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Enzymatic degradation of polyhydroxybutyrate, electrochemical monomer recovery and upcycling by *Pseudomonas alloputida*

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Abstract

An integrated enzymatic–electrochemical system for PHB degradation and upcycling has been developed. Different bacterial strains of *Priestia megaterium*, *Alcaligenes faecalis subspecies faecalis and Comamonas terrigena* were screened for extracellular PHB depolymerase production. The most promising strains were selected for fermentation medium optimization to induce enzyme production. *Priestia megaterium* NRRL B-3254 and *Alcaligenes faecalis subspecies faecalis* NRRL B-1695 were further selected for utilization of the crude extracellular enzymes and optimization of PHB hydrolysis, including the effects of substrate concentration, pH, and buffer composition. Enzymatic degradation yield reached 67%. Subsequently, an electrochemical membrane extraction cell was integrated with the enzymatic reactor to enable simultaneous PHB degradation and monomer separation. The effect of high pH in the electrochemical cell alone, resulted in 80% degradation yield, with 32% content of 3-hydroxybutic acid in the soluble fraction, while the integrated enzymatic–electrochemical process resulted in 72% degradation yield, with 46% content of 3-hydroxybutic acid in the soluble fraction. Finally, the recovered monomer was upcycled as an additional carbon source for polyhydroxyalkanoate production by *Pseudomonas alloputida*, demonstrating the feasibility of a circular bioprocess for polymer degradation and valorization.

Keywords: Polyhydroxybutyrate, enzymatic degradation, electrochemical membrane extraction, upcycling

1 Introduction

The global production and consumption of plastics have increased dramatically over the past decades, driven by population growth and rising demand for materials. More than 8,300 million tons of plastics have been produced since the onset of large-scale plastic production in the 1950s. Approximately 60% are accumulating in the environment due to insufficient waste management. In 2024 alone, global plastic production reached 430.9 million tons, while recycling rates remain low (~9%), and a significant fraction of plastic waste is either landfilled or mismanaged (Geyer et al. 2017; Rosenboom et al. 2022; Plastics Europe 2025). The packaging sector is the main contributor to plastic production (35.9%) and waste generation (due to its short lifespan) (OECD 2023). As a result, large quantities of plastics accumulate to the environment where they undergo fragmentation through mechanical, photochemical, and biological processes, forming microplastics and nanoplastics that pose serious risks to living beings. These particles can induce cytotoxic effects, act as carriers of pathogens and absorb hazardous compounds such as persistent organic pollutants (POPs), amplifying their environmental burden (Ali et al. 2021).

In response to these challenges, there is an urgent need for the transition from the current linear fossil-based economy to a circular bio-based economy with emphasis in sustainability, resource efficiency, and waste valorization (Bachmann et al. 2023). Bio-based and biodegradable polymers such as polylactic acid (PLA) and polyhydroxyalkanoates (PHAs), are gaining increasing attention as alternatives to conventional plastics. Although their current production accounts for less than 1% of global plastics, it is expected to grow significantly in the coming years (Rosenboom et al. 2022). The development of circular systems involves the use of renewable feedstocks, including agricultural and food waste, within biorefinery concepts to produce monomers and polymers via microbial fermentation and chemical conversion. Among biopolymers, PHAs are especially promising due to their biodegradability and tunable properties, with poly(3-hydroxybutyrate) (PHB) being a rigid material suitable for packaging applications, and its copolymers offering enhanced flexibility. The integration of sustainable production, efficient end-of-life strategies, and innovative recycling or degradation technologies is essential to reduce environmental impact and enable the development of closed-loop systems for plastic materials (Ali et al. 2021). Moreover, PHAs have been proved to be non-cytotoxic and can be used as additional feed source for marine organisms (Wang et al. 2019).

Polyhydroxyalkanoates (PHAs) are produced intracellularly by various microorganisms (*Cupriavidus necator*, *Priestia megaterium*, *Paucimonas lemoignei*) as a result of carbon excess

and nutrient limitation, where high energy ester bonds serve as carbon and energy storage. The microorganisms that are able to produce PHAs are also able to degrade them with intra- or extra-cellular enzymes, named PHA depolymerases. The polyester hydrolysis into oligomers and/or monomers is triggered by carbon limitation conditions as a survival strategy that provides readily utilized carbon source (Anderson and Dawes 1990; Khanna and Srivastava 2005). Among PHAs, poly(3-hydroxybutyrate) (PHB) is the most widely studied short-chain-length polyester. Its physicochemical properties like high crystallinity (60–80%) and melting temperature of approximately 170–180 °C, while advantageous for material applications, significantly influence its biodegradability, as enzymatic hydrolysis is primarily a surface-mediated process and is strongly affected by polymer crystallinity, molecular weight, and morphology.

Both intracellular and extracellular PHB depolymerases belong to the broader class of α/β-hydrolase fold enzymes and share a common catalytic mechanism based on a Ser–His–Asp catalytic triad, similar to lipases and carboxylesterases. Intracellular PHB depolymerases act on native polyester (nPHB) in its amorphous state that has a surface layer consisting of proteins and phospholipids. Extracellular depolymerases act on partially crystalline denatured PHB (dPHB), which has lost its surface layer by physical or chemical stress, producing soluble oligomers and monomers such as 3-hydroxybutyrate (3-HBA) (Jendrossek and Handrick 2002; Chen et al. 2009). Extracellular PHB depolymerases typically consist of a substrate-binding domain, and, in many cases, a hydrophobic binding domain that facilitates adsorption onto the polymer surface. The degradation of PHB is a heterogeneous, surface-mediated process that proceeds in two distinct steps: (i) adsorption of the enzyme onto the hydrophobic PHB surface and (ii) catalytic hydrolysis of ester bonds within the polymer chain. Upon adsorption, the active-site performs a nucleophilic attack on the carbonyl carbon of the ester bond, forming a tetrahedral intermediate stabilized by an oxyanion hole, followed by cleavage into an acyl–enzyme intermediate and release of the first product. Subsequent hydrolysis of the acyl–enzyme complex releases soluble oligomers or monomers such as 3-hydroxybutyrate (3-HBA) (Jendrossek and Handrick 2002; Martínez-Tobón et al. 2018).

Due to the semicrystalline nature of PHB, enzymatic attack occurs preferentially in the amorphous regions, while crystalline domains are more resistant to degradation, leading to incomplete hydrolysis and the accumulation of intermediate oligomers. Enzyme adsorption is a critical rate-limiting step and depends on surface hydrophobicity, enzyme charge, and environmental conditions such as pH and ionic strength. Additionally, product inhibition by soluble oligomers and 3-HBA, as well as limited enzyme accessibility due to polymer

aggregation at higher substrate concentrations, further reduce hydrolysis efficiency (Mukai and Doi 1995; Tokiwa and Calabia 2004).

Furthermore, the mode of action of PHB depolymerases can vary between endo-type and exo-type mechanisms. Endo-type depolymerases randomly cleave internal ester bonds, rapidly reducing molecular weight and generating oligomers, whereas exo-type enzymes progressively release monomers or dimers from the polymer chain ends. Many bacterial extracellular depolymerases exhibit a combination of both activities, which explains the coexistence of oligomeric and monomeric products during hydrolysis. The relative contribution of these mechanisms, together with enzyme specificity and stability, ultimately determines the product distribution and limits the overall monomer yield observed in enzymatic PHB degradation systems (Kasuya et al. 1996; Jendrossek and Handrick 2002).

In contrast, thermochemical degradation of PHB proceeds via non-specific ester bond cleavage and is strongly dependent on pH and temperature. Under alkaline conditions (pH > 10), hydroxide ions promote a nucleophilic attack on the ester bonds, leading to rapid chain scission and high polymer conversion. At temperatures between 50 and 100 °C, hydrolysis rates increase substantially, and near-complete degradation (>90%) has been reported. However, the process lacks selectivity, as primary hydrolysis products such as 3-HBA can undergo secondary reactions, including β-elimination to crotonic acid, resulting in complex product mixtures and reduced monomer recovery. Therefore, while thermochemical methods enhance degradation efficiency, they do not provide effective control over product distribution (Kunioka and Doi 1990; Iwata et al. 1999; Yu et al. 2005; Saito et al. 2023).

Electrochemical technologies offer a promising alternative for process intensification by coupling reaction and separation in a single system that has not been applied to PHAs degradation yet. The electrochemical membrane extraction cell consists of a cathode compartment, where hydroxide ions (OH^-) are generated through water electrolysis, potentially facilitating alkaline hydrolysis reactions, where the 3HBA monomer is produced. An anion exchange membrane (AEM) enables the selective transport and recovery of organic acids under an applied electric field towards the anode compartment where the accumulation of organic acid occurs. Such systems have been successfully applied for the extraction of carboxylic acids including succinic, fumaric, and butyric acids, achieving continuous separation with reduced chemical input (Pateraki et al. 2019a; Tatsi et al. 2022).

This work focuses on optimization of enzyme production from wild type bacteria and subsequent enzymatic hydrolysis optimization of PHB. The enzymatic-electrochemical reactor

was developed for PHB hydrolysis and simultaneous monomer extraction and its subsequent upcycling via fermentation for the production of PHA with *Pseudomonas alloputida*.

2 Materials and Methods

2.1 List of microorganisms

Priestia megaterium NRRL B-3254, B-3255, B-3701, B-3702, B-3703, B-3710, *Alcaligenes faecalis subspecies faecalis* NRRL B-170, B-1695, *Comamonas terrigena* B-781 that were screened for PHB depolymerase production were received from ARS (NRRL) Culture Collection. *Pseudomonas alloputida* DSM 6125 that was used for polyhydroxyalkanoate production was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. All microorganisms were grown in 250 mL shake flasks containing 50 mL of 5 g/L peptone and 3 g/L meat extract, for 14-16h at 30 ºC at 220 rpm. Long term preservation occurred in 50% glycerol stocks at -80 ºC.

2.2 Screening for their ability to produce PHB depolymerases

Initial screening for their ability to produce PHB depolymerases was carried out in double layered square petri dishes containing a layer of mineral medium 457 with 20 g/L of agar and a second layer of sonicated mineral medium 457 with 20 g/L of agar and 2g/L commercial PHB powder (100 micron). 20 μL of grown cultures were used for the inoculation on cellulose discs (diameter: 6 mm) incubated at 30 ºC for 10 days. The presence of a hallow zone around the disc indicated depolymerase production. Mineral medium 457 contained (in g per L): 2.44 Na_2HPO_4, 1.52 KH_2PO_4, 0.5 $(NH_4)_2SO_4$, 0.2 $MgSO_4 \cdot 7H_2O$, 0.05 $CaCl_2 \cdot 2H_2O$, 0.005 EDTA, 0.002 $FeSO_4 \cdot 7H_2O$, $1x10^{-4}$ $ZnSO_4 \cdot 7H_2O$, $3x10^{-5}$ $MnCl_2 \cdot 4H_2O$, $3x10^{-4}$ H_3BO_3, $2x10^{-4}$ $CoCl_2 \cdot 6H_2O$, $1x10^{-5}$ $CuCl_2 \cdot 2H_2O$, $2x10^{-5}$ $NiCl_2 \cdot 6H_2O$, $3x10^{-5}$ $Na_2MoO_4 \cdot 2H_2O$.

2.3 Production of PHB depolymerase

P. megaterium NRRL B-3254, B-3702, B-3703 and *A. faecalis subsp. faecalis* NRRL B-1695 were selected for further investigation of PHB depolymerase production in shake flask fermentations. Minimal growth medium contained 2 g/L of succinic acid, 0.96 g/L of NH_4Cl, 0.49 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $FeCl_3 \cdot 6H_2O$ and 0.005 g/L $CaCl_2$ in 33mM KH_2PO_4-Na_2HPO_4 buffer at pH 6.8. Minimal secretion medium contained 1.5 g/L of succinic acid, 0.96 g/L of NH_4Cl and 0.25 g/L $MgSO_4 \cdot 7H_2O$ in 33mM KH_2PO_4-Na_2HPO_4 buffer at pH 7.4. The minimal growth medium (50 mL) was inoculated with 1 mL of the stock in cryotubes and was incubated at 30°C and 220 rpm in an orbital shaker incubator for 24 h. The minimal growth medium

cultures were centrifuged, and the biomass pellet was washed twice with phosphate buffer at pH 7.4 and then it was transferred in the minimal secretion medium (50 mL). It was incubated at 30°C and 220 rpm in an orbital shaker incubator for 48 h, where the production of extracellular and intracellular PHB depolymerases was monitored throughout the fermentation.

PHB depolymerase production was also evaluated in minimal growth/secretion medium supplemented with 1 g/L of yeast extract and in mineral medium 457. Glucose and PHB were also evaluated as potential carbon sources for PHB depolymerase production. All experiments were carried out in duplicates.

2.4 Enzyme activity assays and protein determination

Samples during the fermentation were taken at various intervals and centrifuged for 10 min at 4 °C and 10,000 rpm. The supernatant contained extracellular crude enzymes. The biomass pellet was resuspended in 100mM Tris-HCl / 1mM $CaCl_2$ buffer, pH 8 and was probe sonicated on ice bath for 40 sec followed by 20 sec resting time, for six intervals, aiming to release the intracellular enzymes. The enzymatic reactions started with the addition of 0.5 mL extracellular or intracellular enzymes in 2.5 mL final reaction volume that contained 0.3 g/L commercial PHB in 100 mM Tris-HCl/1 mM $CaCl_2$ at pH 8. The enzymatic reaction was carried out for 30 minutes at 37 °C in a water bath. One unit (U) of PHB depolymerase activity was expressed as the enzyme quantity needed to reduce the turbidity at 650nm in 1-cm-light-path cuvettes by 0.001 OD units per minute (Stinson and Merrick 1974). Enzymatic reactions were terminated by boiling at 95 °C for 5 min.

2.5 Enzymatic degradation optimization

The enzymatic hydrolysis of commercial PHB was optimized at two buffer solutions (Tris-HCl/$CaCl_2$ buffer and phosphate buffer) at different pH values (6, 7, 8 and 9) and different initial PHB concentrations (3, 5 and 10 g/L). The experiments were carried out in Duran bottles, at 37 °C and agitation at 250 rpm over 48 hours. Extracellular enzymes produced by *A. faecalis subsp. faecalis* NRRL B-1695 and *P. megaterium* NRRL B-3254 were added to the mixture at enzyme loading of 3U/mL of hydrolysis. The supernatant was further analyzed using HPLC to determine the degradation products and the precipitated PHB was washed, dried at 100 °C and stored in a desiccator until constant weight. The final weight of the dried precipitate was used to calculate the reduction in PHB concentration. The hydrolysis yield was calculated to assess the efficiency of the process using the following formula:

$$Y_{PHB} = \frac{M_0 - M_x}{M_0} \times 100\%$$

where M_0 is the initial PHB mass in the mixture and M_x is the remaining PHB mass.

Moreover, the hydrolysis yield leading to the monomer was also calculated to assess the efficiency of the process using the following formula:

$$Y_{3-HBA} = \frac{M_{PHB} * 1.21}{M_{3-HBA}} \times 100\%$$

2.6 Enzymatic reactor coupled with electrochemical membrane extraction

An electrochemical extraction cell coupled with an enzyme reactor was employed for the degradation of PHB and the hydroxybutyric acid (3HBA) monomer purification. The enzymatic hydrolysis was carried out in wide mouth, baffled Duran bottles (0.5L), GLS 80, stirred reactor system with 500 mL working volume. Initial PHB concentration was 5 g/L in 100 mM Tris-HCl/1mM $CaCl_2$ buffer with crude extracellular enzyme loading of 100 U/g of PHB. The temperature (37 ºC) and agitation (150 rpm) were controlled by a hot plate magnetic stirrer. The pH was controlled by automatic addition of 1M NaOH or 5M H_3PO_4 with a peristaltic pump connected with a pH meter.

The electrochemical cell comprised of two compartments, an anode and a cathode, the dimensions of which were 20 cm (l) × 5 cm (w) × 1 cm (d) cm and 20 cm (l) × 5 cm (w) ×2.5 cm (d) cm respectively (Stylianou et al. 2023). The two chambers were divided by a single anion exchange membrane (AEM), with operating surface dimensions of 20 cm (l) × 5 cm (w) × 0.3 cm (thickness) (AMI 7001, Membrane International, New Jersey, United States). The cathode was a stainless-steel mesh (20 cm × 5 cm), and the anode an iridium oxide-coated titanium electrode, IrO_2/TaO_2: 0.65/0.35, (20 cm × 5 cm). Two spacers were placed between the surface of the electrodes and the anion exchange membranes to prevent electrode contact with the AEM and promote convection at the membrane surface. The distance between the two electrodes was around 1 cm. The cathode was connected to the enzyme reactor and the anode contained 5 mM K_2HPO_4. Both solutions were circulated in parallel flow across the membrane at a rate of 3.7 L/h in the cathode and 4.2 L/h in the anode, using two peristaltic pumps. The solution of the extract chamber was recycled in an external vessel, with a total liquid volume of 100 mL and a hydraulic retention time of 0.02 h^{-1}, was maintained constant throughout the experiments. The temperature of the extraction vessel was controlled at 37 °C using a water bath. A power supply unit was used to apply current to the cell. The electric current was controlled at 1.03 A, and the voltage was monitored throughout the experiments. Three sets of

experiments were conducted, in order to determine whether degradation was a product of enzymatic hydrolysis, the electrochemical treatment of a combination of both: (i) without any enzyme addition and without pH control, (ii) without enzyme addition at controlled pH 8 with 5M H_3PO_4 and (iii) with extracellular PHB depolymerases produced by *P. megaterium* NRRL B-3254, at a constant pH of 8. Samples collected over the duration of the experiment were analyzed using HPLC to determine the degradation products formed.

2.7 Hydrolysate upcycling by *Pseudomonas alloputida* for the production of PHAs

Fed-batch fermentation was carried out in 250 mL baffled shake flasks containing 50 mL medium at 30 ºC, pH 6.8 and 180 rpm. The fermentation broth consisted of (per liter): 40 glucose 1.1 g $(NH_4)_2SO_4$ for a molar ratio of C/N equal to 80, 3.88 g K_2HPO_4, 1.6 g NaH_2PO_4, 0.1 $MgCl_2{\cdot}6H_2O$ 10 mg EDTA, 2 mg $ZnSO_4{\cdot}7\ H_2O$, 1 mg $CaCl_2{\cdot}2H_2O$, 5 mg $FeSO_4{\cdot}7\ H_2O$, 0.2 mg $Na_2MoO_4{\cdot}2H_2O$, 0.2 mg $CuSO4{\cdot}5\ H_2O$, 0.4 mg $CoCl_2{\cdot}6H_2O$, and 1 mg $MnCl_2{\cdot}2H_2O$. The monomer 3-hydroxybutyric acid that derived from the electrochemical cell was added frequently to keep the monomers concentration lower than 2 g/L to avoid inhibition. Samples were taken to monitor carbon source consumption, biomass and PHB accumulation, while pH was adjusted to 6.8 with 2 M HCl and 5 M NaOH.

2.8 Analytical methods

Cell growth was monitored by measuring the optical density (OD) at 660 nm in a spectrophotometer. Fermentation samples were collected in pre-weighted Eppendorf tubes and centrifuged (9000 g, 10 min, 4 ºC). The supernatant was stored at -20 ºC for further analysis, while the precipitated cells were washed twice with deionized water and once with acetone to remove any impurities, before drying at 65 ºC. The dried samples were left in a desiccator, until constant weight to determine the dry cell weight (DCW). The precipitated biomass obtained from fermentation samples was also used for PHA determination via extraction. Briefly, cell membrane disruption occurred using sodium hypochlorite and chloroform. For each gram of DCW, equal volume of sodium hypochlorite solution (20% v/v) and chloroform were added and left for 2 hours at room temperature under 150 rpm agitation. Three distinct phases were formed after centrifugation. The lower organic phase was collected via a separatory funnel, followed by concentration through evaporation and overnight precipitation at -20 ºC recovering PHA after drying at 50 °C.

Biomass content was also analyzed with Gas Chromatography (GC) after propanolysis. Briefly, 1,2-dichloroethane (2mL), 25% acidified propanol (2mL) and 200 μL of benzoic acid,

were added at maximum 40 mg of biomass and boiled at 100°C for 2 h in a water bath. Equal volume of water was added at room temperature assisting the formation of two immiscible liquid phases. The organic phase was collected and analyzed through a gas chromatographic analyzer (Shimadzu) at a Flame Ionization Detector (FID) and a Mega-Wax column (30 m × 0.25 mm, film thickness 0.25 μm). Helium was used as carrier gas at a flow rate of 1 mL/min. Oven initial temperature was set at 100°C for 1 min, followed by a temperature ramp of 25°C/min at 160°C, held constant for 1 min, and then increased to 188°C at a rate of 10°C/min. The temperature was further increased to 250°C at 25°C/min with a final isothermal period of 5 min. The injector and detector temperatures were 230°C and 250°C, respectively. Commercial PHB was used for qualitative analysis of composition.

HPLC (Jasco), equipped with a Phenomenex Rezex ROA-Organic acid H^+ column (300 mm×7.8 mm) coupled to an ultraviolet detector, was used for quantification of 3-hydroxybutyric acid. The analysis was conducted at 65 ◦C with a mobile phase of 10 mM H_2SO_4 at 0.6 mL/min. Sugar consumption was determined using DNS method (Miller 1959). Free amino nitrogen (FAN) was identified according to the ninhydrin method (Lie 1973) and inorganic phosphorous (IP) was identified according to a method developed by (Harland and Hraland 1980). Inorganic Nitrogen was determined via the phenate method (Scheiner 1976).

3 Results and Discussion

3.1 Screening of bacterial strains for PHB depolymerase activity

Out of the nine bacterial strains that were screened for PHB depolymerase production, six showed the potential to depolymerize PHB in solid agar media by forming a hallow zone at the perimeter of the cellulose disc (Figure 1). Specifically, *P. megaterium* NRRL B-3254, B-3701, B-3702, B-3703 *A. faecalis* subspecies *faecalis* B-1695 and *Comamonas terrigena* B-781 showed the ability to depolymerize commercial PHB and were selected for further optimization. Hallo zone increased up to 25 mm on the 10th day of incubation. The bacteria that showed weak or no depolymerase activity were *A. faecalis subsp. faecalis* NRRL B-170, *P. megaterium* NRRL B-3710, B-3255. Halo zone up to 40mm in width has also been reported with microbial isolates from different environmental samples at the 12th day of incubation (Manna and Paul 2000). PHB depolymerase activity has been detected to the genera *Bacillus*, *Clostridium*, *Streptomyces*, *Alcaligenes*, *Comamonas*, *Pseudomonas* and *Zoogloea* (Jendrossek et al. 1993a).

3.2 Medium optimization in shake flask fermentations

The composition of fermentation medium greatly affects the enzyme production performance. Therefore, different media were evaluated at five selected bacterial strains for the production of extracellular degradation enzymes. The highest enzyme activity was observed in minimal growth/secretion medium (MM) for all bacterial strains. *P. megaterium* B-3254 and *A. faecalis subsp. faecalis* B-1695 showed the highest extracellular enzyme activity of 3.95 U/mL and 3.73 U/mL, respectively at 24 h of fermentation (Figure 2). Minimal growth/secretion medium supplemented with 1 g/L yeast extract (MMY) showed slightly increased biomass concentration but lower enzyme activity for all strains except B-3702. Enzyme production is a result of catabolite repression and substrates that result in slower growth tend to produce more enzymes as shown in *Paucimonas lemoignei* (Delafield et al. 1965). Mineral medium 457 clearly inhibited enzyme production despite the presence of $NH_4^+$and Mg^{+2} in the medium that are necessary for enzyme secretion, as shown to play a role on the secretion mechanism in *Paucimonas lemoignei* (Stinson and Merrick 1974).

3.3 Effect of carbon source on PHB depolymerase production

Succinic acid has been evaluated as the main carbon source for PHB depolymerase production in various microorganisms (TANIO et al. 1982; Jendrossek et al. 1993b; Handrick et al. 2001). Carbon source concentration must be low (1.5 – 2 g/L) to trigger enzyme secretion through catabolite repression (Delafield et al. 1965). Glucose and PHB were also tested as carbon sources and showed significant enzyme production, but succinic acid was the preferred carbon source for PHB depolymerase production in both microorganisms. Specifically, *P. megaterium* NRRL B-3254 reached a maximum enzyme activity of 3.95 U/mL at 24 h of fermentation when succinic acid was the carbon source, while the maximum enzyme activity in PHB and glucose were 2 U/mL and 4.17 U/mL, respectively. *A. faecalis subsp. faecalis* NRRL B-1695 showed a similar pattern, with PHB depolymerase production of 3.73 U/mL, 5.5 U/mL and 2 U/mL in succinic acid, PHB and glucose, respectively, at 48 h of fermentation (Figure 3). Enzyme secretion in glucose was slightly higher than succinic acid at 48h of fermentation.

Succinate was also confirmed to be a requirement for enzyme secretion in *Paucimonas lemoignei* enzymes (Delafield et al. 1965) as it triggers cell starvation due to inability of its transport into the cell at pH above 7 (Jendrossek et al. 1993b). Contrary, in our case, succinate was consumed by both microorganisms and the pH reached 8.8 in both cases by the end of the fermentation. Interestingly when PHB was used as the carbon source, the extracellular enzyme

activity was lower in both microorganisms. *Alcaligenes faecalis* T1 showed maximum extracellular enzyme activity (0.3 U/mL) at the end of the exponential on 1.5 g/L PHB as the sole carbon source, while in succinate the enzyme activity decreased by 1/20 and in the monomer (3HBA) or the dimeric ester the enzyme was not produced (TANIO et al. 1982).

3.4 Enzymatic hydrolysis optimization with crude extracellular PHB depolymerases

Crude extracellular enzymes from *P. megaterium* NRRL B-3254 and *A. faecalis subsp. faecalis* NRRL B-1695 were used for PHB hydrolysis optimization. Substrate concentration (3, 5 and 10 g/L of PHB) was the first parameter that was evaluated in Tris-HCl/$CaCl_2$ and phosphate buffer. Impact of substrate was similar for both microorganisms with the maximum yield being 67% at 3 g/L of PHB (Figure 4A). Higher PHB concentration of 5 g/L resulted in a slight decrease of hydrolysis yield in Tris-HCl/$CaCl_2$ buffer (60% for both strains) but significant decrease in phosphate buffer (50% for 1695 and 17% for 3254). Severe substrate inhibition occurred at 10 g/L of PHB, in both buffer solutions and in both microorganisms, leading to hydrolysis yield lower than 10%. This can be attributed to the heterogeneous nature of PHB depolymerization, which is governed by enzyme adsorption onto the polymer surface rather than classical soluble substrate kinetics. PHB depolymerases possess a substrate-binding domain that facilitates attachment to the polymer surface, and hydrolysis occurs at the solid–liquid interface. As a result, the reaction is limited by the available surface area rather than the bulk substrate concentration. At elevated PHB concentrations, particle aggregation reduces the accessible surface area, while enzyme adsorption becomes saturated, leading to non-productive binding and decreased catalytic efficiency (Kasuya et al. 1996; Hiraishi et al. 2010).

A. faecalis subsp. faecalis NRRL B-1695 was slightly affected by the pH changes in Tris-HCl/$CaCl_2$ buffer, with hydrolysis yields being higher than 55%, leading to the conclusion that these enzymes are more stable in these conditions than the enzymes produced by *P. megaterium* NRRL B-3254, which resulted in significant drop at pH 6 (40%) and pH 9 (20%). Phosphate buffer did not offer enzyme stability at pH 6 and pH 9. The more pronounced inhibition in phosphate buffer suggests an additional role of ionic environment, potentially affecting enzyme adsorption and stability, while the presence of Ca^{2+} ions in Tris-HCl buffer is known to enhance PHB depolymerase activity. The effect of pH on PHB depolymerase activity is primarily attributed to changes in the ionization state of catalytic residues and overall enzyme structural stability.

PHB depolymerases are typically serine hydrolases, and their catalytic efficiency depends on the protonation state of the catalytic triad, resulting in optimal activity within a neutral to

slightly alkaline pH range. For example, depolymerases from *Pseudomonas stutzeri* exhibit maximum activity at pH 7.0, while enzymes from *Streptomyces* spp. and other *Pseudomonas* strains show optima in the range of pH 8.0–9.0, often retaining more than 70–90% of their maximal activity within this interval. (Kasuya et al. 1995, 1996; Jendrossek and Handrick 2002). Complete biodegradation of PHB by extracellular depolymerases has been reported in the literature, with studies demonstrating up to 100% degradation of PHB films under optimized conditions (e.g., *Pseudomonas* sp. DSDY0501) within short timeframes. However, such high degradation efficiencies are typically achieved at low effective substrate loadings and high surface accessibility (Di et al. 2019).

Phosphate buffer resulted in little or no monomer production, while in Tris-HCl/$CaCl_2$ buffer with 3 g/L initial PHB, *P. megaterium* NRRL B-3254 resulted in 0.9 g/L of 3-HBA (corresponding to 40% of the soluble hydrolysis products) and *A. faecalis subsp. faecalis* NRRL B-1965 resulted in 0.7 g/L of the monomer 3-HBA (corresponding to 30% of the soluble hydrolysis products). A final hydrolysis was carried out at larger scale (500 mL) in an enzymatic bioreactor using a 5 g/L initial substrate concentration and 100 U/g of substrate from extracellular enzymes produced by *P. megaterium* in Tris-HCl/$CaCl_2$ buffer at pH 8, resulting in 60 % hydrolysis yield with 1.13 g/L of 3-HBA. Enzymatic degradation of PHB did not lead to a full degradation of the polymer, and the monomer was only 38% of the soluble hydrolysis products (Figure 5).

3.5 PHB degradation: enzymatic hydrolysis and simultaneous 3-HBA separation using an electrochemical membrane extraction cell

Poly-hydroxybutyrate degradation can occur at high pH and temperatures leading to the production of 3-HBA and crotonic acid or other volatile compounds. Enzymatic degradation offers targeted degradation to its monomer, dimer or oligomers at mild temperatures. Use of chemicals and high temperature for hydrothermal alkaline treatment will lead to high energy demands, solvent/catalyst recovery and possible emissions. Moreover, product separation can be the main limitation.

The use of the electrochemical cell alone led to PHB degradation due to elevated pH values at room temperature. At the cathode compartment the pH can reach values of 12-14 due to water electrolysis and the generation of hydroxide ions (OH^-). Moreover, when the monomer is produced and reaches the cathode electrode, it dissociates and moves through the anion exchange membrane towards the anode compartment, where it accumulates. It is obvious that 3-HBA started being produced when PHB concentration was reduced by 80%, leading to the

conclusion that it is first converted to soluble oligomers, and these are later converted to 3-HBA (Figure 5A). Extraction of 3-HBA starts almost immediately at 0.3 g/L in the cathode compartment and is accumulated and concentrated in the anode compartment (Figure 5B). At 72h, PHB hydrolysis yield was 80% w/w with 3-HBA being 2.95 g/L in the anode compartment The concentration of the monomer in solubles was 32% w/w, leading to a 3-HBA to PHB yield of 21%.

Addition of enzymes in the enzymatic reactor that is connected to the cathode compartment requires a stable pH at 8 since enzyme activity is lower at higher pH values. For this reason, 5 g/L of PHB in Tris-HCl-$CaCl_2$ buffer were connected to the cathode compartment without enzyme addition but with pH regulation at 8, where PHB hydrolysis was not observed. The current supply alone does not have an impact on PHB degradation. After enzyme addition, hydrolysis occurred at the cathode compartment leading to 72% hydrolysis yield (Figure 5C). and simultaneous separation of 3-HBA in the anode compartment reaching a value of 5.9 g/L at 72h (Figure 5D). The concentration of the monomer in solubles was 46% w/w, leading to a 3-HBA to PHB yield of 28%.The system requires optimization in order to efficiently hydrolyze and separate the monomer. The system was operating at 1.03 A within a voltage range of 12-25 V in both cases. Coulombic efficiency of 3-HBA extraction was lower than 1% and the energy that was required to hydrolyze PHB was 484 kWh/kg of PHB.

The target of applying the electrochemical membrane extraction cell was to separate the monomer 3-hydroxybutyric acid (3-HBA) in one unit operation. The utilization of an electrochemical membrane extraction cell has already been used for the separation and purification of different organic acids like succinic acid, fumaric acid, butyric acid caproic acid and others (Pateraki et al. 2019b; Tatsi et al. 2022). The extraction of organic acids is driven by the current supply from the cathode compartment towards the anode compartment through an anion exchange membrane.

3.6 Production of PHA by *Pseudomonas alloputida*

The extract was then concentrated via evaporation and was used as an additional carbon source to *P. alloputida* fermentation to produce PHAs. The fermentation with *P. alloputida* was carried out with glucose as the main carbon source with small doses of 3-HBA feeding to avoid inhibition and ensure complete consumption of the monomer. Total DCW was 4.4 g/L with 40% PHA accumulation and a yield of 4.3 % w/w (Figure 6). In total, 40 g/L of glucose and 3 g/L of 3-HBA were consumed by *P. alloputida* KT2440 with a consumption rate of 0.33 g/L/h for glucose and 0.04 g/L/h for 3-HBA on average. A significant quantity of 59,2 mg/L of inorganic

nitrogen was also consumed, while inorganic phosphorus remained stable at around 1200 mg/L throughout the fermentation. Despite the continuous feeding of 3-HBA, incorporation of C4 monomers into the polymer was minimal (1.2% of total PHA composition). These results indicate that glucose was the main carbon source converted to PHA and *P. alloputida* channels carbon flux towards β-oxidation-derived intermediates, leading to the synthesis of medium-chain-length PHAs. Nevertheless, *P. alloputida* KT2440 has the ability to incorporate fatty acids into PHA composition directly related to the chain length of the supplied substrate. Odd-chain fatty acids typically yield odd-chain-length 3-hydroxyalkanoates, while even-chain fatty acids lead to the formation of even-chain length 3-hydroxyalkanoates. More specifically the number of carbon atoms in C7, C8 and C9 fatty acid substrates was mainly incorporated in its derivative hydroxy-fatty acid in the PHA (heptanoate resulted in 98% C7-chain-length PHA, octanoate resulted in 90% of C8-chain-length PHA and nonanoate resulted in 73% C9-chain-length PHA) (Sikkema et al. 2023).

Conclusion

This work demonstrates a novel integrated approach for the degradation and valorization of PHB through the combination of enzymatic hydrolysis and electrochemical membrane extraction, followed by microbial upcycling. Extracellular depolymerases produced by *Priestia megaterium* and *Alcaligenes faecalis* subsp. *faecalis* enabled efficient PHB hydrolysis, with maximum yields achieved at low substrate concentrations (3-5 g/L) in Tris-HCl/$CaCl_2$ buffer, The results highlighted the strong influence of substrate concentration as hydrolysis was inhibited at high substrate concentrations. The integration of an electrochemical membrane extraction cell allowed simultaneous degradation and recovery of 3-HBA, while electrochemical treatment alone achieved high polymer degradation. Finally, the recovered 3-HBA was successfully utilized as a secondary carbon source by *Pseudomonas alloputida* for polyhydroxyalkanoate production, confirming the potential of this system for circular bioprocessing.

Declaration of competing interests

The authors declare no conflict of competing interests

Acknowledgments

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development for the production and enzymatic recycling of bio-based poly(3-hydroxybutyrate)" with Acronym BioWaste2Plastics and ID No 7526.

Author Contributions Statement

L.Z.: Methodology, Data curation, Writing – original draft, **V.P.R.:** Methodology, **P.K.:** Methodology, **C.P.:** Conceptualization, Data curation, Validation, Supervision, Writing – review and editing

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5 Figure legends

Figure 1: Hallo zone around the cellulose disc after 10 days of incubation indicates PHB degradation by microorganisms grown on solid agar mineral medium with PHB as the sole carbon source. Strains lacking enzymatic activity do not exhibit hallo zone. *Comamonas terrigena* B-781*, Alcaligenes faecalis subspecies faecalis* NRRL B-170, B-1695, *Priestia megaterium* NRRL B-3701, B-3702, B-3703, B-3254, B-3255, B-3710

Figure 2: Extracellular enzyme activity of *P. megaterium* NRRL B-3254, B-3701, B-3702, B-3703 and *A. faecalis subsp. faecalis* NRRL B-1695 in different media (MM, MMY and 457). MM: Minimal growth/secretion medium, MMY: Minimal growth/secretion medium supplemented with 1g/L yeast extract and 457: Mineral medium DSM 457.

Figure 3: Extracellular enzyme activity of *P. megaterium* NRRL B-3254 and *A. faecalis subsp. faecalis* NRRL B-1695 in minimal growth/secretion medium and different carbon sources. SA: succinic acid (■), Glu: Glucose (●), PHB: Polyhydroxybutyrate (▲).

Figure 4: PHB hydrolysis yield with extracellular enzymes produced by *P. megaterium* NRRL B-3254 and *A. faecalis subsp. faecalis* NRRL B-1695 in Tris-HCl and phosphate buffer at pH 3 g/L initial PHB concentration and different pH values and initial substrate concentrations of 3, 5 and 10 g/L. PHB hydrolysis yield with extracellular enzymes produced by *P. megaterium* NRRL B-3254 and *A. faecalis subsp. faecalis* NRRL B-1695 in Tris-HCl buffer and phosphate buffer at pH 8 and initial substrate concentrations of 3, 5 and 10 g/L.

Figure 5: Use of electrochemical membrane extraction cell (i) without enzyme addition and uncontrolled pH (A: cathode and B: anode) and (ii) combined with the enzyme reactor at pH 8 (C: cathode and D: anode), PHB (○), 3-HBA (●), pH (⁕)

Figure 6: Fermentation with *Pseudomonas alloputida* for the production of PHA using glucose as the main carbon source, supplemented with 3-HBA monomer derived from PHB hydrolysis. Glucose (□), 3-HBA (○), DCW (■), PHA (●)

6 List of figures

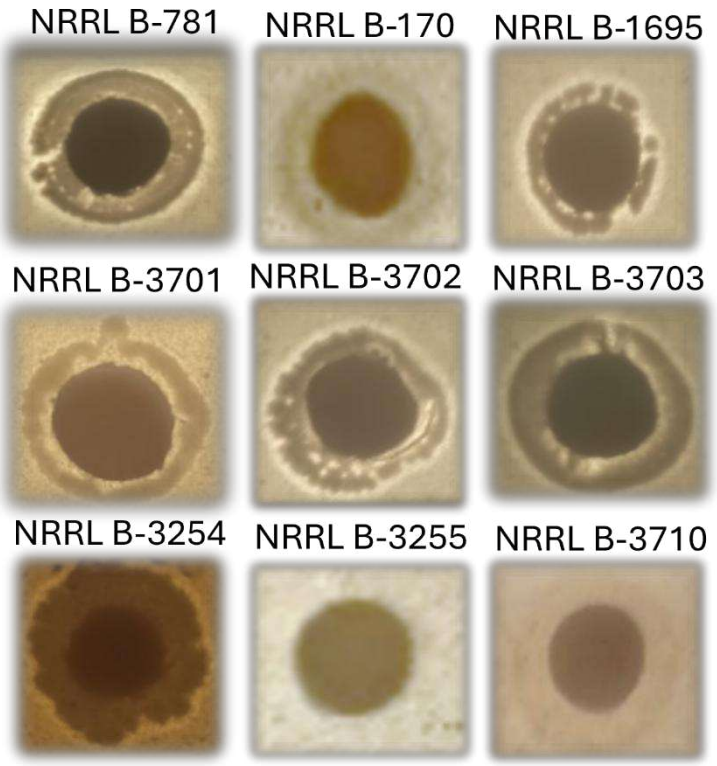

Figure 1

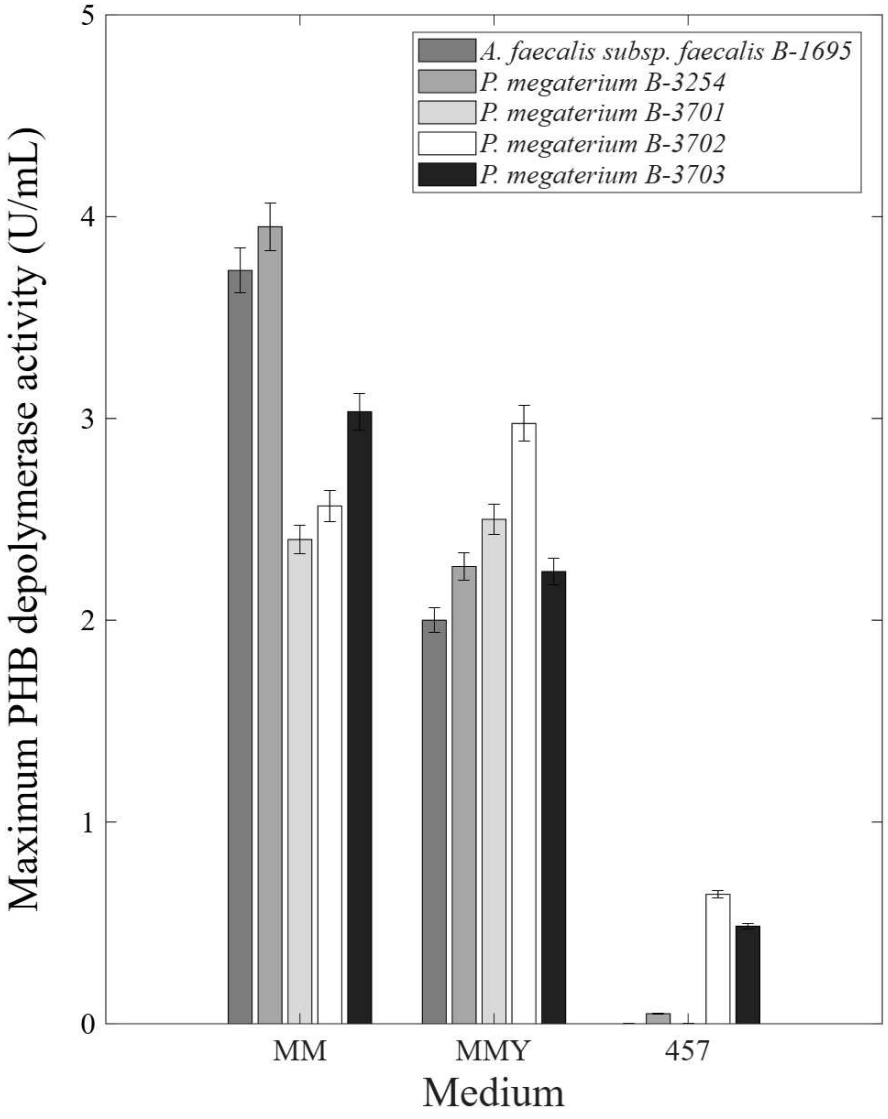
A. faecalis subsp. faecalis B-1695
P. megaterium B-3254
P. megaterium B-3701
P. megaterium B-3702
P. megaterium B-3703
Maximum PHB depolymerase activity (U/mL)
0
1
2
3
4
5
MM
MMY
457
Medium

Figure 2

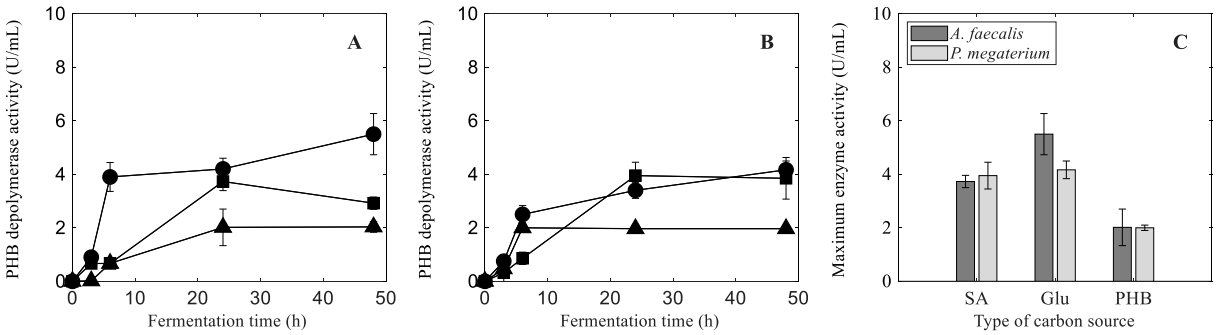

Figure 3

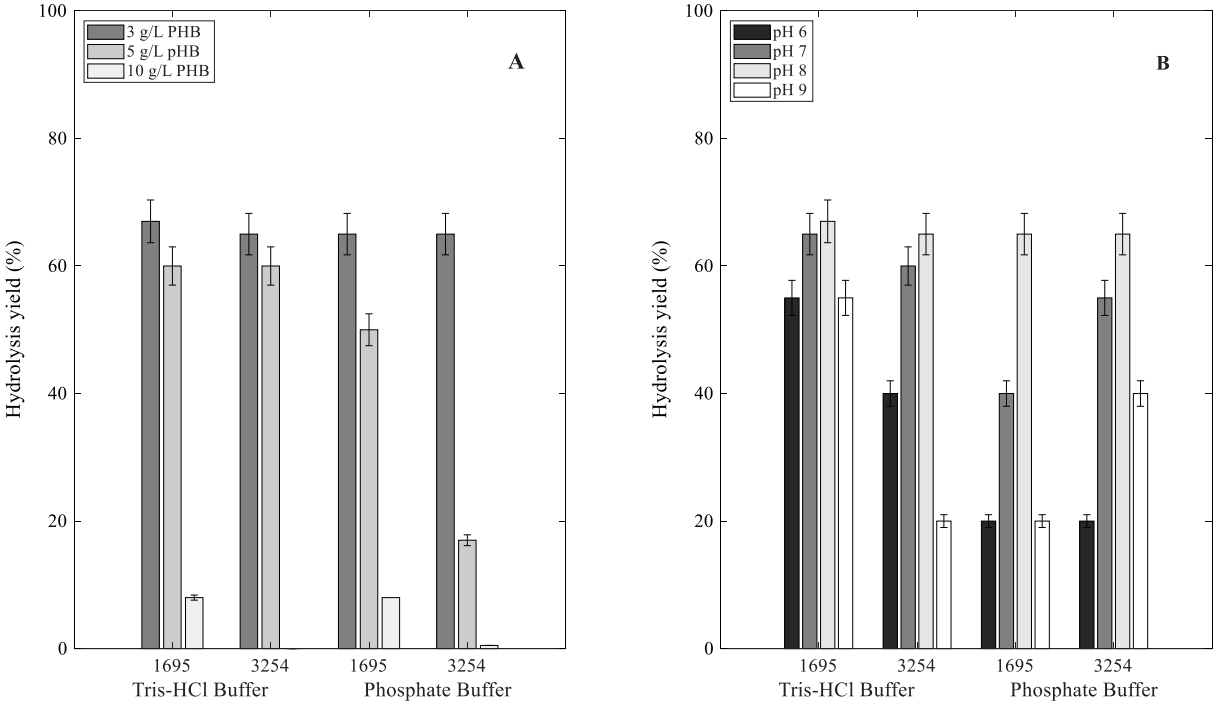

Figure 4

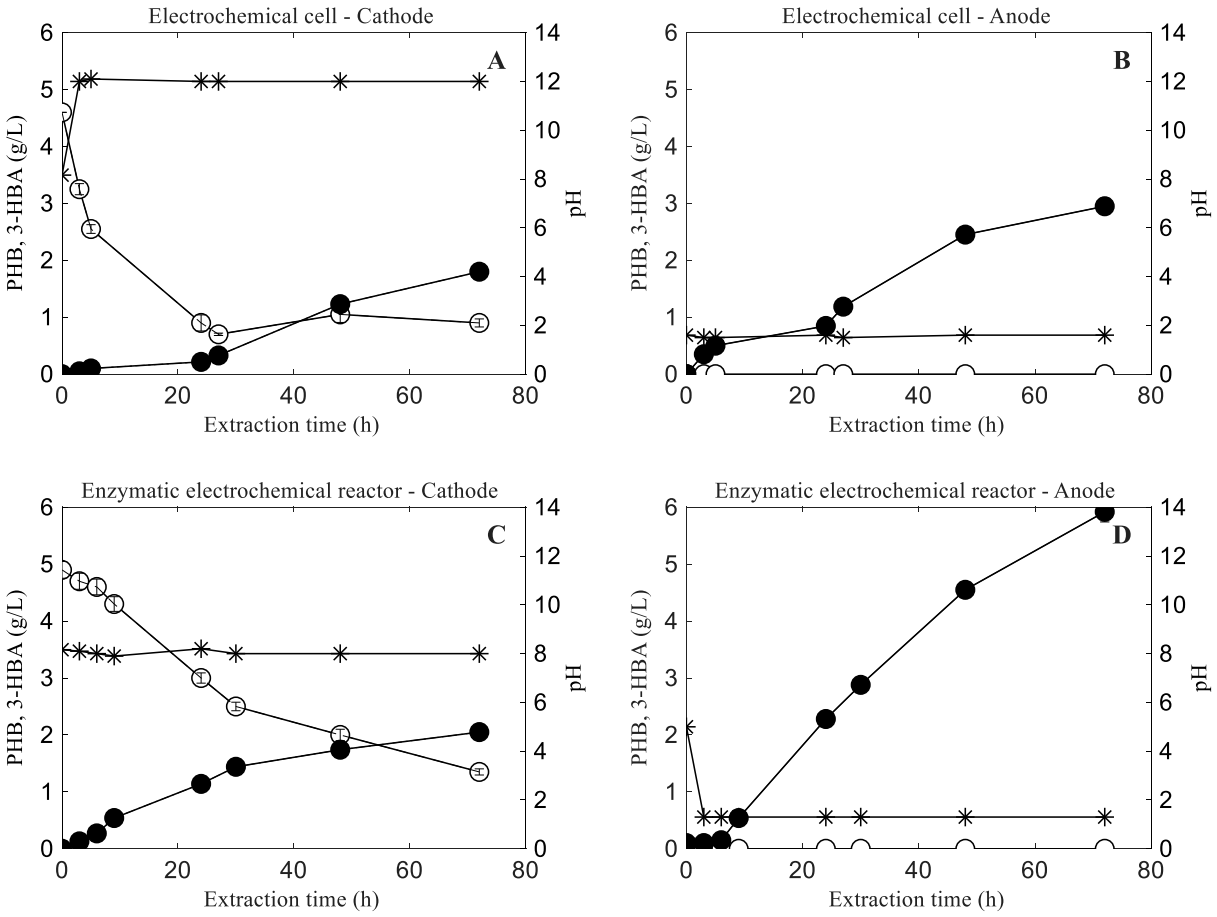
Electrochemical cell - Cathode
A
Electrochemical cell - Anode
B
Enzymatic electrochemical reactor - Cathode
C
Enzymatic electrochemical reactor - Anode
D
PHB, 3-HBA (g/L)
pH
Extraction time (h)

Figure 5

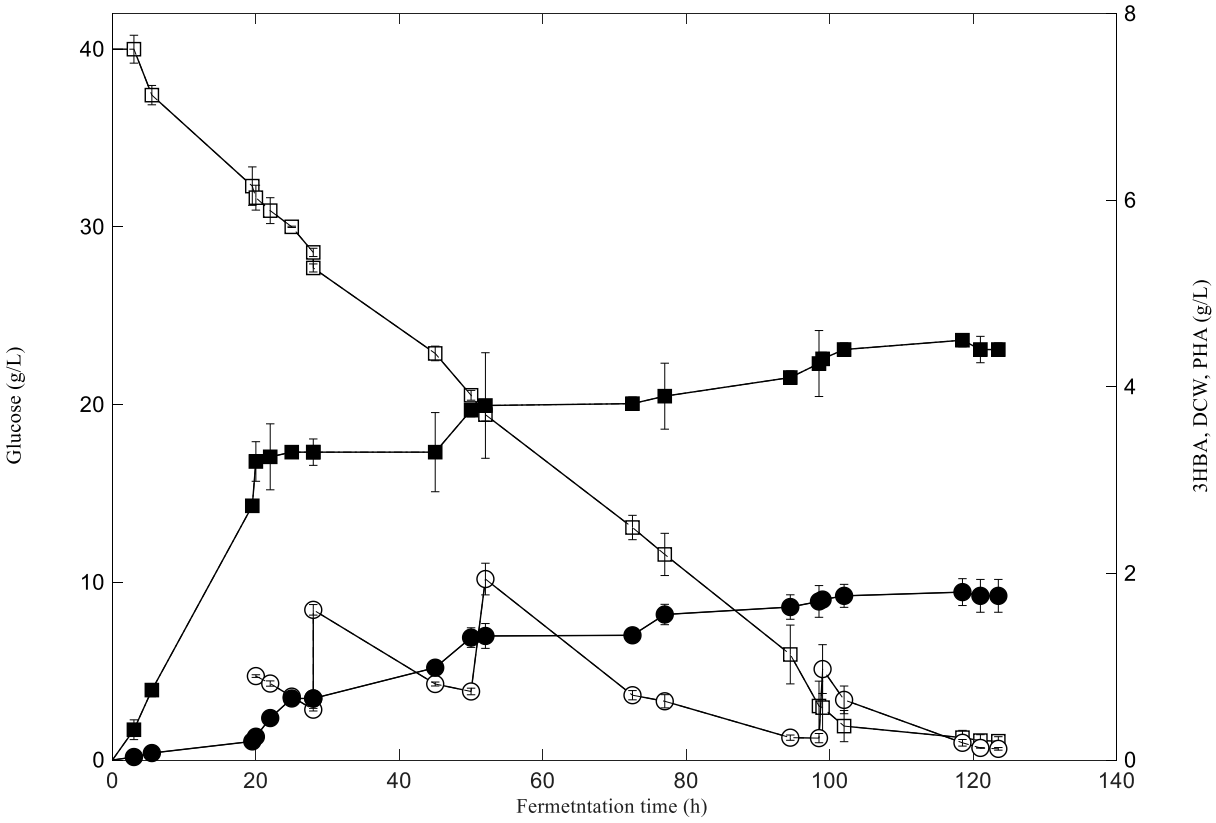
Glucose (g/L)
3HBA, DCW, PHA (g/L)
Fermetntation time (h)
0
10
20
30
40
0
2
4
6
8
0
20
40
60
80
100
120
140

Figure 6