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Influence of inorganic carbon on purple phototrophic bacteria polyhydroxyalkanoates production under high reductive stress environment☆

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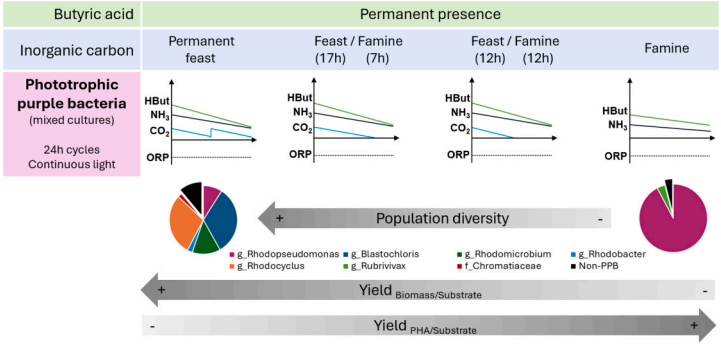
HIGHLIGHTS

- PPB mixed cultures were selected under reductive stress and four IC availabilities.
- IC presence primarily promoted biomass growth over PHA production.
- PPB selected without IC balanced redox stress *via* pathways alternative to IC fixation.
- IC absence promoted PHA production in cultures selected under feast/famine of IC.
- *Rhodopseudomonas palustris* showed strong adaptability to high reductive environments.

GRAPHICAL ABSTRACT



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ABSTRACT

Fermented wastes rich in reduced compounds challenge anaerobic purple phototrophic bacteria (PPB) systems by causing redox imbalances. This study evaluated polyhydroxyalkanoates (PHA) production and CO_2 fixation by PPB mixed cultures as means to balance internal redox, under four inorganic carbon (IC) availability conditions in a sequencing batch reactor. Culture selection under permanent IC presence promoted higher microbial diversity, but, strongly dependent on IC to balance internal redox, even when more oxidized substrates were supplemented. Increasing IC limitation favoured *Rhodopseudomonas palustris*, revealing its redox balancing capability independently of IC fixation. PHA contents of 20–29% gPHA/gVSS were achieved across all IC availabilities, however, when IC was present, growth was promoted over PHA production, indicating a preferable electron balance through CO_2 fixation. Nevertheless, cultures selected under feast-famine IC exhibited a preference for PHA accumulation under IC limitation, suggesting IC tuning as a potential growth-controlling strategy to boost phototrophic PHA production using reduced feedstocks.

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1. Introduction

The metabolic flexibility of purple phototrophic bacteria (PPB) has an increasing significance in the field of sustainable resource recovery, primarily due to their capacity to produce economically valuable bioproducts. Their ability to process complex organic streams into valuable bioproducts like polyhydroxyalkanoates (PHA), single-cell proteins (SCP), hydrogen, and fertilizers highlights their potential in environmental and industrial biotechnology (Capson-Tojo et al. 2020; Fradinho et al. 2021a). PPB's photosynthesis ability allows them to thrive in anaerobic illuminated environments, providing the necessary energy in the form of ATP and ensuring the means to support the uptake and utilization of organic compounds for growth. As a core metabolic pathway, the tricarboxylic acid (TCA) cycle plays a vital role in the assimilation of organic compounds. Substrates such as organic acids are metabolized into acetyl-CoA, allowing them to enter the TCA cycle and generate the electrons as well as production of essential metabolites and NADH for complementary biosynthetic routes (Petushkova et al. 2019). However, when considering carbon sources more reduced than biomass (e.g., propionate, butyrate, valerate), there is an expected increase in reduced cofactors that presents a challenge for maintaining redox balance (Alloul et al. 2023). Nevertheless, several metabolic pathways have been identified in PPB that play an important role in mitigating reductive stress, like the Calvin-Benson-Bassham (CBB) cycle, hydrogen production, isoleucine pathway, and PHA production (Bayon-Vicente et al. 2021; Hädicke et al. 2011; Fradinho et al. 2021b). One of the key electron sink pathways is CO_2 fixation and reduction through the CBB cycle, which has often been recognized as essential for growth with reduced substrates (Wang et al. 1993; Richardson et al. 1988). As well, PHA production can also serve as an electron sink in PPB mixed cultures operated under permanent carbon presence through the oxidation of NADH cofactors during PHA formation, thus balancing the reductive power (Fradinho et al., 2016). In PPB mixed cultures, the maximum PHA productivity reported to date is 0.111 g PHA/L·hour (Almeida et al., 2021). This remains significantly lower than the productivities achieved by aerobic cultures, which range from 1-2 g PHA/L·hour (Sali & Mackey, 2021). This underscores the importance of advancing research in phototrophic PHA production to enhance its feasibility and competitiveness. Nevertheless, PPB can attain PHA contents comparable to aerobic bacteria, and indeed, PHA production with PPB mixed cultures selected under the permanent presence of carbon, have reached promising PHA contents of 60 % g PHA/g VSS when using synthetic acetate as the sole substrate (Fradinho et al. 2016). However, when considering fermented real wastes, used as a means of valorizing wastes and decreasing PHA production costs, lower PHA contents were attained, resulting in 20 % g PHA/g VSS with fermented mixtures of wastewater and molasses (Almeida et al., 2021) and 42 % g PHA/g VSS with fermented urban organic waste (Allegue et al., 2022). Despite the possible inhibitory effect of unknown compounds present in real fermented wastes, the redox level of each organic carbon present in the fermented substrate has also been mentioned as having an impact on the PPB mixed cultures performance. In fact, a decrease in the uptake rate of more reduced compounds was frequently noted after acetate was depleted, and studies suggested that acetate, because of its lower reductive state, may function as a co-substrate, improving the uptake rate of the more reduced compounds (Fradinho et al. 2014; Almeida et al. 2023). Similarly, studies in wastewater treatment with PPB mixed cultures have also highlighted that bicarbonate played a critical role in supporting electron dissipation, enabling PPB to efficiently metabolize reduced organics such as butyrate and ethanol, pointing to bicarbonate as a key parameter for optimizing carbon and nutrient removal as well as biomass growth in PPB systems in wastewater treatment (Nairn et al. 2020). Regarding the impact of bicarbonate on PHA production, a recent study using a phototrophic mixed culture (PMC) highly enriched in *Rhodopseudomonas palustris* demonstrated that bicarbonate presence positively influenced the consumption of reduced compounds (medium-chain acids), biomass production, and PHA productivity (Montiel-Corona & Buitrón, 2023).

While IC availability can enhance the uptake of reduced compounds and PHA production, the long-term impact of IC presence on the selection of a PMC and its subsequent PHA production performance remains unclear. Specifically, it is uncertain whether IC fixation directly competes with PHA storage for reducing power or if its presence simply promotes substrate uptake, thereby indirectly stimulating PHA production. Aiming to improve the knowledge of how IC availability shapes culture selection and PHA productivity in PPB mixed cultures, as well as to overcome the slower intake of reduced compounds, four enriched PPB mixed cultures were selected under different inorganic carbon (IC) availability. The chosen IC conditions – always available, feast-famine with different extensions of the famine phase, and absence – were designed to reflect real-world scenarios, such as those found in wastewater treatment and biorefineries, where IC availability often fluctuates due to varying organic and inorganic carbon inputs or gas waste streams (e.g., CO_2 from fermentation processes) (Nairn et al. 2020; Liu et al. 2019; Tobin et al. 2020). These conditions will allow to explore how PPB cultures adapt to environments with steady IC supply, periodic IC availability, or IC limitation, which could be critical for optimizing PHA production in practical applications. Furthermore, the four PPB mixed cultures were submitted to reductive stress, ensured by an anaerobic environment and synthetic butyrate as the single organic carbon substrate. In such conditions, both pathways of PHA production and IC fixation via the CBB cycle are expected to be active. Therefore, this study proposes to evaluate the impact of IC on the selection of a PPB mixed culture enriched in PHA-accumulating PPB and the respective PHA production capacity under high-reducing stress environments. Coupling such findings will allow the development of PHA production with PPB, potentially improving its competitiveness with current aerobic PHA production systems.

2. Materials and methods

2.1. Photobioreactor operation

Aerobic sludge collected from the Beirolas wastewater treatment plant (Lisbon, Portugal) was used to enrich a PPB mixed culture under photoheterotrophic conditions using a synthetic feed comprising a mixture of acetate, propionate, and butyrate. Although no inorganic carbon was supplemented during the inoculum enrichment, measured IC values ranged between 4 and 9 Cmmol/L. This enriched culture served as the inoculum for cultivating the PPB mixed cultures in this study. A sequencing batch reactor (SBR) with a working volume of 4.2 L was operated anaerobically at a temperature of 30 °C and under constant illumination. The continuous illumination of the reactor was provided by four external halogen lamps (100 W) and a UV–visible light absorbing filter (Lee Colour Filter 299 1.2 N.D.) placed around the reactor wall, ensuring a volumetric light intensity of 2.3 W/L (with an incident light intensity of 90.9 W/m^2) inside the reactor, and limiting the light inputs to near-infrared (NIR) wavelengths (see supplementary material for reactor dimension and lamp positioning). Throughout 24-hour cycles, the PPB mixed culture was selected under the permanent presence of organic carbon, using synthetic butyric acid as the sole organic substrate to ensure reductive stress in the system, and four different operational conditions were sequentially set according to the IC availability, which was gradually decreased along the SBR operation: condition 1) permanent feast of IC; condition 2) feast and famine of IC (Feast hours/Famine hours (F/F) = 2.3 ± 0.2); condition 3) feast and famine of IC (F/F = 1.2 ± 0.1); and condition 4) IC famine. The reactor worked under continuous magnetic stirring, with a hydraulic retention time (HRT) equal to the solids retention time (SRT), which were 3 days in conditions 1, 2, and 3, and 6 days in condition 4. Both parameters were maintained by withdrawing 1.4 L or 0.7 L, respectively, of mixed liquor at the end of each cycle. The withdrawn volume was then restored at cycle onset through dump feeding (3 min long) of the feedstock

solutions (mineral media, organic, and IC media). During the withdrawal and feeding processes, nitrogen was flushed into the reactor's headspace and sparged in the feedstock solutions to prevent the entrance of oxygen. pH was controlled at 6.5 using HCL (1 M) and NaOH (1 M). PPB mixed cultures characterization (cycle monitorization and accumulation assays) was performed at the end of each condition, at least after 3 SRT, to guarantee the stability of the cultures. Table 1 summarizes the reactor operational settings throughout the conditions.

2.2. Feedstock supplementation: preparation and methodology

At the beginning of each cycle, the SBR was fed with the feedstock solutions of organic carbon, mineral medium, phosphate medium, and IC by dump feeding. The organic carbon solution consisted of butyric acid (133 mL of solution was fed to the reactor in conditions 1, 2, and 3; 90 mL was fed in condition 4), and the organic loading rates (OLR) were adjusted according to the culture performance at each condition, and to maintain a permanent presence of butyrate in the system (Table 1). The mineral medium solution contained per liter 3.1 g $MgCl_2{\cdot}6H_2O$, 0.4 g $MgSO_4{\cdot}7H_2O$, 8.4 g NaCl, 9.3 g NH_4Cl, 1.1 g $CaCl_2{\cdot}2H_2O$, 105 mL iron citrate solution (1.0 g/L), 21 mL trace element solution (see supplementary material for composition details) (133 mL of feeding volume in condition 1, 2 and 3). In condition 4, concentrations of ammonia and sulfate were adjusted to 0.11 g $MgSO_4{\cdot}7H_2O$ and 2.32 g NH_4Cl per liter (90 mL of feeding volume in condition 4). The phosphate medium contained per liter 0.16 g KH_2PO_4 and 0.2 g K_2HPO_4 in conditions 1, 2, and 3 (1 L of feeding volume), and 0.05 g KH_2PO_4 and 0.07 g K_2HPO_4 in condition 4 (500 mL of feeding volume). Both C/N and C/P ratios were set according to the standard biomass chemical formula $CH_{1.8}O_{0.5}N_{0.2}P_{0.02}$ to avoid limitations in the reactor. The IC feeding was performed with a solution of Na_2CO_3. In condition 1, the IC solution was fed twice in the cycle to ensure a permanent presence of IC, with the first feeding occurring at the beginning of the cycle and the second feeding after 12 h of the cycle (66 mL per feeding, with a total loading rate of 5.0 ± 0.6 ICmmol/L·day). In conditions 2 and 3, the IC feeding was performed only at the beginning of the cycle (133 mL per feeding, with a loading rate of 4.4 ± 0.8 and 2.0 ± 0.7 ICmmol/L·day, respectively), while in phase 4, no IC was supplied to the SBR.

2.3. Batch tests

Batch tests were conducted at the end of each condition to assess the adaptive mechanisms of each selected culture to conditions different from those imposed in the SBR while simultaneously evaluating the best accumulation strategy to maximize the PHA production. Control tests were carried out under the same IC availability and light intensity of the SBR (2.2 W/L) to establish the standard performance of the culture under a different system operation (configuration of the batch test reactors is described in supplementary material). The culture performance was assessed under higher light intensity (10.5 W/L), under several IC availabilities, as well as under the presence of butyrate and acetate as substrate. Exclusively during condition 4, light intensity was maintained at 2.2 W/L due to lower biomass concentrations. A summary of all the batch accumulation tests performed at each condition and the respective operational conditions applied can be found in the supplementary material.

At the end of each cycle, the biomass was directly collected into the batch reactors, with the headspace being continuously flushed with nitrogen to guarantee anaerobic conditions. The volume introduced into the batch reactors was restricted to the volume of the SBR purge (1.4 L for conditions 1 to 3 and 0.7 L for condition 4). This resulted in approximately 700 mL of biomass mixed liquor being present in each batch reactor across all tests. Before initiating each test, the broth was supplemented in the same proportions as in the SBR with the mineral media, the organic, and the IC feedstock (conditions 1, 2, 3 – total feeding volume of 0.35 L; condition 4 – total feeding volume of 0.14 L). At a controlled temperature of 30 °C, in conditions 1, 2, and 3 (conditions with HRT of 3 days), duplicates of each test were carried out in parallel in identical reactors (working volume ~ 1 L). In condition 4, tests were performed with a working volume of approximately 0.8 L, and duplicates were performed in sequential days due to the lower volume of biomass withdrawn from the SBR during this condition (HRT 6 days). The reactor walls were covered with a UV-light absorbing filter, similar to the SBR. A light intensity of 10.5 W/L was set by two external halogen lamps of 100 W each, while the 2.23 W/L intensity was set by one external halogen lamp of 30 W and another of 60 W. The pH was controlled by HCl (1 M) addition once the culture achieved a pH of 6.8, oscillating between the pH of 6.4 to 6.8.

2.4. Microbial community analysis

The characterization of the microbial culture was performed by DNA sequencing. The biomass samples for this analysis were collected from the reactor at the end of each condition and stored at −20 °C. The DNA extraction and sequencing were performed by DNA Sense (Aalborg, Denmark), and details can be found in the supplementary material.

Table 1
SBR operation and feedstock solutions settings throughout the four IC conditions.

		SBR conditions			
		1	**2**	**3**	**4**
IC setting		Permanent feast	Feast and famine F/F = 2.3 ± 0.2	Feast and famine F/F = 1.2 ± 0.1	Famine
Light		Continuous light (2.3 W/L; 90.9 W/m^2)			
Temperature / pH		30 / 6.5			
HRT = SRT		3	3	3	6
Butyrate	**Feeding mode**	Dump feed	Dump feed	Dump feed	Dump feed
	Timing	Pulse at 0 h	Pulse at 0 h	Pulse at 0 h	Pulse at 0 h
	OLR	30.1 ± 3.2	29.6 ± 3.9	31.2 ± 3.0	5.3 ± 2.5
IC	**Feeding mode**	Dump feed	Dump feed	Dump feed	–
	Timing	Pulse at 0 h and 12 h	Pulse at 0 h	Pulse at 0 h	–
	ICLR	5.0 ± 0.6	4.4 ± 0.8	2.0 ± 0.7	0
Nutrients	**Feeding mode**	Dump feed	Dump feed	Dump feed	Dump feed
	Timing	Pulse at 0 h	Pulse at 0 h	Pulse at 0 h	Pulse at 0 h
	NLR	5.5	5.5	5.5	0.9
	PLR	0.55	0.55	0.55	0.01
C:N:P		1: 0.2: 0.02			

Temperature – °C; **HRT and SRT** – days; **OLR** – Cmmol/L·day; **ICLR** – Inorganic carbon loading rate, in Cmmol/L·day; **NLR** – ammonia loading rate, in Nmmol/L·day; **PLR** – phosphate loading rate, in Pmmol/L·day.

2.5. Analytical methods

Butyric acid, acetic acid, and glucose were determined by high-performance liquid chromatography (HPLC). Detection was performed with a refractive index detector and an Aminex HPX-87H column (Bio-Rad). The eluent used was 0.01 N sulfuric acid, with a flow rate of 0.5 mL/min and an operating temperature of 30 °C.

IC was analyzed in a TOC-VCSH Analyser (Shimadzu) with a pre-acidification of the sample followed by a combustion catalytic oxidation at a temperature of 680 °C and high purity air as a carrier gas at a flow rate of 150 mL/min. Samples for this method were basified with NaOH (6 M), thus preventing IC losses prior to analysis and enabling a representative IC analysis.

Total carbohydrates hydrolysable to glucose were determined using the method described by Lanham et al. (2012), with small adjustments described in Fradinho et al. (2013). Polyhydroxyalkanoates content was established by gas chromatography, according to Lanham et al. (2013), with minor modifications (see supplementary material for details on the analytical method).

Ammonia and phosphate were analyzed in a Segmented Flow Analyser (Skalar 5100, Skalar Analytical, The Netherlands) through an implemented colorimetric method. Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) were determined according to standard methods (Apha et al., 2012). Light measurements were carried out using a Li-COR light meter LI-250 A equipped with an LI-200SA pyranometer sensor.

2.6. Calculations

Calculations for the active biomass (X) considered the standard biomass chemical formula $CH_{1.8}O_{0.5}N_{0.2}P_{0.02}$, typically applied for mixed microbial cultures. The active biomass concentration was determined by subtracting the total carbohydrates and PHA content from the VSS. The biomass specific growth rate (μ, in day^{-1}) was determined by dividing the ammonium uptake rate by the N:C molar biomass ratio of 0.2 (as per the above specified biomass chemical formula) and then dividing by the initial active biomass concentration. The PHA and total carbohydrate content were expressed as a percentage of VSS on a mass basis (% g PHA/g VSS; % g Carbs/g VSS). The IC feast to famine ratio (F/F) was determined by dividing the number of hours in the presence of IC (feast phase) by the total hours of famine of IC. Details on the calculations of specific uptake rates of substrate and IC, specific production

Table 2
Characterization of the PPB mixed cultures at each operating condition during steady state. Average values registered in the SBR cycles, with n = 3 in conditions 1, 2, and 3, and n = 4 in condition 4.

	Biomass (X_0)	PHA		Carbohydrates
	g/L	% g PHA/g VSS	g PHA/L·day	% g Carbs/g VSS
Condition 1 *Permanent feast IC*	1.03 (0.06)	29.4 (7.5)	0.15 (0.05)	9.4 (0.9)
Condition 2 *Feast / famine ICF/F = 2.3 ± 0.2*	0.83 (0.01)	23.0 (1.0)	0.09 (0.00)	9.9 (1.7)
Condition 3 *Feast / famine ICF/F = 1.2 ± 0.1*	0.94 (0.14)	20.1 (3.8)	0.10 (0.03)	4.3 (0.4)
Condition 4 *IC famine*	0.44 (0.04)	29.6 (5.1)	0.03 (0.01)	7.5 (0.8)

X_0 – initial active biomass; PHA and Carbohydrates – values registered at the end of the cycle before withdrawal. The standard deviation is presented under brackets.

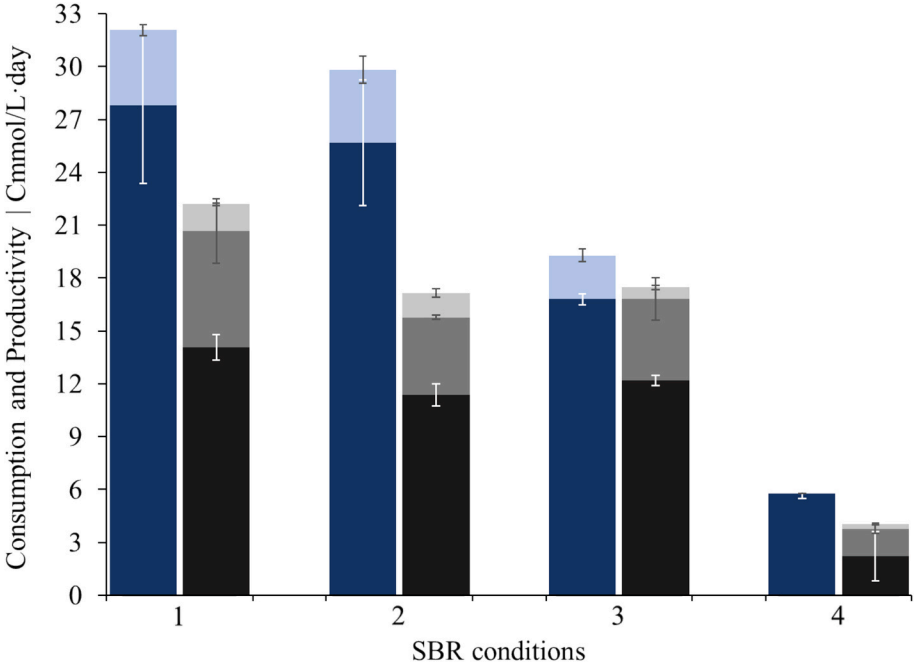

Fig. 1. Overall consumption of butyrate and IC, and production of biomass, PHA and carbohydrates throughout the SBR conditions Average values registered in the SBR cycles, with n = 3 in condition 1, 2 and 3, and n = 4 in condition 4. **1** – permanent feast IC; **2** – feast and famine of IC, F/F = 2.3 ± 0.2; **3** – feast and famine of IC, F/F = 1.2 ± 0.1; **4** – IC famine; Butyrate consumption (■); IC consumption (■); Biomass productivity (X) (■); PHA productivity (■) and Carbohydrates productivity (■).

rates of PHA and carbohydrates, yields, PHA and biomass productivity can be found in supplementary material.

3. Results and discussion

3.1. Phototrophic mixed cultures characterization

The IC influence on the selection of PPB mixed cultures enriched in PHA accumulating PPB under reducing environments was evaluated. The permanent presence of butyrate, a compound more reduced (degree of reduction $\gamma = 5$) than biomass ($\gamma = 4.3$) and PHA ($\gamma = 4.5$) ensured the reductive stress in the system, and by setting four different conditions of IC availability, four PPB mixed cultures were attained. Fig. 1 presents the global carbon consumption (butyrate and IC) and material production (biomass, PHA, carbohydrates) along the four operating conditions, while Table 2 presents the average values of PHA, carbohydrates, and biomass that were achieved at each set of conditions.

Regarding the active biomass concentration, a decrease in biomass production was observed, which followed the decrease in IC availability (Fig. 1 and Table 2). The higher biomass concentration was attained in condition 1, under the constant presence of IC in the medium (1.05 ± 0.04 g/L), followed by the feast and famine conditions (0.83 ± 0.01 g/L in condition 2 and 0.94 ± 0.14 g/L in condition 3). Once in the absence of IC, in condition 4, the biomass values suffered a considerable decrease, attaining only 0.44 ± 0.13 g/L despite the SRT adjustment in this condition from 3 to 6 days (to prevent biomass washout). The lower biomass in IC-limited cultures inherently led to higher light availability per cell, which likely contributed to the higher PHA accumulation observed in those cultures. Nevertheless, such condition can eventually translate what would happen in real operation systems (with a fixed incident light intensity), if a feedstock with low IC content was applied. When considering the PHA production, the produced polymer was mostly composed of 3-hydroxybutyrate monomer (99 % Cmmol HB/Cmmol PHA and 1 % Cmmol HV/Cmmol PHA). The presence of a small fraction of 3-hydroxyvalerate (HV), despite butyrate being an even-carbon substrate, could be attributed to minor metabolic activity involving internal glycogen reserves (Brdjanovic et al. 1998). Although glycogen consumption was not observed, the incorporation of odd-carbon units into the polymer suggests that glycogen-derived precursors might have contributed to the formation of HV. The highest PHA content was achieved in conditions 1 and 4, with the permanent presence of IC leading to 29.4 ± 7.5 % g PHA/g VSS and the absence of IC registering a similar content of 29.6 ± 5.1 % g PHA/g VSS. Yet, the highest productivity was attained at condition 1, 0.15 ± 0.05 g PHA/L·day, mostly due to the higher biomass concentration registered in that condition. In contrast, in condition 4, biomass growth was limited due to the absence of IC, and while PHA content remained similar to condition 1, the lower biomass concentration resulted in lower PHA productivity. Nevertheless, despite the increased PHA content registered in condition 1, an interesting behavior was observed throughout the decreasing availability of IC, where the selected PPB mixed cultures started to show a greater reduction in biomass growth compared to PHA accumulation, indicating that while growth was strongly affected by IC limitation, PHA production was comparatively less impacted (Fig. 1).

As for carbohydrates production, lower contents were consistently registered compared to the PHA production, fluctuating from 4 to 10 % g Carbs/g VSS (Table 2). Despite not being observed any specific tendency regarding the carbohydrates content along the SBR conditions, the decrease in carbohydrates productivity is in line with the decreasing availability of IC in the system (Fig. 1). This carbohydrates production decline was an expected profile since carbohydrates, in the form of glycogen, are a product of the IC fixation through the CBB cycle. Likewise, in the absence of IC, PHA consumption was never observed, suggesting that carbohydrates production was mainly produced through IC fixation.

Regarding the total yields obtained, values lower than 1 were consistently attained (0.90 in condition 1, 0.59—–0.86 in condition 2, 0.75 – 0.84 in condition 3, and 0.82 in condition 4, in Cmmol X + PHA + Carbs/Cmmol S + IC). Parallelly, increasing expression of excretion products was detected in the HPLC analysis (see supplementary material). Despite the lack of identification of these compounds, their increasing expression, along with the increase of the redox stress, could justify the decreasing yields observed and suggest that the selected PPB mixed cultures were activating other metabolisms besides PHA production or IC fixation to balance the reducing power in the cells. Additionally, considering the possibility of excretion products, the production of extracellular polymeric substances (EPS) could be a possibility, which may also contribute to the observed carbon discrepancies. While EPS production in PPB systems has not been extensively studied, it has been documented in several studies (Panwichian et al., 2011; Hubas et al., 2011; Zhang et al., 2024; Stegman et al., 2020). EPS production is particularly plausible under the highly stressful conditions applied in the system, which are known to stimulate EPS production in microbial communities as a protective response (Ates, 2015).

3.2. PPB mixed cultures performance under different inorganic carbon availabilities

Monitorizations of the PPB mixed cultures were carried out at the end of each condition to evaluate the performance of the culture upon selection under different IC availabilities (Fig. 2, Table 3).

The assessment of the results showed that under constant IC availability (Fig. 2.A), the culture was able to easily consume the butyrate as well as grow, presenting a specific butyrate uptake rate of 0.70 ± 0.07 Cmmol/Cmmol X·day and specific growth rate of 0.57 ± 0.01 day^{-1}. The majority of the butyrate consumed was towards biomass growth, presenting a yield of biomass per substrate consumed of 0.80 Cmmol X/Cmmol S. In opposite, only 9 % of the total butyrate was used for PHA production, being concordant with the low production rate of PHA observed during this condition (0.08 ± 0.02 Cmmol/Cmmol X·day, Table 3). As for the carbohydrates production, a constant accumulation was observed throughout the cycle, presenting an accumulation rate of 0.04 ± 0.02 Cmmol/Cmmol X·day and a yield of carbohydrates produced per IC consumed of 0.36 Cmmol Carbs/Cmmol IC.

In conditions 2 and 3 (Fig. 2. B and C), the loading rate of IC was reduced, and the PPB mixed cultures were selected with a period under the absence of IC (around 4 h in condition 2 and 12 h in condition 3). The selected PPB mixed cultures presented higher consumption rates of butyrate and higher biomass growth under the presence of IC (Table 3, Feast IC). Once IC was totally consumed, a decay in butyrate uptake rate was observed, followed by a decline in biomass growth (Fig. 2, Table 3, Famine IC). Concerning the accumulation of biopolymers, in conditions 2 and 3, PHA consumption was observed under the presence of IC, and only after IC became depleted was PHA produced, presenting a PHA storage yield of 0.43 and 0.59 Cmmol PHA/Cmmol S during the famine phase of IC in condition 2 and 3, respectively. Contrary to the accumulation of PHA, carbohydrates were only accumulated during the feast phase of IC, presenting a steady profile after IC became depleted. Nevertheless, both cultures (conditions 2 and 3) were able to achieve similar production rates of carbohydrates, as the ones registered in condition 1 (Table 3), presenting a yield of IC per carbohydrates produced of 0.26 and 0.40 Cmmol Carbs/Cmmol IC in condition 2 and 3, respectively.

Throughout conditions 1 to 3, the presence of IC allowed butyrate to be consumed with rates that are comparable to the consumption rates of acetate (less reduced compound), as previously reported for PMC selected under anaerobic conditions, permanent feast of acetate and similar specific light intensities (around 2 W/g X) (0.69 Cmmol Acetate/Cmmol X·day, Fradinho et al. 2016). However, it should be noted that the light path and light availability profile may differ from one study to another due to variations in reactor design.

The low biomass growth rates registered during the absence of IC in

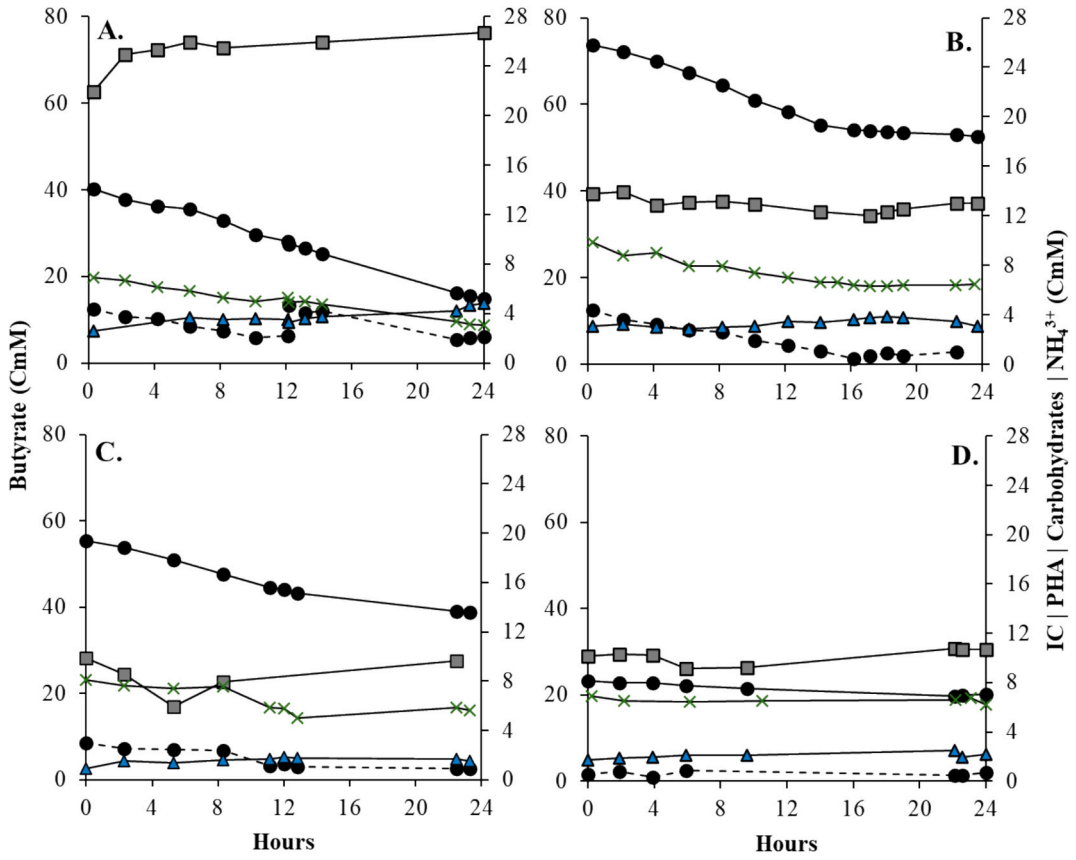

Fig. 2. PPB mixed cultures profile upon selection under **A.** permanent presence of IC (condition 1); **B.** feast and famine of IC (F/F = 2.3 ± 0.2) (condition 2); **C.** feast and famine of IC (F/F = 1.2 ± 0.1) (condition 3); **D.** absence of IC (condition 4). Butyrate (●); IC (– ● –); PHA concentration (■); Carbohydrates concentration (▲); Ammonia concentration (X).

Table 3
Kinetic values of the PPB mixed cultures in all conditions. With n = 3 in conditions 1, 2, and 3, and n = 4 in condition 4.

		- *q*S	- *q*IC	*q*PHA	*q*Carbs	μ	Yields	
		Cmmol/Cmmol *X*·day				day^{-1}	PHA/S	X/S
Condition 1		0.70 (0.07)	0.11 (0.02)	0.08 (0.02)	0.04 (0.02)	0.57 (0.01)	0.08 (0.01)	0.82 (0.07)
Condition 2	**Feast IC**	1.28 (0.24)	0.19 (0.01)	-0.02 (0.03)	0.05 (0.01)	0.76 (0.17)	-	0.59 (0.02)
	Famine IC	0.24 (0.10)	-	0.09 (0.05)	-	0.08 (0.03)	0.45 (0.22)	0.41 (0.27)
Condition 3	**Feast IC**	0.66 (0.12)	0.10 (0.01)	-0.03 (0.10)	0.04 (0.01)	0.53 (0.10)	-	0.75 (0.15)
	Famine IC	0.19 (0.03)	-	0.12 (0.03)	-	0.05 (0.02)	0.59 (0.13)	0.25 (0.10)
Condition 4		0.22 (0.07)	-	0.15 (0.06)	0.01 (0.02)	0.03 (0.01)	0.68 (0.08)	0.14 (0.08)

Condition 1 – permanent feast IC; **Condition 2** – feast and famine of IC, F/F = 2.3 ± 0.2; **Condition 3** – feast and famine of IC, F/F = 1.2 ± 0.1; **Condition 4** – IC famine. The standard deviation is presented under brackets.

conditions 2 and 3 projected a decline of the PPB mixed culture growth under the absence of IC (condition 4), and therefore, the SRT of condition 4 was adjusted to 6 days to prevent biomass washout. Indeed, selection under the absence of IC resulted in a drastic decrease in both substrate consumption as well as biomass growth (0.22 ± 0.07 Cmmol/Cmmol *X*·day and 0.03 ± 0.01 day^{-1}, respectively). Nonetheless, carbon retrieved to PHA production was able to attain a yield of 0.69 Cmmol PHA/Cmmol S, and the PHA production presented an accumulation trend throughout the cycle, achieving the highest PHA production rate across all conditions, 0.15 ± 0.06 Cmmol/Cmmol *X*·day.

Overall, the monitorization of the selected PPB mixed cultures suggests that PHA can be produced in the presence of IC in a culture selected under its permanent presence and that in cultures selected with limited IC, PHA production can thrive over biomass growth in the absence of IC. Nevertheless, it is still necessary to understand the resilience of the selected PPB mixed cultures when subjected to different operational conditions and how it can impact PHA production.

3.3. PPB mixed culture's adaptative mechanisms

To evaluate the PPB mixed culture's capability to accumulate PHA when subjected to operational conditions different than the ones applied during selection, several batch tests were performed. Table 4 summarizes the kinetic values obtained in each test. For results interpretation, note that the effect of higher light intensity should be assessed by comparing the tests in presence of IC to the control tests (in supplementary material), while all other tests should be benchmarked against the tests in the presence of IC, rather than the control.

Table 4
Kinetics of the batch tests performed with the PMCs selected under each condition. Tests in conditions 1, 2, and 3 were performed under increased light intensity (10.5 W/L), and in condition 4 (2.2 W/L) with or without IC, or acetate. Batch tests were performed in duplicate (n = 2). Kinetics of the control tests can be found in supplementary material.

	Condition		−qS		qPHA	qCarbs	μ
			− qHBut	− q HAc			
			Cmmol/Cmmol *X*·day				day^{-1}
Butyrate	**1**	**With IC**	0.67 (0.12)	–	0.19 (0.01)	0.13 (0.02)	0.48 (0.05)
		Without IC	0	–	− 0.13 (0.01)	− 0.10 (0.05)	0
	2	**With IC**	0.94 (0.06)	–	− 0.06 (0.01)	0.17 (0.07)	0.73 (0.03)
		Without IC	0.44 (0.07)	–	0.40 (0.06)	− 0.03 (0.06)	0.08 (0.03)
	3	**With IC**	1.11 (0.12)	–	−0.04 (0.04)	0.18 (0.00)	1.10 (0.06)
		Without IC	0.33 (0.02)	–	0.25 (0.05)	0.03 (0.01)	0.04 (0.01)
	4	**With IC**	0.56 (0.07)	–	− 0.54 (0.03)	0.03 (0.01)	1.08 (0.32)
		Without IC	0.24 (0.10)	–	0.10 (0.02)	0	0.12 (0.10)
Butyrate + Acetate	**1**	**With IC**	0.24 (0.22)	1.20 (0.81)	0.58 (0.19)	0.25 (0.03)	0.86 (0.04)
		Without IC	0.04 (0.02)	0.13 (0.06)	0.14 (0.02)	0.09 (0.07)	0
	2	**With IC**	0.13 (0.02)	0.41 (0.01)	0.23 (0.08)	0	0.31 (0.07)
		Without IC	0.16 (0.09)	0.35 (0.02)	0.35 (0.06)	0	0.17 (0.03)
	3	**With IC**	1.00 (0.09)	0.35 (0.01)	0.54 (0.34)	0.13 (0.03)	0.31 (0.00)
		Without IC	1.15 (0.52)	0.33 (0.13)	0.74 (0.43)	0.25 (0.12)	0.31 (0.10)
	4	**Without IC**	1.85 (0.00)	0.45 (0.00)	1.40 (0.36)	0.08 (0.17)	0.44 (0.02)

1 – permanent feast IC; **2** – feast and famine of IC, F/F = 2.3 ± 0.2; **3** – feast and famine of IC, F/F = 1.2 ± 0.1; **4** – IC famine; HAc – acetate; HBut – butyrate; IC – Inorganic carbon; standard deviation presented under brackets.

The first conditions to be tested were the permanent presence of butyrate with and without IC in all SBR conditions. In a culture selected under the permanent presence of IC (condition 1), the batch tests performed under the presence of IC demonstrated that the culture was able to grow and produce PHA simultaneously but exhibited a preference for growth over PHA production ($Y_{X/S}$ 0.72 Cmmol *X*/Cmmol S; $Y_{PHA/S}$ 0.28 Cmmol PHA/Cmmol S). On the other hand, the cultures selected with decreasing availability of IC (conditions 2, 3, and 4) demonstrated that, in tests under the presence of IC, biomass growth was highly stimulated ($Y_{X/S}$ 0.78 Cmmol *X*/Cmmol S in condition 2, $Y_{X/S}$ 0.99 Cmmol *X*/Cmmol S in condition 3, $Y_{X/S}$ 1.93 Cmmol *X*/Cmmol S in condition 4) while PHA was consumed, a behavior that was previously observed in the SBR in condition 2 and 3. These results indicate that, regardless of the selection condition applied, the presence of IC consistently prioritizes growth over PHA production. The observed preference for growth over PHA production in the presence of IC can be attributed to the role of IC fixation in easing the balance of reducing power, enabling the culture to prioritize biomass synthesis. This is even more poignant for cultures of conditions 2 to 4, where IC presence also enabled PHA consumption, highlighting the metabolic flexibility in adapting to varying carbon availability, utilizing both IC fixation and PHA production to maintain redox balance. A more detailed interpretation of these metabolic shifts, including the relative efficiencies of IC fixation and PHA production as redox balancing mechanisms, is provided in Section 4.

The tests carried out in the permanent presence of butyrate but under IC limitation showed that the PPB mixed culture selected with a permanent presence of IC (condition 1) became highly inhibited with no capacity for butyrate consumption nor growth (Table 4, zero values). Contrary, the PPB mixed culture selected under IC limitation (condition 4) was able to simultaneously accumulate PHA (0.10 Cmmol/Cmmol *X*·day) and grow (0.12 day^{-1}), with the consumed butyrate being equally divided between PHA production and biomass growth ($Y_{PHA/S}$ 0.42 Cmmol PHA/Cmmol S and $Y_{X/S}$ 0.50 Cmmol *X*/Cmmol S). This suggests that the condition 4 culture possesses alternative strategies to mitigate excess reducing power besides IC fixation (further discussed in section 4). As for the cultures selected under FF of IC, the absence of IC seemed to promote PHA production, displaying a preference to accumulate PHA over biomass growth ($Y_{PHA/S}$ 0.91 Cmmol PHA/Cmmol S and $Y_{X/S}$ 0.18 Cmmol *X*/Cmmol S in condition 2, $Y_{PHA/S}$ 0.76 Cmmol PHA/Cmmol S and $Y_{X/S}$ 0.12 Cmmol *X*/Cmmol S in condition 3).

To further understand the importance of IC in the redox balance throughout the SBR conditions, tests were carried out in the permanent presence of butyrate and acetate, a less reduced compound, with and without IC (batch test profiles can be consulted in supplementary material). In the culture selected with a permanent presence of IC (condition 1), a preference for acetate consumption over butyrate consumption was observed, and PHA production was stimulated in both the presence and absence of IC when compared with the previous tests with butyrate only. Nevertheless, in the presence of IC, growth was still preferred over PHA accumulation ($Y_{PHA/S}$ 0.40 Cmmol PHA/Cmmol S and $Y_{X/S}$ 0.59 Cmmol *X*/Cmmol S).

In cultures selected with lower IC availability (conditions 2, 3, and 4), while in condition two, the culture still presents a preference for acetate over butyrate (with or without IC), in conditions 3 and 4, acetate was no longer the preferable organic acid, and its presence did not lead to a decrease in butyrate consumption. However, acetate was important for the cultures of conditions 3 and 4 when IC was absent since the presence of acetate increased the butyrate consumption rate, as well as biomass growth when compared with the tests without acetate (see butyrate without IC tests). This suggests that acetate may play a role beyond simply providing CO_2, potentially through additional metabolic mechanisms (e.g., PHA production). Regarding PHA, across conditions 2, 3, and 4, acetate addition promoted PHA production over growth, either in the presence or absence of IC. In particular, acetate addition avoided PHA consumption in tests with IC and further improved PHA storage in tests without IC, culminating in the highest PHA production rate of 1.40 Cmmol/Cmmol *X*·day for the condition 4 culture.

Overall, results have shown, once again, that IC had a major impact on biomass growth and that PHA production could be stimulated in the absence of IC when the culture was selected with decreasing IC availability. As for the presence of acetate, tests showed that PHA production was stimulated, independently of the selection conditions applied, and that acetate could play a role in the redox balance in PPB mixed cultures selected with the lowest availability of IC (conditions 3 and 4).

Additionally, in Fig. 3, it is possible to follow the PHA content obtained at each test, as well as the respective productivities obtained. According to the results, the highest PHA productivities were attained in the presence of acetate, agreeing with the previous results where acetate presence led to an increase in PHA production rates across all conditions (see Table 4). The test carried out in condition 4 under the absence of IC and using butyrate and acetate as organic carbon sources achieved the highest PHA content (47.6 % g PHA/ g VSS). Nonetheless, the highest productivity was obtained in condition 1 (0.26 g PHA/L·day), in the presence of IC, butyrate, and acetate, where PHA and biomass growth

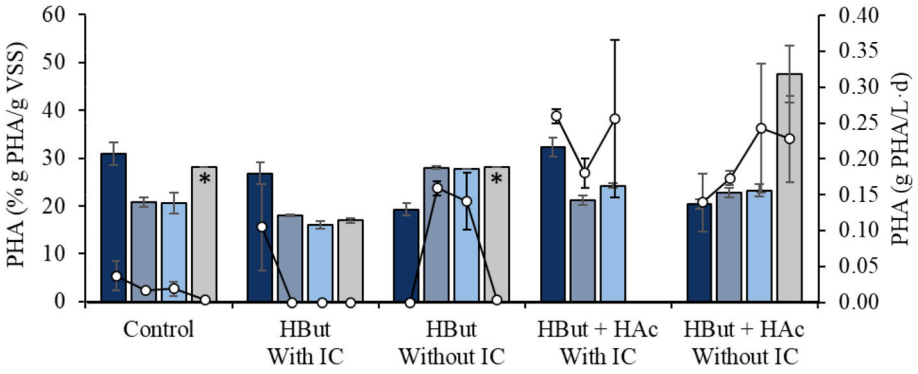

Fig. 3. PHA content and productivities registered in the duplicated batch accumulation tests (n = 2). Permanent feast IC (condition 1) (■); feast and famine of IC, F/F = 2.3 ± 0.2 (condition 2) (■); feast and famine of IC, F/F = 1.2 ± 01 (condition 3) (■); IC famine (condition 4) (■); Productivity (○).*same test. Zero values of PHA productivity correspond to PHA consumption.

occurred simultaneously.

Furthermore, when considering the tests performed only with butyrate, the highest productivities were attained in condition 2 and condition 3 under the absence of IC, attaining 0.16 and 0.14 g PHA/L·day, respectively. The culture selected in condition 1 also presented a considerable increase in the PHA production, but only in the presence of IC, with a productivity of 0.11 g PHA/L·day. While in conditions 2 and 3, PHA productivity improved 7 to 10 times in comparison to the control test, condition 1 culture was only able to increase its productivity by 3 times. Therefore, the cultures selected with a famine phase of IC presented a greater aptitude to maximize PHA production under reduced environments when exposed to increased illumination and IC limitation in PHA accumulation steps.

3.4. Bacterial communities

Analysis of the microbial population was performed at the end of each selecting condition (see supplementary material for a heatmap representation of the most abundant taxonomic groups identified). Results show that despite the different availability of IC in the medium, the selected cultures were majorly composed of microorganisms belonging to Alphaproteobacteria and Betaproteobacteria classes and that PPB content represented more than 89 % of the total population across all the conditions. The photosynthetic culture selected under the permanent presence of IC (condition 1) presented the greatest variability of microorganisms across all conditions. The most abundant species was *Rhodocyclus tenuis,* which represented 28.8 % of the total population, followed by a variety of *Blastochloris* that represented an overall content of 32.4 %, and *Rhodomicrobium* species (13.1 %). In condition 2 and 3, the existence of a famine phase of IC decreased the population variability, with both *Rhodopseudomonas palustris* and *Blastochloris viridis* species showing a considerable increase, while *Rhodocyclus tenuis* species presented comparable contents to the ones in condition 1. Nevertheless, the increase of the inorganic famine period between condition 2 and 3 did not reckon a significative change in the population when compared with one another. The most drastic shift of the population occurred in condition 4 when the PPB mixed culture was selected without IC. In these conditions, *R. palustris* represented 89.6 % of the total population, and *Rubrivivax gelatinosus* appeared for the first time, presenting a content of 3.2 %, indicating the ability of these organisms to grow in adverse conditions. Such results suggest that *R. palustris*, in particular, might have the capacity to engage alternative metabolic pathways to alleviate excessive reducing power (besides IC fixation).

3.5. Process overview

PPB mixed cultures were selected in a reduced environment under four different IC availability conditions, to assess the impact of IC on PHA accumulation as well as in selection of a PPB mixed culture for PHA accumulation. It is important to note that, across all four conditions, ammonia was consistently maintained at high concentrations. This approach was chosen not only to prevent the activation of hydrogen production and ensure consistency throughout the study, but also because ammonia limitation has been demonstrated to negatively affect the selection of PPB mixed cultures for PHA accumulation (Almeida et al., 2023).

Throughout the four conditions, the PHA content registered was consistently higher than carbohydrates (Table 2), with 3 to 7 times more PHA being produced than carbohydrates in Cmmol/L·day (Fig. 1). Although this could suggest that PHA production was the favorable pathway for balancing reducing power, it must be taken into consideration that, per C-mol of glucose produced through the CBB cycle, 2 mol of reduced co-factors (like $NADH_2$) can be oxidized, while per C-mol of HB produced, only 0.25 mol of $NADH_2$ are oxidized (Martin et al. 2000; Wang et al. 2018). Overall, glucose production allows the oxidation of 8 times more reduced co-factors than HB production, per C-mol of molecule produced, suggesting that despite the lower carbohydrates content when compared to PHA, the majority of the reduced power was being balanced through IC fixation. In fact, previous studies by De Meur et al. (2020) and Cabecas Segura et al. (2021) showed that PHA production through butyrate assimilation does not completely fill the electron sink purpose since not all the reduced co-factors generated during butyrate assimilation can be oxidized through this pathway (Fig. 4). Nevertheless, by diverting butyrate to PHA storage, cells can limit the carbon flow and prevent further production of reducing power. Indeed, butyrate storage as PHA prevents the additional production of up to 9 reduced co-factors if, for example, butyrate was instead directed to the TCA cycle (Fig. 4). As such, while PHA production acts by limiting the further release of reducing power, it could be said that IC fixation acts posteriorly, mitigating the reducing power that has been produced, and the presence of PHA and carbohydrates in the system demonstrate that both routes played an active role in balancing the reductive power. Interestingly, along with the decreasing of IC availability in the SBR, the yield of produced PHA over consumed butyrate increased (Table 3), indicating that the selected cultures began to favor the pathway that limits the production of reducing power, and possibly triggered alternative pathways to mitigate the reducing power that was being produced. In conditions 2 and 3, PHA was indeed consumed in the presence of inorganic carbon (IC) and only began to accumulate once IC was depleted. Therefore, independently of the growth slowdown, a notable increase in PHA production was observed compared to when IC was available. This suggests that the metabolic shift toward PHA accumulation was not merely a consequence of growth limitation but also a result of the cultures adapting to IC scarcity by favoring pathways that reduce the production of excess reducing power.

Furthermore, when considering the yields of carbohydrates

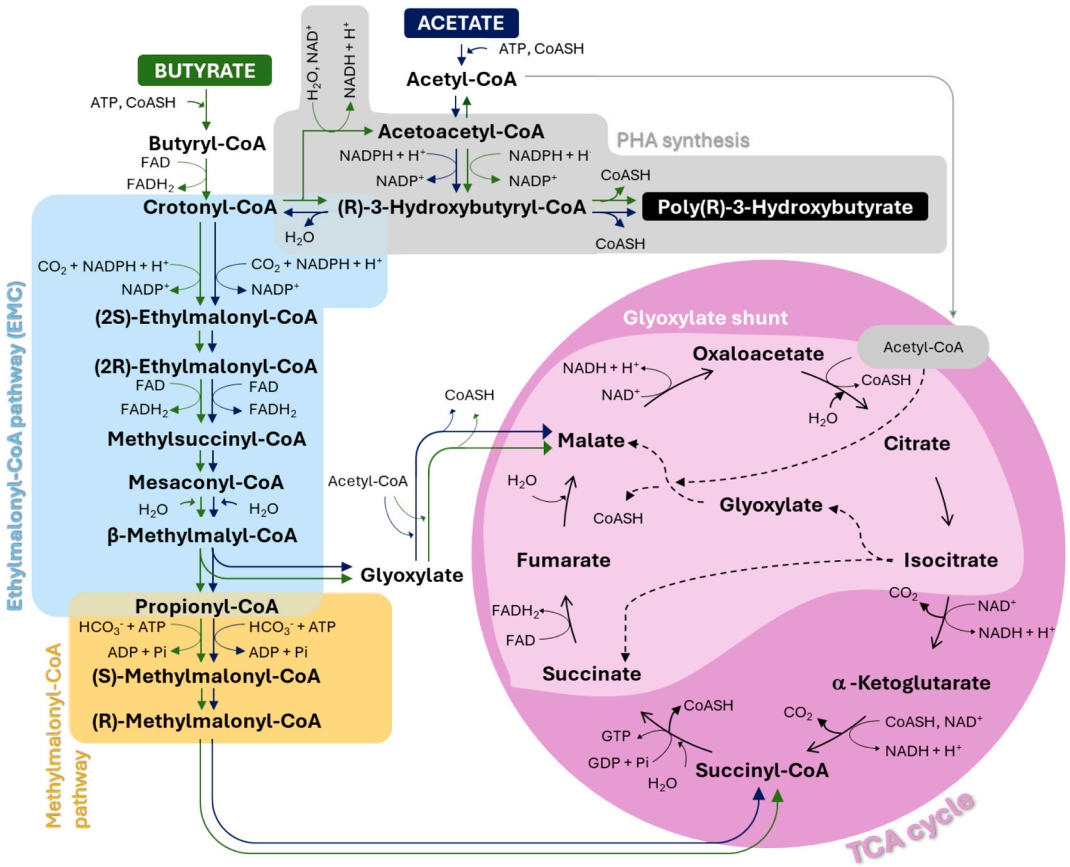

Fig. 4. Representation of metabolic pathways for the assimilation of acetate and butyrate potentially use by PPB mixed cultures, based on the proposals by Leroy et al. (2015) and De Meur et al. (2020) for Rhodospirillum rubrum, and McKinlay and Harwood (2010) for Rhodopseudomonas palustris. CoASH – reduced form of CoA. Green arrows – butyrate pathway; Blue arrows – acetate pathway; Dashed arrows – glyoxylate shunt. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

produced per IC consumed, low values were attained throughout the conditions. The ethylmalonyl-CoA (EMC) pathway (Fig. 4) was recently identified as being utilized in butyrate assimilation by purple bacteria (De Meur et al. 2020), and besides playing a role in IC fixation, it also contributes to the reoxidation of reduced cofactors (Alloul et al. 2023). Despite the brief coverage of the EMC pathway in PPB microorganisms (Petushkova and Tsygankov, 2017; Erb et al., 2007; De Meur et al., 2020), its existence must be acknowledged as it may dissipate reductive power and compete with carbohydrate production from IC via CBB cycle, which could possibly justify the low yield ($Y_{Carbs/IC}$) attained in the present work. While carbohydrate production in this study was only observed in the presence of IC (Fig. 2), it is important to emphasize that carbohydrate synthesis in purple phototrophic bacteria (PPB) is not exclusively dependent on IC assimilation. The carbohydrate contents attained in this study, ranging from 4.3 to 9.9 % g Carbs/g VSS, are consistent with values previously reported for both mixed cultures (PMC) and single-strain purple bacteria (2–15 %) under conditions without IC supplementation (De Philippis et al. 1992; Fradinho et al. 2013; Fradinho et al. 2016; Allegue et al. 2022; Almeida et al. 2023; Marchetti et al. 2024). This suggests that carbohydrate production in PPB is a multifaceted process influenced by multiple metabolic pathways and is not solely reliant on IC assimilation. For instance, other carbon sources, including the utilization of stored PHA, can also contribute to carbohydrate synthesis through the polysaccharide biosynthesis pathway (Fradinho et al. 2021a).

Regarding the PHA production, the highest contents were registered under the operation with the PPB mixed cultures selected in the permanent presence of IC and the absence of IC (conditions 1 and 4), although the greatest productivity was attained with selection under the permanent presence of IC (condition 1). These results were expected since the lowest biomass growth rate was recorded in the absence of IC, and the biomass concentration would ultimately have an impact on the overall biopolymer production. It is well-known that IC is necessary for bacterial growth when considering heterotrophic growth with highly reduced compounds (Richardson et al., 1988; Ormerod, 1956). This necessity is primarily due to its key role in the CBB cycle, which plays a crucial task in redox balance (McKinlay and Harwood, 2010), explaining the lower biomass concentration registered in condition 4. Nonetheless, although greater IC availability during PPB mixed culture selection resulted in higher biomass concentration as well as PHA content, batch accumulation tests showed that the culture's adaptability to thrive without IC declined, being strongly limited to grow in its absence, even if a more oxidized compound like acetate is supplied (Table 4, tests without IC, condition 1). On the opposite, as IC limitation increases during the selection, the cultures are no longer exclusively dependent on IC to balance the reducing power, and the presence of a more oxidized compound, such as acetate, can function as a reducing power reliever, ensuring the production of PHA and biomass growth, with a predominance of PHA production (Table 4, tests without IC, condition 3 and 4). Additionally, when considering the cultures selected with alternating periods or permanent absence of IC, a dissociation of biomass growth and PHA production was observed, and PHA production could be improved in the absence of IC. Indeed, imposing IC limitations led to the highest PHA productivities registered in batch tests with butyrate as the sole carbon, and the cultures were capable of improving their PHA productivity up to 10 times when compared with the control tests. In the batch tests, it seems that the limiting growth conditions imposed by the absence of IC can boost PHA production by the PPB mixed culture,

mimicking a similar behavior widely explored in PHA accumulating aerobic bacteria through the uncoupling of ammonia from the organic carbon (Oliveira et al., 2017; Matos et al., 2021, Cruz et al., 2022). As such, IC limitation should be considered in future studies since it could be an advantageous strategy when targeting improved phototrophic PHA production while limiting growth.

With respect to the microbial communities, the most significant shift was observed in condition 4, where the PPB mixed culture was predominantly composed of *R. palustris*, a species that had already shown considerable increase in conditions 2 and 3. Undoubtedly, *R. palustris* thrived in high reductive stress environments and did not depend exclusively on the CBB cycle to manage excessive reducing power. Besides PHA production, which, as discussed above, limits butyrate flow towards the TCA cycle and thus decreases the production of reductive power, hydrogen production could be one of the alternative pathways that could help alleviate the reductive stress. In fact, hydrogen production has been described in this species (McKinlay and Harwood, 2010; Touloupakis et al., 2021), and despite the high levels of ammonium in the reactor (see supplementary material), studies have shown that hydrogen can be produced up to 100 mg N/L, although with very low productivities (Capson-Tojo et al., 2020). Likewise, the glyoxylate shunt pathway has also been described in members of *R. palustris* (McKinlay et al., 2014). As such, bypassing the TCA cycle could function as a way to prevent further production of reductive power (3 NADH and 1 $FADH_2$ are obtained in the TCA cycle per acetyl-CoA while 2 acetyl-CoA are consumed in the glyoxylate shunt producing 2 NADH and 1 $FADH_2$), without the need for IC, contrasting with the EMC pathway discussed above. Despite the less extensive research in *R. palustris*, there are two pathways that must also be taken into consideration, and that could play a role in maintaining redox homeostasis. Those are the isoleucine production pathway, which has been previously linked to the regulation of redox power in PPB (Yen & Gest, 1974; Alloul et al., 2022), as well as the EPS production, which has been reported as a protection mechanism of several bacteria, including PPB when submitted to stress conditions (Nunkaew et al. 2014; Zhang et al. 2019; Ates, 2015). Likewise, it is also noteworthy to mention that with the decrease in the IC availability in the medium, an increased concentration of excretion products was observed (see supplementary material), which could also play a role in dissipating reductive power. Furthermore, while the metabolic pathways have an important role in the redox-balancing of PPB, it is also important to note that different species may exhibit varying elemental compositions, which could influence their reduction levels and capacity to handle reductive stress and, therefore, present higher adaptability to the higher reductive environments (Alloul et al. 2022).

Overall, operation under different IC availabilities had its most significant impact on the population selection, and within each selected culture, IC was a direct effector of biomass growth, stimulating growth when present and inhibiting when absent. Also, although all four different operating conditions allowed the selection of PPB mixed cultures enriched in PHA accumulating bacteria, the absence of IC seemed to significantly favor PHA accumulation in batch tests and should be considered in further testing. Nevertheless, to explore this feature, a previous culture selection with IC limitation periods is required to select PPB with such capability.

4. Conclusions

This study highlights the impact of IC availability on the selection and PHA production performance of PPB mixed cultures. Experiments showed that IC presence favors biomass growth over PHA production. On the opposite, IC absence can potentially boost PHA production in cultures previously selected under alternating IC availability or absence of IC, suggesting that IC limitation might impair growth, thereby enhancing PHA production. Additionally, sequencing results unveiled the potential for *Rhodopseudomonas palustris* to grow in highly reductive stress environments with limited IC, suggesting its capacity to engage alternative metabolic pathways to alleviate excessive reducing power while maintaining PHA accumulation capacity.

CRediT authorship contribution statement

Juliana Roda Almeida: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Maria Ascensão Miranda Reis:** Validation, Supervision, Resources, Conceptualization. **Joana Costa Fradinho:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Conceptualization.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2025.132462.

Data availability

Data will be made available on request.

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