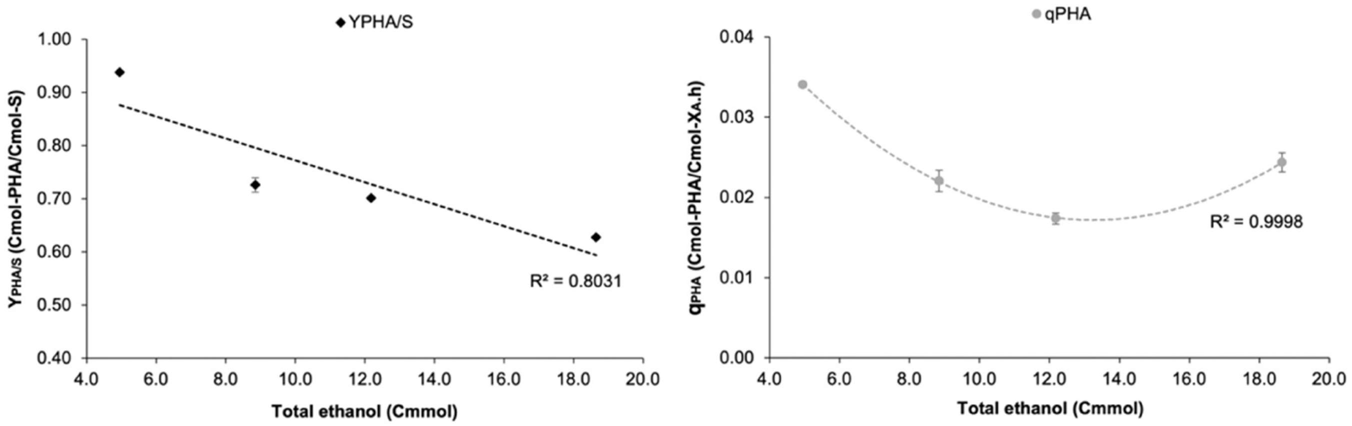

Fig. 4. Representation of overall PHA yield and specific PHA storage rate in function of the total ethanol supplied for tests A, B, C and D. The values presented are mean ± standard deviation from the replicates performed.

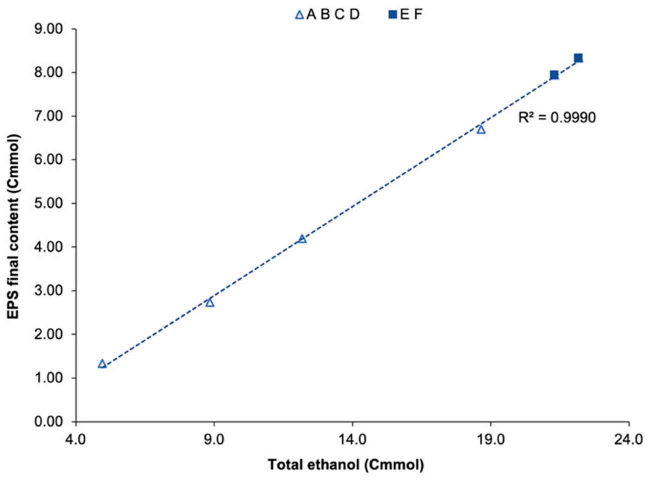

Fig. 5. EPS final content in assays A, B, C, D in function of the total ethanol supplied. Also the EPS content for the previous tests E and F (with a mixed of substrates) is depicted.

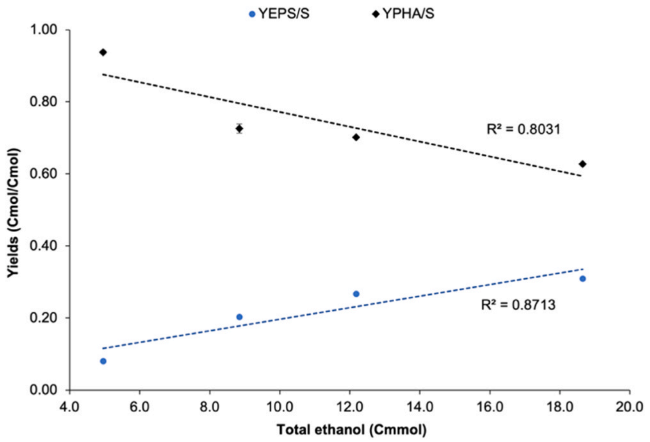

Fig. 6. Evolution of PHA and EPS production yields in function of the total ethanol being supplied in assays A, B, C and D.

by a similar decrease in PHA yield. Since both PHA and EPS production need extracellular carbon sources and energy, it is expected that EPS synthesis will translate in a reduced PHA production. On the other hand, the increase in EPS content seems to have resulted in a protection strategy for the biomass allowing it to produce PHA with a higher storage rate for the maximum ethanol content tested (Fig. 4). EPS does not seem to act as energy reserve, and microorganisms are usually unable to catabolize the EPS produced and accumulated extracellularly. In this way, the main role of EPS is to protect the cells inside its environment [24]. Because of this, we assume that no EPS consumption occurred during these assays and that the EPS produced resulted in a protection strategy for the biomass to cope with ethanol increasing content.

Apparently, simultaneous PHA and EPS production using an ethanol rich feed was never investigated before. Devi et al. (2012) [25] focused on the simultaneous production of intra- (PHA) and extra-cellular (EPS) polymers by using *Sinorhizobium meliloti* MTCC 100 and rice bran in hydrolyzed form, obtaining a PHA production yield of 0.14 g-PHA/g-sugar and an EPS production yield of 0.47 g-EPS/g-sugar. In the current study, PHA production surpassed EPS production but the low EPS production yield does not preclude its extraction and valorization, since the biopolymer is extracellular and its recovery is compatible with the extraction of PHA. Moreover, the potential economic impact of the co-production process is not only dependent on the production yields but also on the quality of the produced biopolymers.

After EPS extraction and quantification, the elemental and sugar composition of the samples were analysed. Elemental analysis results (Table I, Supplementary Material) showcased that the EPS produced by the selected culture presented a protein fraction of 13.9 ± 0.82%, meaning that it was mainly composed by exopolysaccharides. The polymers' sugar composition is available in the Supplementary Material, Figure III. The EPS produced by the culture in the different assays showed a similar sugar composition, being mainly composed of glucose (93.8 ± 13.2 mg.L^{-1}), galactose (65.2 ± 12.3 mg.L^{-1}) and rhamnose (45.7 ± 12.8 mg.L^{-1}). As previously reported in the literature, in aerobic granules-derived EPS, the sugar fraction of EPS usually includes neutral sugars (such as glucose, mannose, galactose, etc.), galacturonic acid, glucuronic acid and amino sugars (glucosamine and galactosamine) [26]. EPS-based biomaterials can be used as replacements to synthetic polymers and present several industrial and environmental potential applications such as biosorbents, gel-forming materials for the paper industry, flame retardant materials and cement curing materials [26].

To the best of our knowledge, simultaneous PHA and EPS production in a way that EPS serves as a protecting strategy allowing for the culture to produce PHA, even with in the presence of high contents of microbial inhibitory compounds, was never investigated before. The primordial objective of this work was to maximize PHA production, but when using an ethanol enriched feedstock, concomitant EPS production could not be avoided. The fact that carbon is being deviated from PHA production metabolism can initially be considered as an inconvenient, but if no carbon is lost in the process and the production of another value-added product such as EPS occurs, this emerges as a way to further valorise feedstocks enriched in ethanol. Contrarily to PHA, EPS is usually released extracellularly, and when not recovered can end up being rejected with the waste stream. In the current process no carbon is lost, and two products are produced: one intracellular and other extracellular. Overall, EPS-based materials are eco-friendly and sustainable, presenting great potential for several industrial applications. The recovery of EPS as a value-added co-product of the PHA process follows a biorefinery concept, where a multiproduct is obtained from a single feedstock through a circular bioeconomy principle where carbon sources are converted into biodegradable polymers that can be further converted to carbon to generate new products.

4. Conclusions

In this work, the impact of ethanol on the carbon flux of the PHA production process was investigated for the first time. Simultaneous production of PHA and EPS was observed, and EPS was produced as a protection strategy allowing the biomass to deal with increasing contents of ethanol and still produce PHA. This work further discloses the strategies that allowed the selected bacterial consortium to cope with increasingly inhibitory levels of ethanol and to still manage to produce PHA in these conditions. Moreover, the recovery of other products in the PHA production process can help in establishing the principles of a circular bioeconomy.

CRediT authorship contribution statement

Nidia Dana Lourenco: Conceptualization, Validation, Writing – review & editing. **Maria AM Reis:** Conceptualization, Funding acquisition, Project administration, Resources, Validation, Writing – review & editing. **Catarina Rangel:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Gilda Carvalho:** Conceptualization, Validation, Writing – review & 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.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2024.112609.

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