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Valorization of aromatic hydrocarbons into polyhydroxyalkanoates: advances towards sustainable waste gas treatment

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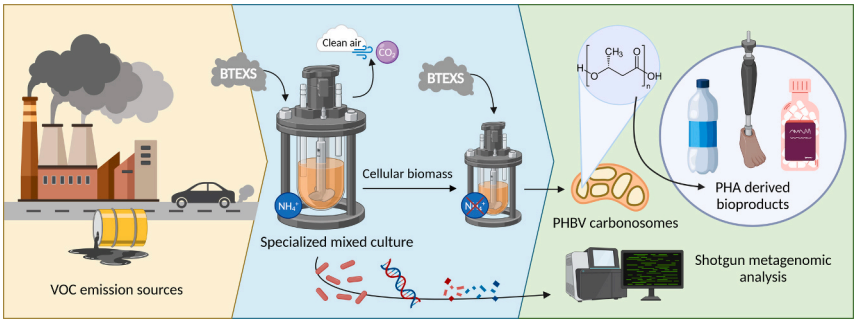
HIGHLIGHTS

- First demonstration of the continuous bioconversion of a BTEXS gas stream into PHA.
- Two-stage system removed toluene/ethylbenzene and synthesized PHA continuously.
- PHBV was produced with variable 3HB and 3HV monomer composition over time.
- Shotgun metagenomics revealed key bacteria, enzymes, and metabolic pathways.
- First-time identification of toluene degradation routes by *Pseudonocardia*.

GRAPHICAL ABSTRACT



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ABSTRACT

Benzene, toluene, ethylbenzene, xylene and styrene (BTEXS) are priority gaseous pollutants due to their widespread release and health risks. This study demonstrates an efficient BTEXS bioconversion process into polyhydroxyalkanoates (PHA) using a specialized mixed microbial culture dominated by *Pseudonocardia* and *Rhodococcus*. The consortium achieved simultaneous degradation rates of 15.1 ± 3.9 g $m^{-3}h^{-1}$ for toluene and 17.6 ± 5.7 g $m^{-3}h^{-1}$ for ethylbenzene, with removal efficiencies over 90 %. The operating strategy promoted PHA accumulation up to 21.4 % g_{PHA} g_{DCW}^{-1}. A two-step process was successfully implemented consisting of an initial reactor for biomass growth followed by a second reactor under nitrogen deprivation. PHA analysis revealed the synthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer with a dynamic increase in 3-hydroxyvalerate content under prolonged nitrogen starvation. Metagenomics provided insights into the microbial networks and metabolic pathways involved in the process. This research offers a sustainable solution for mitigating BTEXS pollution while producing valuable bioplastics.

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1. Introduction

Air pollution stands as one of the greatest challenges facing world society today. Volatile organic compounds (VOCs) are harmful pollutants which pose an extreme risk to human health due to their chemical structure (Akmirza et al., 2017). They are ubiquitous in the environment and can potentially lead to numerous health issues, including respiratory tract and nervous system damage, as well as carcinogenic, teratogenic, and mutagenic effects (Liao et al., 2022; Wu et al., 2023b). Additionally, VOCs contribute to adverse environmental impacts by promoting the synthesis of photochemical ozone and deteriorating air quality, particularly in urban areas (Tani & Mochizuki, 2021). VOCs are predominantly emitted by the petrochemical industry, but also by transport sources and chemical, pharmaceutical and paper manufacturing (Gao et al., 2022). Benzene, toluene, ethylbenzene, xylenes and styrene (BTEXS), which account for over 60 % of non-methane VOCs emitted globally (Liao et al., 2022; Yu et al., 2022), are classified as persistent organic pollutants because of their long half-life and their significant adverse impacts on human health and the environment (Mishra et al., 2023).

BTEXS treatment technologies are based on either the recovery or the removal of these pollutants. Physical methods like adsorption, absorption, condensation, and membrane separation have been widely commercialized for VOC recovery, transferring them to a liquid or solid medium. Their effectiveness is limited to scenarios with high pollutant loads, where the recovery process can justify the high associated costs. On the other hand, main destruction technologies include combustion, photocatalysis, and biological treatments (Gao et al., 2022). They are based on chemical or biochemical reactions that degrade pollutants into non– or less hazardous inorganic substances such as CO_2 and H_2O. While physicochemical technologies typically exhibit high removal efficiencies, their significant operational costs and environmental impacts undermine both their cost-effectiveness and sustainability. In contrast, biological treatment technologies (i.e. biofiltration, bioscrubbing, or membrane bioreactors) have gained recognition as environmentally friendly alternatives which offer economic competitiveness due to their low cost (Yu et al., 2022). Despite the significant biotechnological advances in BTEXS treatment, current biological approaches predominantly focus on their destruction rather than exploring their potential for bioconversion and circularity. Therefore, new strategies aimed at developing innovative biotechnological processes to valorize BTEXS into substances of economic and social value could revolutionize the landscape of biological waste gas remediation.

With the growing demand for sustainable bioplastics, polyhydroxyalkanoates (PHA) have emerged as one of the most promising alternatives to conventional plastics (Berezina et al., 2015). These biodegradable polyesters, produced by microorganisms under nutrient-limited conditions (e.g. nitrogen deficiency) and accumulated as intracellular granules known as carbonosomes (Dalton et al., 2022), are capable of forming biodegradable thermoplastics with valuable properties such as biodegradability and biocompatibility, and a wide versatility across various industrial sectors, including packaging and pharmaceutical manufacturing (Tan et al., 2015). The type and structure of the PHA will depend on the microorganism and the building block used for its synthesis (Koller & Mukherjee, 2020). Poly-3-hydroxybutyrate (PHB) is probably the most studied PHA due to its physical properties, resembling petroleum-based plastics like polyethylene terephthalate (Nanda et al., 2021). Several microorganisms are able to incorporate 3-hydroxyvalerate (3HV) units into the PHB molecule, forming a poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) copolymer, which exhibits improved flexibility and mechanical strength compared to PHB, making it attractive for medical and biomaterial applications (Jin et al., 2023). Despite these merits, PHA commercialization remains limited by the lack of competitiveness with conventional plastics, mainly resulting from the high production costs, with carbon substrates accounting for 70–80 % of these expenses (Li & Wilkins, 2020).

In this context, the valorization of BTEXS into PHA offers significant untapped potential in terms of cost-effectiveness and sustainability. However, scarce investigations have explored this alternative and its optimization in continuous bioreactors. *Pseudomonas* strains are the most investigated bacteria due to their VOC biodegradation and PHA synthesis capabilities (Tan et al., 2015; Tobin and O'Connor, 2005). Although a minority of previous studies have demonstrated that aerobic bacteria, such as *Pseudomonas* and *Rhodococcus*, can convert BTEXS compounds into PHA, most of this research has been limited to batch or short-term experiments (Hori et al., 2009; Nikodinovic et al., 2008). Consequently, the feasibility and effectiveness of applying this process in continuous operation remains largely unexplored. Gaining a deeper understanding of these microbial communities could not only uncover novel efficient PHA-producing strains but also enhance bioremediation strategies for the efficient removal of BTEXS compounds (Yoshikawa et al., 2016).

In light of the increasing interest and necessity for understanding BTEXS biodegradation and PHA synthesis under the circular bioeconomy principles, the present study examined the potential of a mixed culture for the synthesis of PHA from toluene and ethylbenzene as sole carbon sources. Following an initial batch screening phase, the process was optimized in a continuous reactor to enhance cell growth, BTEXS removal efficiency and PHA synthesis. The composition of the synthesized biopolymer was also analyzed to gain insight into its potential applications. Furthermore, a metagenomic analysis was performed to determine the microbial communities present in the process and the metabolic pathways involved.

2. Materials and methods

2.1. Bacterial strains and culture medium

A mixed culture previously isolated from a toluene stirred tank bioreactor, under mesophilic and aerobic conditions, was selected for further enrichment in PHA-producing microorganisms, owing to the high VOC-degrading performance demonstrated in earlier experiments. The mixed culture was grown in autoclaved 120 mL serum bottles with 50 mL of M9 mineral medium (Sambrook et al., 1989). The composition of M9 was as follows: 7.5 g L^{-1} (33.7 mM) Na_2HPO_4-$2H_2O$, 3 g L^{-1} (22 mM) KH_2PO_4, 0.5 g L^{-1} (8.6 mM) NaCl, 0.5 g L^{-1} (9.4 mM) NH_4Cl, 246.5 mg L^{-1} (1.0 mM) $MgSO_4$-$7H_2O$, 44.1 mg L^{-1} (0.3 mM) $CaCl_2$-$2H_2O$, 1 μg L^{-1} biotin, 1 μg L^{-1} thiamine-HCl, 50 mg L^{-1} EDTA, 8.3 mg L^{-1} $FeCl_3$-$6H_2O$, 0.84 mg L^{-1} $ZnCl_2$, 0.13 mg L^{-1} $CuCl_2$-$2H_2O$, 0.10 mg L^{-1} $CoCl_2$-$2H_2O$, 0.10 mg L^{-1} H_3BO_3 and 0.016 mg L^{-1} $MnCl_2$-$4H_2O$. When specified, D-glucose was added at a concentration of 4 g L^{-1} (22.2 mM). The bottles were closed with butyl septa and aluminum caps and inoculated at 5 % v/v of the mixed culture. Incubation was conducted at 25 °C and 150 rpm for 5 days in an IKA KS 4000i orbital shaker (Staufen, Germany). Once the culture reached an OD_{650} of 0.734 (0.71 ± 0.03 g L^{-1}), the inoculum was transferred to autoclaved 1.2 L Schott Duran Afora V-62897 bottles (Mainz, Germany) sealed with bromobutyl septa. The bottles contained 200 mL of M9, 5 mL of the previous inoculum and a concentration of 4 g m^{-3} of the corresponding BTEXS in the gas phase. The concentration of NH_4Cl was adjusted based on the specific nitrogen (N) requirements of each experiment: 0× (M9-NF), 1× (1.31 g N-NH_4Cl L^{-1}), 2× (2.62 g N-NH_4Cl L^{-1}) or 10× (13.09 g N-NH_4Cl L^{-1}). BTEXS were added in liquid phase to the bottles using a 10 μL Hamilton 800 Series liquid syringe (Reno, NV, USA). Growth was maintained for 1 week at 25 °C and 250 rpm under continuous agitation using a Thermo Fisher Scientific Cimarec i Multiple 6 magnetic stirrer plate (Waltham, MA, USA).

2.2. Chemicals and reagents

All the reagents for the preparation of M9 were acquired from

PanReac AppliChem (Barcelona, Spain) or Merck (Darmstadt, Germany). Aromatic volatile organic compounds were purchased from Sigma-Aldrich (Madrid, Spain) in their liquid form, at purities of toluene ≥99.5 %, ethylbenzene ≥99.8 % and styrene ≥99 %. For PHA analyses, chloroform (≥99 %), 1-propanol (99.7 %), hydrochloric acid (37 % v/v) and benzoic acid (≥99.5 %) were supplied by PanReac AppliChem (Barcelona, Spain). PHB and PHBV (molar ratio 88/12, ≥99.99 %) standards were purchased from Sigma-Aldrich (Madrid, Spain).

2.3. Aromatic hydrocarbons biodegradation in batch assays

For the batch assays, autoclaved 1.2 L bottles containing 200 mL of M9 were inoculated at 5 % v/v of the previously acclimated inoculum. The biodegradation potential of toluene, ethylbenzene and styrene was studied individually and in a mixture containing all of them. The corresponding amount of liquid pollutant was added as the sole carbon and energy source at a final concentration of ~4 g m^{-3} in the gas phase. Each condition was tested in duplicate, together with an abiotic control to rule out any physical–chemical removal of the pollutants. The assays were maintained at 25 °C under continuous agitation at 250 rpm. Pollutants were replenished in the gas phase upon depletion. The liquid medium from assays that successfully completed at least three removal cycles was then centrifuged, and the biomass was resuspended in 200 mL of M9-NF to repeat the test under *N*-deficiency conditions. The experiments concluded when no further degradation of the contaminants was observed.

During the batch assays, VOC concentrations in the gas phase and optical density in the liquid phase were daily analyzed. Additionally, liquid samples were withdrawn by the end of the assays to determine optical density (OD), total nitrogen (TN), total organic carbon (TOC), inorganic carbon (IC), and total suspended solids (TSS) concentrations. Biomass samples were also taken during the *N*-deficiency phase for PHA analysis. The average daily removal of the batch tests was calculated through Eq. (1).

$$\overline{x} = \frac{C_{removed}}{t} \quad (1)$$

where $\overline{x}$ is the average daily removal of BTEXS (g m^{-3} d^{-1}), $C_{removed}$ is the total concentration of BTEXS eliminated during the assay (g m^{-3}), and t is the total number of days of the assay (d). The results from the batch assays regarding contaminant removal performance and biomass productivity guided the selection of the conditions for subsequent continuous experiments.

2.4. Optimization of toluene and ethylbenzene valorization process

The optimization of the biodegradation process and simultaneous production of PHA was conducted in a chemostat consisting of a 2.5 L stirred tank bioreactor (R1) controlled by a Sartorius Stedim Biostat®A unit (Goettingen, Germany) (see Supplementary Materials 1). The bioreactor was equipped with an automated system for pH and temperature control, with continuous supply of toluene and ethylbenzene in the gas phase. The polluted stream was prepared by injecting the liquid contaminant using a Fisher Scientific Fisherbrand 78-0100l Single syringe pump (Madrid, Spain) and a 5 mL Hamilton 1005 RN liquid syringe (Reno, NV, USA) into an air stream. The flow rate of the air stream was controlled at 304.1 ± 16.5 mL min^{-1} by means of an Aalborg rotameter (Orangeburg, NY, USA), corresponding to an empty bed residence time (EBRT) of ~6.6 min. After homogenization in a 2-L mixing chamber, the gas mixture was introduced into the reactor through a 2 μm-pore stainless steel diffuser. The bioreactor was maintained at 25 °C using a heating blanket and continuously agitated at 350 rpm with a six-blade Rushton turbine. The system was operated with a 2-L working volume of M9 with varying nitrogen concentration, as explained below. The pH was maintained at 7 using a 4 N NaOH solution through a Hamilton EasyFerm Bio HB MS 225 probe (Reno, NV, USA).

The bioconversion process was tested in three different experimental phases (Table 1). Briefly, toluene was supplemented as the sole carbon source in the first stage, while a mixture of toluene and ethylbenzene (50 % v/v) was used in stages II and III. Additionally, in stage III, a 0.5 L tank reactor (R2) with magnetic stirring was interconnected to R1 in order to evaluate the performance of a two-stage process. The liquid dilution rate (D), expressed as the liquid inlet flow rate (Q_L) divided by the working volume (V), was adjusted according to the needs of the culture. In addition to the pollutant removal efficiency and the biomass productivity, the carbon mineralization rate, N consumption, and productivity of the synthesized PHA were calculated.

2.4.1. Stage I

The autoclaved bioreactor R1 was operated for 63 days with a continuous supply of toluene at a concentration of 3.5 ± 0.5 g m^{-3} with a syringe pump velocity of 0.05 mL h^{-1}. After inoculation, the initial TSS and TN concentrations were 11 mg L^{-1} and 165 mg L^{-1}, respectively. Nitrogen concentration was monitored periodically, and 500 mL of the cultivation liquid medium was exchanged with fresh M9 when TN levels dropped below 30 mg L^{-1} to support biomass growth. This procedure was repeated until day 21, when seven nitrogen-deprivation cycles with a duration between 3 and 9 days were performed to assess the synthesis of PHA (days 21, 34, 46, 53, 56, 59 and 62). After each limitation period, nitrogen was replenished by exchanging 500 mL of the cultivation broth with fresh 1 × N M9 or 2 × N M9, depending on culture growth conditions. Daily, 100 mL liquid samples were withdrawn for TN, TOC, IC, OD_{650}, TSS and PHA analysis; and replaced with either fresh M9 or M9-NF in the *N*-deprivation phase.

2.4.2. Stage II

In stage II, a similar operating procedure to that of the previous phase was implemented, with the exception that a mixture of toluene and ethylbenzene at ~50 % v/v was fed to the reactor at individual concentrations of 1.7 ± 0.4 and 2.1 ± 0.6 g m^{-3} for toluene and ethylbenzene, respectively. After inoculation, TSS and TN concentration values of 18 mg L^{-1} and 142 mg L^{-1} were recorded. The cultivation medium was replenished more frequently to prevent culture inhibition resulting from the accumulation of toxic metabolites, by replacing 200 mL of the liquid broth with fresh 1 × N or 2 × N M9 every two days. At this stage, only a final 14-days nitrogen deprivation cycle was carried out. The same analytical procedure as the one described in stage I was followed.

2.4.3. Stage III

A two-stage system, consisting of a growth reactor unit (R1) and a subsequent PHA-accumulating reactor (R2) was tested. Both reactors were operated in parallel, with the main air flow divided into two streams of approximately 230 mL min^{-1} for R1 and 66 mL min^{-1} for R2, corresponding to EBRT of ~8.7 and ~6.0 min, respectively. Toluene and ethylbenzene inlet concentrations were similar to those of stage II.

Initially, after inoculation, reactor R1 had a TSS and TN concentration of 210 mg L^{-1} and 169 mg L^{-1}, respectively. The liquid dilution rate was 0.05 d^{-1} during the lag phase and was adjusted in the progressive phases according to the concentration and state of the biomass. When the exponential phase was achieved on day 6, the dilution rate was increased to 0.1 d^{-1} until day 17. Once the stationary phase was reached (day 18), the dilution rate in R1 was maintained at 0.175 d^{-1} throughout the complete experimentation period by daily replacing 350 mL of the culture broth with 1 × N M9. On day 38, R2 was inoculated using 400 mL from R1 (stage III.1). After centrifugation at 7800 rpm for 10 min, the supernatant was discarded, and the biomass pellet was resuspended in 400 mL of fresh 1 × N M9. Nitrogen limitation in R2 was maintained for one week. One additional run of the R2 bioreactor was performed by day 52 (stage III.2), although this time the biomass pellet was resuspended in M9-NF prior inoculating the R2. Daily, 20 mL of the

Table 1
Experimental conditions during the different continuous operational stages.

Stage	Duration (days)	VOC	VOC concentration (g m^{-3})	EBRT (min)		*N*-deprivation cycles (number/duration)	
				R1	R2	R1	R2
I	63	T	T: 3.5 ± 0.5	6.7	–	7/3–9 days	–
II	73	T + EB	T: 1.7 ± 0.4 EB: 2.1 ± 0.6	6.4	–	1/14 days	–
III	77	T + EB	T: 1.8 ± 0.2 EB: 2.3 ± 0.3	6.7/8.7		No N deprivation	
III.1	9	T + EB	T: 1.8 ± 0.2 EB: 2.3 ± 0.2		6.3		1/9 days
III.2	9	T + EB	T: 1.7 ± 0.1 EB: 2.2 ± 0.1		5.8		1/9 days

cultivation broth of R2 were withdrawn and exchanged with fresh M9-NF, resulting in a dilution rate of 0.05 d^{-1}. The same analytical procedure as described earlier was followed.

2.5. Analytical procedures

To analyze the concentration of gaseous pollutants, 250 μL samples were taken from the headspace of the bottles or from the gas sampling ports using a 250 μL Hamilton gas syringe (Reno, NV, USA) and manually injected into an Agilent 8860 GC-FID (Santa Clara, CA, USA) equipped with a HP-5 column (30 m × 320 μm × 0.25 μm). The injector, oven and detector temperatures were maintained at 150, 80 and 250 °C, respectively, while helium was used as a gas carrier at 2 mL min^{-1}. The concentration of O_2 and CO_2 was determined using a 100 μL Hamilton gas syringe (Reno, NV, USA), manually injecting the samples into a Bruker 430 GC-TCD (Palo Alto, CA, USA) equipped with a CP-Molsieve 5A column (15 m × 0.53 μm × 15 μm) and a CP-PoraBOND Q column (25 m × 0.53 μm × 10 μm). Injector, column and detector temperatures were maintained at 150, 45 and 175 °C, with helium as the carrier gas.

Liquid samples of 50 mL were filtered through 0.45 μm filters to analyze the TN, TOC, and IC concentrations in a Shimadzu TOC-L TNM-L unit (Kyoto, Japan). TSS analysis was performed by filtration of 5 to 10 mL of culture broth with 0.45 μm previously weighed filters. The filter was then dried for 24 h at 80 °C, cold in a desiccator and weighed to calculate the biomass concentration. OD_{650} was measured in a Shimadzu UVmini-1240 spectrometer (Kyoto, Japan). An OD_{650}-TSS curve was also performed to estimate biomass concentration from the OD values. Finally, a Ifm Electronic PN709s7 sensor (Essen, Germany) was used to analyze the pressure in the gaseous inlet stream, while gas flowrates were measured using the water displacement method with a graduated cylinder.

For the qualitative and quantitative analysis of the composition of the PHA, a 7820A GC coupled to a 5977E Agilent mass spectrometer (GC–MS) (Santa Clara, CA, USA) equipped with a DB-WAX column (30 m × 250 μm × 0.25 μm) was used. Injector and column temperatures were set at 250 and 40 °C, respectively, increasing the oven temperature at 8 °C min^{-1} to a final value of 200 °C. MS source and MS quad temperatures were 230 and 150 °C, respectively. Helium was used as the carrier gas at 1 mL min^{-1}. The PHA extraction methodology was adapted from the one followed by Riis & Mai (1988). Briefly, 2 mL of chloroform, 1 mL of propanol-HCl 80:20 v/v and 10 μL of benzoic acid internal standard were added in glass tubes containing the freeze-dried biomass to carry out the digestion in a Thermo Fisher Scientific Thermoblock Digital Dry Bath (Waltham, MA, USA) at 100 °C for 4 h. Subsequently, after cooling the samples, 1 mL of distilled water was added to separate the dense organic phase from the inorganic one. Approximately 1.5 mL of the organic phase were collected with a pipette and subsequently filtered through a 0.22 μm filter into a chromatography vial. Calibration curves for PHB and PHBV were also performed.

PHA quantification was also carried out using a gravimetric method. A sample of 6 mL of the liquid broth was withdrawn and washed twice with a NaCl solution (0.9 %). The pellet was dried at 80 °C for 24 h in a vial and weighed to obtain the microbial mass. The pellet was then grinded, resuspended in 4 mL chloroform and incubated for 4 h at 100 °C in a Thermoblock. The incubated sample was filtered through 0.45 μm filters and dried for 24 h. Finally, the specific mass of PHA (mg_{PHA} $mg_{biomass}^{-1}$) was determined.

2.6. Metagenomic analysis

Shotgun metagenomics was performed to investigate genes associated with toluene and ethylbenzene metabolism and the genes related to PHA production in the enriched consortium, and to analyze the microbial community. To this aim, samples of biomass were taken from the final stage of the bioreactor (stage III) on day 77 and the DNA was extracted and sent for Illumina shotgun sequencing (NovaSeq X Plus-PE150) to Novogene (Germany) according to manufactureŕs protocol. The obtained genomes from each sample were quality checked and assembled according to Marcos-Rodrigo et al. (2024). Taxonomic analysis was conducted by aligning unigene sequences against bacterial, fungal, archaeal, and viral sequences from NCBI's NR database (https://www.ncbi.nlm.nih.gov/) using a cut-off e-value of 10^{-5}. MEGAN software was used to retain species annotation information during taxonomic classification. Functional annotation of assembled metagenomes was conducted using DIAMOND (v0.9.9), aligning unigenes against functional databases, including KEGG Orthology (https://www.kegg.jp/kegg/), eggNOG (https://eggnogdb.embl.de/#/app/home), and CAZy (https://www.cazy.org/), with a cut-off e-value of 10^{-5}. Functional abundance differences were assessed using ANOSIM, while comparative analyses of metabolic pathways and functional differences were performed via MetaStat and LefSe. Annotated genes were identified within the BTEXS oxidation pathways (toluene, ethylbenzene, styrene and xylene isomers) and PHA/PHB biosynthesis. The specific genes associated with each pathway were then mapped to their corresponding bacterial taxa using the NCBI database (https://www.ncbi.nlm.nih.gov/). This whole genome shotgun project has been deposited in GenBank under Bioproject number PRJNA1252081.

3. Results and discussion

3.1. Aromatic hydrocarbons biodegradation in batch assays

Although the majority of studies on BTEXS degradation have focused on pure microbial strains, recent findings indicate that mixed microbial consortia possess synergistic metabolic interactions that significantly enhance the biodegradation of BTEXS compounds (Hernández-Ospina et al., 2024). In the series of batch experiments, the mixed culture exhibited efficient toluene removal at a rate of 3.9 ± 0.1 g m^{-3} d^{-1}, even in the absence of nitrogen in the medium (see Supplementary Materials 2). On the other hand, ethylbenzene was degraded with an average removal rate of 2.2 ± 0.2 g m^{-3} d^{-1} under nitrogen-sufficient conditions. However, the time required for its depletion under nitrogen

deprivation significantly increased, even inhibiting cell growth after two degradation cycles. This led to a decrease in the removal rate to 0.8 ± 0.3 g m^{-3} d^{-1}. Since nitrogen is essential for enzymatic synthesis, its limitation can significantly hinder the biodegradation of BTEXS compounds (Delhoménie et al., 2008). While toluene can be metabolized through multiple pathways by a wide range of microorganisms, potentially allowing its degradation to persist longer under nitrogen-limited conditions, ethylbenzene has a rather more restricted degradation capacity, which may have amplified the inhibitory effects of nitrogen scarcity. Finally, the culture was unable to complete more than one cycle of styrene degradation (0.5 ± 0.3 g m^{-3} d^{-1}), requiring at least 5 days for styrene depletion and subsequently resulting in growth inhibition. When styrene was supplemented in a mixture with toluene and ethylbenzene, it hindered the degradation of the rest of contaminants, yielding a combined removal rate of 0.9 ± 0.4 g m^{-3} d^{-1}. Although PHA synthesis was detected under nitrogen-deprived conditions when cultures were supplemented with toluene and ethylbenzene, only the presence or absence of PHA could be assessed. Quantification was not possible, as PHA concentrations fell below the calibration curve, yielding unreliable data. This limitation was likely due to the low carbon concentrations used in the batch tests to prevent toxicity associated with excessive BTEXS levels. In relation to the abiotic culture, the loss of BTEXS due to sampling or physicochemical degradation was ~0.1 g m^{-3} d^{-1}.

Consistent with this research, several bacterial species exhibit a higher capacity for toluene degradation compared to other pollutants such as ethylbenzene, xylene, or styrene (Cao et al., 2009). This preferential degradation is not solely due to metabolic accessibility, but may also be influenced by the formation and accumulation of toxic metabolic intermediates during de degradation of more complex compounds. For instance, the degradation of methylated aromatics such as xylene or styrene can generate intermediates like 3-methylcatechol, which are known to exert cytotoxic effects and inhibit microbial growth under batch conditions (Yoshikawa et al., 2017). Moreover, the enzymatic pathways required for the degradation of these compounds are often more complex or tightly regulated, which can further limit their biodegradation efficiency. For example, *Pseudomonas* sp. BTEX-30 achieved a higher toluene specific degradation rate of 14.2 mg $L^{-1}h^{-1}$ in batch tests, in contrast to lower rates observed for ethylbenzene and *m*-xylene, with values of 11.2 mg $L^{-1}h^{-1}$ and 0 mg $L^{-1}h^{-1}$, respectively (Khodaei et al., 2017).

While pure cultures can achieve high removal efficiencies, mixed communities offer significant advantages in terms of metabolic capacity and resilience, which is especially desirable in long-term experiments or under non-optimal conditions. For instance, a coculture of *Pseudomonas putida* F1 and *Pseudomonas stutzeri* OX1 was able to synergistically degrade ethylbenzene and *o*-xylene through metabolic complementation (Nagarajan & Loh, 2014). In addition, although toluene was the most efficiently degraded compound in the batch tests, it is also important to highlight that ethylbenzene biodegradation can be enhanced when co-supplemented with other easier assimilable compounds, such as toluene at moderate concentrations, allowing a gradual cellular adaptation to BTEXS and their intermediates (Muñoz et al., 2009; Ni et al., 2010). Although BTEXS compounds can cause inhibition or toxicity, their structural similarity may enhance degradation in mixtures through co-metabolism and enzyme induction. Typically, xylene acts as the strongest inhibitor, while toluene is rapidly degraded and promotes the degradation of other compounds, such as benzene, ethylbenzene, xylene or naphthalene under aerobic conditions (Gülensoy and Alvarez, 1999; You et al., 2012). Thus, a non-adapted mixed culture dominated by *Pseudomonas stutzeri* and *Vibrio mimicus* increased its ethylbenzene degradation from 0.2 mg g^{-1} d^{-1} (ethylbenzene alone) to 0.4 mg g^{-1} d^{-1} when supplemented with benzene, toluene and xylenes (Babaarslan et al., 2003). Based on these proof of concept results, which also allowed for culture specialization, further optimization of the continuous process focused specifically on the bioconversion of toluene and ethylbenzene into PHA, aiming to maximize degradation efficiency and system stability.

3.2. Stage I – Optimization of continuous toluene valorization

During stage I, a toluene inlet concentration of 3.5 ± 0.5 g m^{-3} was continuously supplemented to the reactor, corresponding to an inlet load of 31.4 ± 5.3 g $m^{-3}h^{-1}$. When the TN concentration decreased below 30 mg L^{-1}, 500 mL of the culture broth were exchanged with fresh M9 medium, this means a sporadic dilution rate of 0.25 d^{-1}. Temperature and pH were maintained at 25.5 ± 0.8 °C and 7.0 ± 0.1, respectively. After inoculation at a biomass concentration of 11 mg L^{-1}, an initial 6-days lag phase was recorded. This prolonged lag phase can be attributed to the low biomass concentration of the initial inoculum, which supported gradual development of specialized BTEXS-degrading communities, but mainly to the inherently slow assimilation of BTEXS compounds compared to easily metabolized carbon sources such as sugars. This phenomenon has also been reported in other BTEXS mesophilic aerobic reactors, where acclimation periods of up to one month were required to achieve removal efficiencies greater than 86 % (Mohammad et al., 2007). The toluene removal efficiency (RE) after the lag phase reached an average value of 99.5 ± 0.8 % from day 9 until the end of stage I (Fig. 1a). This removal corresponded to an average specific consumption of 12.5 ± 6.7 mg toluene $g_{DCW}^{-1}h^{-1}$. Similar values were reported for a mixed culture mainly composed of *Pseudonocardia* and *Rhodococcus,* which achieved an average toluene RE of 87 % (61 g $m^{-3}h^{-1}$) at an inlet concentration of 1.25 g m^{-3} in a 14 L compost biofilter (Juteau et al., 1999). However, the specific consumption rate observed in this research is relatively high compared to recent biodegradation studies. For instance, *Pseudomonas putida* F1 exhibited a toluene degradation rate of 1.9 mg g_{DCW}^{-1} h^{-1} in a tubular packed bed reactor (Xu et al., 2023). The high efficiency achieved in the system was primarily attributed to the moderate VOC loading in the bioreactor. While higher concentrations can be tolerated, they may induce toxicity in certain bacteria (i.e. concentrations of 10.9 g m^{-3} of toluene proved to induce negative metabolic effects in *P. putida* mt-2 (Muñoz et al., 2009)). Upon reaching the stationary phase, CO_2 production from cellular metabolism was recorded at 20.0 ± 11.2 mg CO_2 $g_{DCW}^{-1}h^{-1}$, corresponding to outlet CO_2 concentrations of 5.6 ± 1.3 g m^{-3} and a carbon mineralization ratio of 48.5 ± 12.2 %. Although some studies have reported nearly 100 % carbon mineralization from toluene (Xu et al., 2023), mineralization ratios typically range between 34 % and 91 % depending on temperature, humidity, pollutant properties and nutrient concentration or availability (Bordoloi et al., 2019). The IC remained at an average value of 13.0 ± 4.3 mg L^{-1}, while TOC concentration exhibited a rapid increase from day 6 until day 11, corresponding to the exponential phase, probably due to the intermittent dilution rate of 0.25 d^{-1} (Fig. 1b). Given the stable toluene concentration throughout the operation, the increase in TOC may have resulted from the accumulation of secondary metabolites (Muñoz et al., 2009). In fact, a yellowish color was perceived in the reactor, probably due to the accumulation of 2-hydroxymuconic semialdehyde (2-HMS), a metabolite produced by the enzyme activities of catechol 1,2-oxygenase and catechol 2,3-dioxygenase during the degradation of aromatic compounds (Nie et al., 2015). Regarding biomass growth, a progressive increase in TSS concentration was recorded from the beginning of the experiment until day 26, when a concentration of ~1.9 g L^{-1} was achieved. Following a stabilization period, the biomass increased again, eventually stabilizing at a maximum TSS concentration of ~4.5 g L^{-1}. The maintenance of the reactor for a total of 63 days allowed for process optimization and demonstrated its suitability for continuous operation, highlighting its potential for future scale-up.

A total of 7 nitrogen-limiting cycles, each lasting between 3 to 9 days, were conducted for the synthesis of PHA. The highest concentration of 14.1 % g_{PHA} g_{DCW}^{-1} was achieved during the longest cycle (Fig. 1c). Prolonged *N*-limitation cycles can enhance PHA accumulation by

redirecting carbon flux to storage under stress conditions. However, cell density can also have a significant impact on intracellular PHA content. While high biomass concentration may improve BTEXS biodegradation, it can also foster the emergence of different microbial communities less specialized in PHA synthesis. This shift can ultimately result in a reduction of the PHA accumulation under prolonged *N*-limitation as observed after day 45. These results are consistent with the values provided by Hori et al. (2009), who achieved a maximum of 18 % PHBV after 72 h of cultivation in *Rhodococcus aetherivorans* IAR1 fed with toluene. Similarly, Narancic et al. (2012) reported PHA contents from 3 % to 25 % DCW in various *P. putida* strains using a mixture of BTEXS compounds. This initial operating stage indicated that longer *N*-limiting cycles might be necessary to increase the PHA content in the cells.

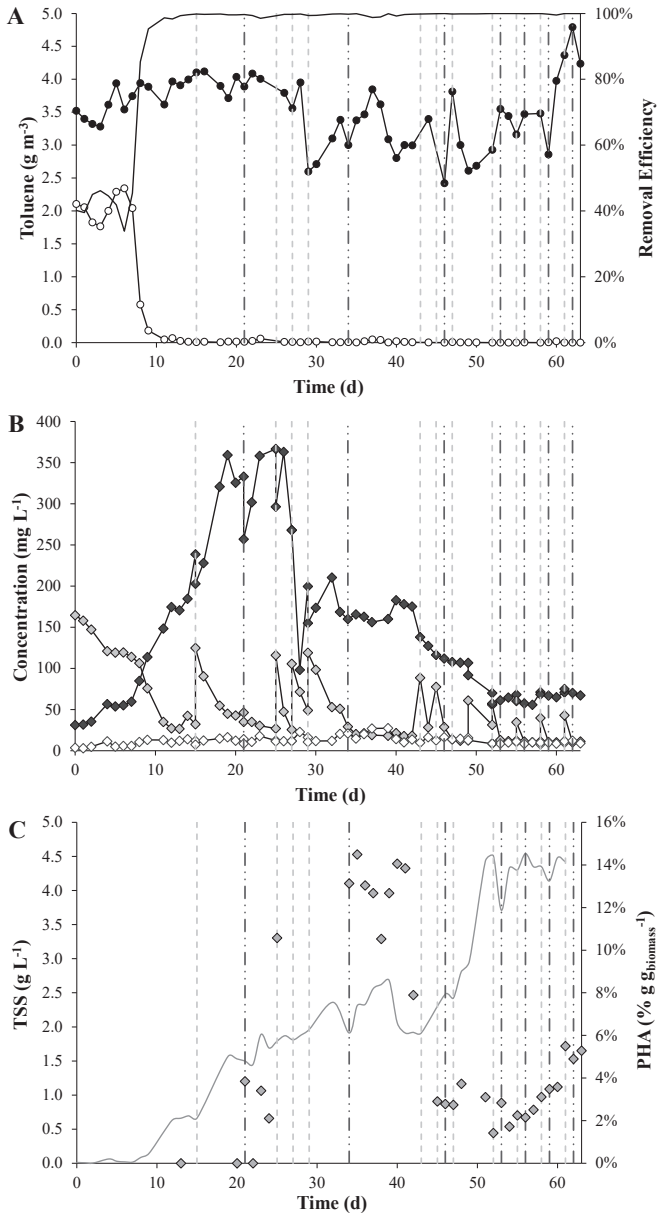

Fig. 1. Operating and performance parameters during Stage I: A) Toluene inlet (black circles) and outlet (white circles) concentrations and removal efficiency (continuous line); B) Total nitrogen (grey diamonds), total organic carbon (black diamonds) and inorganic carbon (white diamonds) concentrations; C) Biomass as total suspended solids (grey continuous line) and poly-hydroxyalkanoates (grey diamonds) concentrations. Vertical lines represent nitrogen feeding days (grey dashed lines) and start of nitrogen deprivation conditions (black dashed lines).

3.3. Stage II – Optimization of continuous toluene and ethylbenzene valorization

Based on the results from the first operating stage, Stage II was set with an inlet polluted gas stream containing a 50 % v/v mixture of toluene and ethylbenzene, with average concentrations of 1.7 ± 0.4 and 2.1 ± 0.6 g m^{-3}, respectively. The average air flow was set at 310 ± 17 mL min^{-1}, corresponding to an EBRT of ~ 6.4 min, resulting in a loading rate of 15.7 ± 4.0 g $m^{-3}h^{-1}$ for toluene and 19.2 ± 5.5 g $m^{-3}h^{-1}$ for ethylbenzene. A constant temperature of 25.5 ± 0.5 °C and a pH of 7.1 ± 0.1 were maintained. Changes from Stage I included consistent liquid dilution rate of 0.1 d^{-1} by daily replacing 200 mL of culture medium and a single extended nitrogen-limiting cycle for PHA synthesis to standardize and stabilize reactor performance. The estimated biomass concentration at the beginning of the assay was 18 mg L^{-1}. The culture exhibited rapid growth; however, a longer adaptation period was necessary for the mixed culture to the new inlet composition, requiring 22 days to reach full ethylbenzene consumption. Despite their structural similarities, toluene has greater metabolic versatility, as more microbial communities can utilize it as a carbon and energy source. Studies have shown that toluene is typically degraded faster than ethylbenzene, both as a single substrate and in mixtures, indicating that more microorganisms possess enzymatic machinery for its removal (Zhang et al., 2012). In contrast, ethylbenzene degradation is more restrictive and is usually limited to specific microbial strains or consortia. This reason may explain why ethylbenzene degradation required more time to achieve similar values to toluene degradation in this stage.

Once the stationary phase was reached, an average RE of toluene and ethylbenzene of 96.6 ± 5.1 % (7.8 ± 3.5 mg $g_{DCW}^{-1}h^{-1}$) and 91.9 ± 13.6 % (9.1 ± 4.4 mg $g_{DCW}^{-1}h^{-1}$) (Fig. 2a) were achieved, respectively. Concomitant degradation of both VOCs has been previously reported by other authors. For instance, You et al. (2012) observed that the specific degradation rate of ethylbenzene in *P. putida* YNS1 was lower than that of toluene, although the strain was capable of degrading both compounds. Likewise, co-degradation of toluene (9.7 ± 0.6 ppmv) and ethylbenzene (8.7 ± 0.6 ppmv) was reported by Pineda et al. (2023) using *R. erythropolis* in a 1 L stirred flask reactor. The CO_2 generated by culture metabolism averaged 3.7 ± 0.8 g m^{-3}, with a mineralization rate of 33.3 ± 8.3 %. This represents a decrease in carbon mineralization of over 10 % compared to the previous phase when only toluene was supplemented. However, specific CO_2 remained almost unchanged at a value of 19.1 ± 9.1 mg $g_{DCW}^{-1}h^{-1}$, probably due to the difference in average biomass concentration during the stationary phase.

Upon reaching a steady-state removal efficiency on day 22, the culture exhibited an average biomass concentration of 1.8 ± 0.7 g L^{-1}. However, TSS continued to increase thereafter, reaching a maximum concentration of 3.4 g L^{-1} by day 51, and stabilizing by the end of the assay at 2.3 ± 0.7 g L^{-1} (Fig. 2c). Both TOC and IC concentrations remained below 58.7 ± 14.9 and 4.7 ± 1.8 mg L^{-1}, respectively. This confirmed that the constant 0.1 d^{-1} dilution rate implemented in this stage was adequate to avoid the accumulation of secondary metabolites in the culture broth, likely inhibitory for the microbial community, although did not accommodate fluctuations in biomass concentration. The PHA concentration in the extracted biomass consistently remained below 10 % g g_{DCW}^{-1}, except for the values recorded 3 days after the start of M9-NF feeding, when a peak concentration of 20.6 % g g_{DCW}^{-1} was achieved (Fig. 2c). Afterwards, PHA concentration declined in parallel with a decrease in BTEXS degradation, likely due to a shift in carbon flux toward PHA consumption, resulting from the limited synthesis of enzymes involved in BTEXS degradation. In fact, the decline of the removal efficiency was also observed in the following days. These results align with the 22 % accumulation reported by Nikodinovic et al. (2008) for *P. putida* F1 using toluene and ethylbenzene in batch assays. However, further optimization was required to achieve consistent PHA synthesis and productivity.

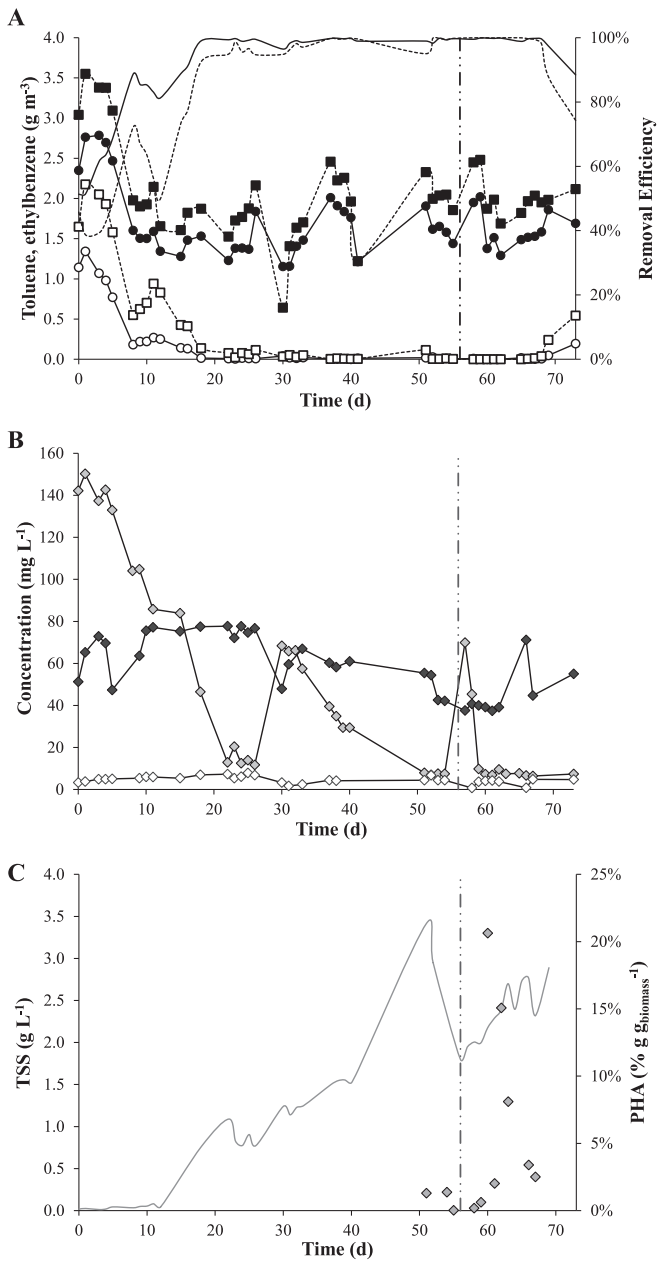

Fig. 2. Operating and performance parameters during Stage II: A) Inlet and outlet concentrations for toluene (black and white circles) and ethylbenzene (black and white squares) and removal efficiency of toluene (continuous line) and ethylbenzene (dotted lined); B) Total nitrogen (grey diamonds), total organic carbon (black diamonds) and inorganic carbon (white diamonds) concentrations; C) Biomass as total suspended solids (grey continuous line) and polyhydroxyalkanoates (grey diamonds) concentrations. Vertical line indicates the start of nitrogen-free M9 medium feeding (black dashed line).

3.4. Stage III – Optimization of continuous toluene and ethylbenzene valorization in a two-stage process

The need for a prolonged *N*-deprivation period to achieve adequate PHA concentrations highlighted the importance of decoupling the growth and accumulation stages. Thus, in stage III, a two-stage system (growth, R1 + accumulation, R2) was evaluated for the continuous conversion of toluene and ethylbenzene into PHA. This two-stage configuration enabled maintaining optimal process conditions at each stage (N content, substrate feed and dilution rate), and relieved R1 from the metabolic instability caused by N limitation, thus improving biomass growth, BTEXS removal and PHA synthesis. This approach also allowed for the steady withdrawal of cultivation broth, facilitating continuous downstream processing. An average inlet concentration of toluene and ethylbenzene of 1.8 ± 0.2 g m^{-3} and 2.3 ± 0.3 g m^{-3}, respectively, both to R1 and R2, was maintained (Fig. 3a). The average flow rate in R1 was set at 297 ± 15 mL min^{-1}, resulting in an EBRT of 6.7 min and a loading rate to the system of 15.9 ± 2.3 g $m^{-3}h^{-1}$ for toluene and 20.2 ± 2.9 g $m^{-3}h^{-1}$ for ethylbenzene. Temperature and pH were maintained at 24.6 ± 1.1 °C and 7.0 ± 0.1, respectively.

The R1 was inoculated at an initial biomass concentration of 210 mg L^{-1}, rapidly increasing the TSS concentration until stabilizing within only 9 days at a value of 0.5 g L^{-1}. In spite of the progressive culture

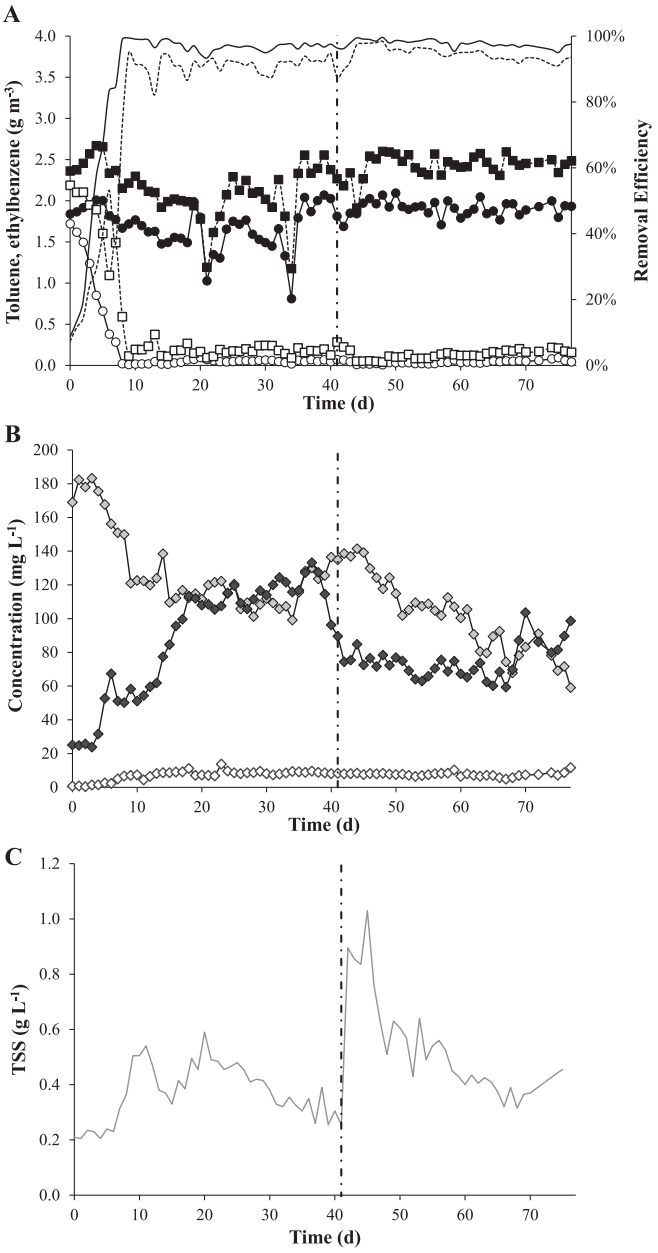

Fig. 3. Operating and performance parameters in reactor R1 during Stage III: A) Inlet and outlet concentrations for toluene (black and white circles) and ethylbenzene (black and white squares) and removal efficiency of toluene (continuous line) and ethylbenzene (dotted lined); B) Total nitrogen (grey diamonds), total organic carbon (black diamonds) and inorganic carbon (white diamonds) concentrations; C) Biomass as total suspended solids (grey continuous line).Vertical lines represent homogenization of the bioreactor biomass (dark dashed line).

media dilution rate, 0.05–0.175 d^{-1}, the maximum TSS concentration recorded in this test, 1.03 g L^{-1} was lower than that observed in previous stages, likely due to the formation of biomass aggregates, which induced significant error in the experimental determination, together with the higher dilution rate during the operation. The formation of aggregates and biofilms is a common phenomenon under certain stress conditions, such as enhancing cellular defense against exposure to UV-A/B/C radiation (De Almeida and Quilty, 2016). This phenomenon could also be triggered by prolonged exposure to VOCs due to their aromatic nature, as these molecules have been identified as inducers of exploratory growth and intercellular communication in certain bacteria, such as *Streptomyces* (Jones & Elliot, 2017). Despite the lower biomass concentration measured, the consumption of toluene and ethylbenzene in R1 gradually increased after inoculation, reaching steady values of 97.4 ± 1.3 % and 92.6 ± 3.8 %, respectively, by day 8 (Fig. 3a). A CO_2 outlet concentration of 4.6 ± 0.6 g m^{-3} was recorded during the complete stage, corresponding to an average mineralization of 37.1 ± 6.2 %, similar to that recorded in Stage II.

Two R2 runs were performed, corresponding to operating days 38 and 52 of R1. In the first run, biomass from the growth reactor was directly inoculated in R2, and N was allowed to steadily deplete. As a result, biomass growth and VOC biodegradation were significant during the first few days. However, once the N was consumed by day 4, the elimination decreased from 95.9 % for toluene and 87.0 % for ethylbenzene by day 3 (maximum values of the assay) to 49.8 % and 35.8 %, respectively, by the end of the stage. The specific PHA content per gram of biomass revealed a dominant profile of short chain length PHA, primarily composed of 3HB. However, this first run of R2 resulted in a very low intracellular concentration of 3HB, likely due to the presence of nitrogen at the start of the assay, with a maximum of only 0.7 ± 0.2 % (Fig. 4a). This may have also hindered the detection of 3HV, which was not identified.

The second R2 assay was started directly with *N*-free culture medium, resulting in a continuous decrease in BTEXS removal, from 93.6 % for toluene and 81.8 % for ethylbenzene on day 0, to 35.4 % and 26.5 %, respectively, on day 9. In contrast to the previous experiment, a considerable cellular concentration of PHA, composed of both 3HB and 3HV, was reached, with a maximum of 21.4 ± 2.4 % g g_{DCW}^{-1} on day 2 (84:16 3HB:3HV) (Fig. 4b), quite similar to stage II values. Therefore, nitrogen limitation proved to be effective only when preceded by a sustained period of cellular *N*-deprivation. Additionally, the composition of the synthesized PHA copolymer (i.e. ratio 3HB:3HV) was shown to be partially adjustable over time, from 89:11 on day 1 to 78:22 on day 8. This is consistent with the findings of Hori et al. (2009), who reported that 3HV fraction increases after 2 days of limitation. However, the synthesis of PHA can vary drastically depending on the bacterial strain and the environmental conditions. The observed changes in the 3HB:3HV ratio were probably due to differences between the metabolic pathways of toluene and ethylbenzene. Toluene is rapidly assimilated and metabolized to acetyl-CoA, which is used directly in the synthesis of 3HB. In contrast, ethylbenzene degradation, which was restricted to specific communities, generates propionate, a key precursor of 3HV that is slowly accumulated due to its high cellular toxicity (Chen et al., 2011). Consequently, 3HV production may have been resulted in a moderate but steady increase that became appreciable as the duration of the *N*-limitation progressed. According to these results, the N limitation required for PHA synthesis inherently leads to reduced BTEXS removal. This underscores the need for a two-stage system, as proposed in this study, where a primary stage ensures complete removal of BTEXS, followed by a second stage dedicated to PHA synthesis.

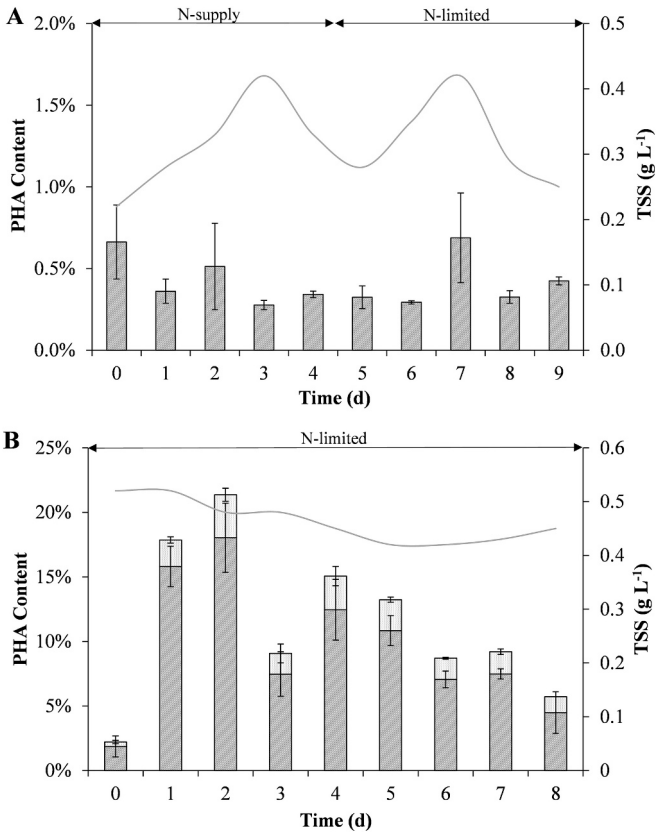

Fig. 4. Polyhydroxyalkanoate specific content and biomass content in R2 during stage III: A) First run starting without nitrogen-limitation: total suspended solids (continuous grey line), 3-hydroxybutyrate (stripped columns); B) Second run started with nitrogen-limitation: total suspended solids (continuous grey line), 3-hydroxybutyrate (stripped columns), 3-hydroxyvalerate (dotted columns).

3.5. Metagenomic analysis

The metagenomic analysis from the mixed culture showed that the microbial community was dominated by the bacterial genus *Pseudonocardia,* which represented more than 37 % of the total genera (see Supplementary Materials 3). Within this genus, the most abundant populations belonged to an uncultured *Pseudonocardia* sp. (13.4 ± 1.8 %), followed by *Pseudonocardia* sp. SCN 72–86 (10.7 ± 1.5 %), and *Pseudonocardia sulfidoxydans* (5.1 ± 0.8 %). *Hydrogenophaga* also represented more than 14 % of the microbial community, being uncultured species of such genus the most abundant. Less abundant taxa included uncultured species of the genus *Sphingopyxis* (7.6 ± 1.1 %), *Kaistia defluvii* (5.1 ± 2.5 %), *Rhodococcus zopfii* (2.5 ± 0.8 %), uncultured species of the genus *Microbacterium* (2.2 ± 0.7 %) and the genus *Acidovorax* (1.4 ± 0.4 %).

Metabolic analysis of the metagenome revealed genes encoding enzymes involved in the degradation pathways of toluene, ethylbenzene, styrene, and all three xylene isomers (*m*-, *p*-, and *o*-xylene), as well as in the metabolic pathways of benzoate, catechol and polyhydroxyalkanoate synthesis (Fig. 5). Unfortunately, although gene-level abundance could be determined, several key genes were associated with uncharacterized or poorly described taxa, and others from critical pathways remain unannotated. As a result, it was not possible to

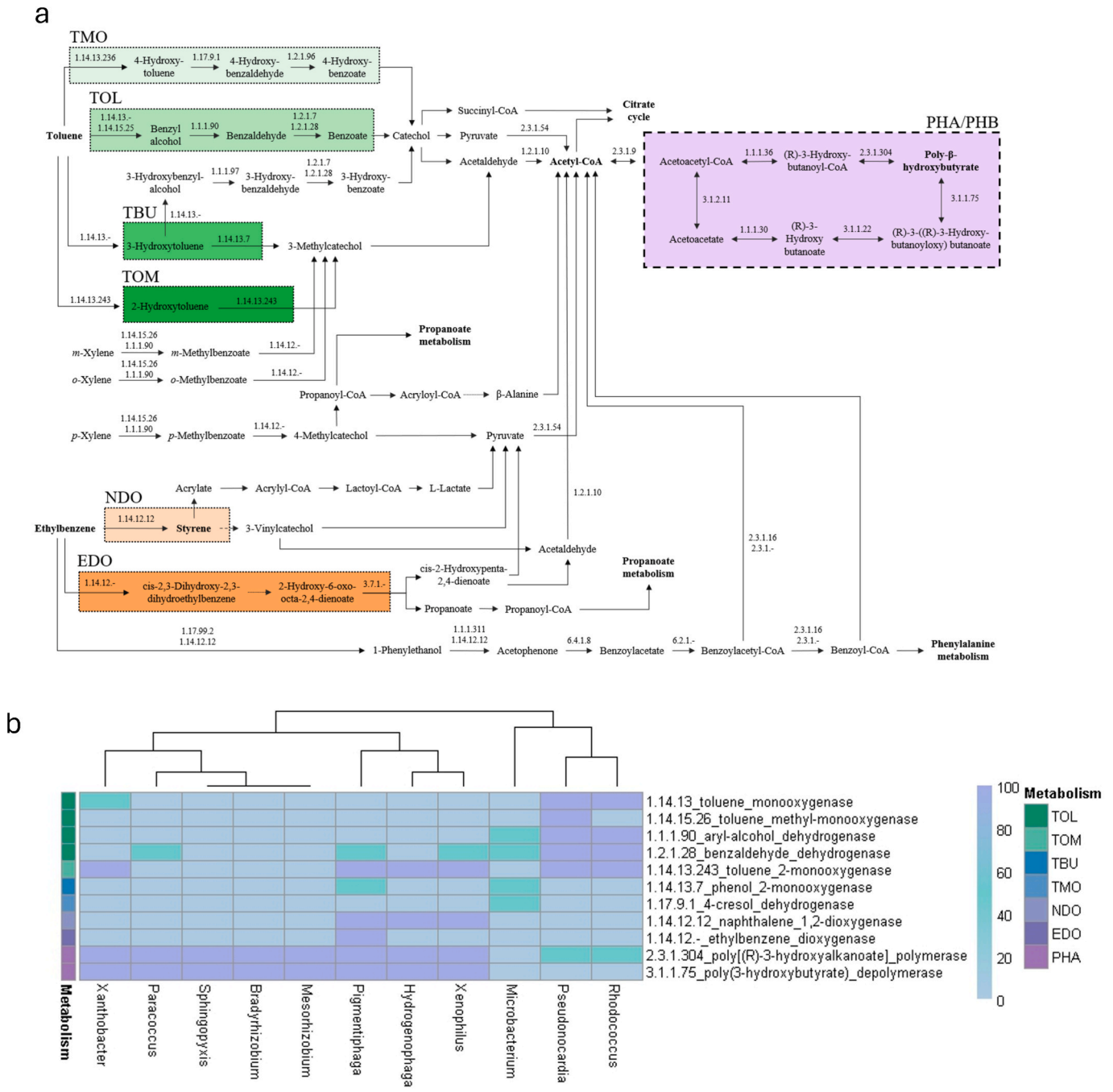

Fig. 5. A) Diagram of the metabolic pathways present in the mixed culture. Pathways of BTEXS degradation, conversion to metabolic intermediates and polyhydroxyalkanoate synthesis are displayed. The main routes for toluene and ethylbenzene degradation and polyhydroxyalkanoate synthesis are highlighted: toluene p-monooxygenation (TMO), toluene deoxygenation (TOL), toluene m-monooxygenation (TBU), toluene o-monooxygenation (TOM), naphthalene 1,2-dioxygenation (NDO), ethylbenzene deoxygenation (EDO), and polyhydroxyalkanoate/poly-3-hydroxybutyrate synthesis (PHA/PHB). B) Heatmap representation of the presence of key enzymes for each of the key pathways. Each column represents a genus, and the colors indicate the presence of genes coding for each enzyme, as well as the genus in which each gene was found in the metagenome. The color scale is as follows: purple indicates a complete set of genes encoding the enzyme, green represents a partial set of genes, and light blue denotes null presence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

precisely determine the contribution of each taxon to the toluene and ethylbenzene degradation pathways, as well as the PHA synthesis route.

Pathway analysis of the specialized mixed culture revealed the presence of all enzymes required for toluene degradation. The primary catabolic pathway for toluene (TOL) involves its conversion into benzyl alcohol by the enzyme toluene monooxygenase (EC:1.14.13.-) or the enzyme toluene methyl-monooxygenase (EC:1.14.15.26, *xylM*) (EC:1.18.1.3, *xylA*), followed by its transformation into benzaldehyde through benzyl-alcohol dehydrogenase (EC:1.1.1.90, *xylB*, *xylE*) (Cao et al., 2009). Benzaldehyde is then converted into benzoate via benzaldehyde dehydrogenase (EC:1.2.1.28, *xylC*), which is subsequently transformed into catechol (Fig. 5a). *Pseudonocardia*, together with *Rhodococcus*, were found to be the genera with the highest number of coding genes for this pathway which was correlated with their high abundance (Fig. 5b). Although some species of the genus *Pseudonocardia* have been identified with potential for toluene degradation (Juteau

et al., 1999), this is the first study that shows that *Pseudonocardia* has the genomic capability to completely degrade toluene. The TOL pathway identified in *Rhodococcus* had been previously reported for the species *Rhodococcus* sp. TG1 (Qiao et al., 2025).

In the toluene *o*-monooxygenation pathway (TOM), the degradation of toluene occurs via 2-hydroxytoluene (*o*-cresol) and 3-methylcatechol, catalyzed by the enzyme 2-monooxygenase (EC:1.14.13.243) (Fig. 5a). Microorganisms identified with the ability to use this route were members from the genera *Pseudonocardia*, *Hydrogenophaga*, *Rhodococcus, Xenophilus, Pigmentiphaga and Xanthobacter* (Fig. 5b). Although *Hydrogenophaga* sp. D2P1^T has been identified as capable of degrading *p*- and *m*-xylene into 3-methylcatechol (Banerjee et al., 2022), there are no previous studies showing that members of the *Hydrogenophaga* genus can degrade toluene.

The toluene *m*-monooxygenation pathway (TBU) is based on the degradation of toluene into 3-hydroxytoluene (*m*-cresol) through toluene monooxygenase (EC:1.14.13.-) and further degradation of *m*-cresol into 3-methylcatechol via phenol 2-monooxygenase (EC:1.14.13.7). Only *Microbacterium* and *Pigmentiphaga* genera were identified with the phenol 2-monooxygenase gene needed for the TBU pathway (Fig. 5b). While there are a few articles pointing to the capability of *Microbacterium* to degrade toluene (Su et al., 2013), comprehensive studies on its genomic and metabolic capabilities related to this process remain limited.

The toluene *p*-monooxygenation pathway (TMO) involves the toluene transformation into 4-hydroxytoluene (*p*-cresol) through toluene monooxygenase enzyme (EC:1.14.13.7) and subsequent 4-hydroxybenzaldehyde production by 4-cresol dehydrogenase (EC:1.17.9.1). Although the necessary enzymes for this pathway were detected in the metagenomes, they could not be attributed to a single species. Moreover, it was not possible to identify all the genes coding for ethylbenzene degradation since many of them are not annotated in genomic databases. Aerobic degradation of ethylbenzene can follow two different pathways: its transformation to styrene via naphthalene 1,2-dioxygenase (NDO) (EC:1.14.12.12, *nah*) or its degradation to propanoate or benzoate via ethylbenzene dioxygenase (EDO) (EC:1.14.12.-, *etb*) (Hernández-Ospina et al., 2024). Although the *etb* genes encoding ethylbenzene dehydrogenase (EDO) were only detected in *Pigmentiphaga*, the *nah* genes responsible for the naphthalene 1,2-dioxygenase synthesis (NDO) were present in *Hydrogenophaga*, *Pigmentiphaga* and *Xenophilus* (Fig. 5b). Despite there is no information on the degradation of ethylbenzene or styrene by these genera, some authors report the presence of the genus *Hydrogenophaga* when treating gasoline and mixed BTEXS (Daghio et al., 2015). Based on the metagenomic analysis of key enzymes involved in toluene and ethylbenzene degradation within the mixed culture (Fig. 5b), it could be concluded that both microbial and metabolic diversity were substantially higher for toluene degradation compared to ethylbenzene degradation.

Similar to other xenobiotic compounds, BTEXS compounds are generally metabolized into derivatives or catechol itself, which is then directed into central metabolism via conversion to acetyl-CoA, propionyl-CoA or other coenzyme A intermediates. The transformation of acetyl-CoA into PHA occurs by the action of the enzyme acetyl-CoA C-acetyltransferase (EC:2.3.1.9), which transforms it into acetoacetyl-CoA. The acetoacetyl-CoA reductase enzyme (EC:1.1.1.36, *phbB*) is responsible for its transformation into (R)-3-hydroxybutanoyl-CoA. Although all these reactions are reversible, PHA synthesis is conditioned by poly [(R)-3-hydroxyalkanoate] polymerase (EC:2.3.1.304, *phaC*, *phaE*). Then, the poly(3-hydroxybutyrate) depolymerase (EC:3.1.1.75, *phaZ*) transforms the PHA into (R)-3-((R)-3-hydroxybutanoyloxy) butanoate, ultimately regenerating acetoacetyl-CoA. Likewise, *phaC* and *phaZ* genes are also involved in the conversion of propionyl-CoA to 3-ketovaleryl-CoA, and subsequently to 3-hydroxyvaleryl-CoA and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate). Specificity for 3HB or 3HV mainly depends on the availability of metabolic precursors and culture conditions. Several microorganisms in the culture were identified with both *phaC* and *phaZ* genes, including some genera in which no BTEXS elimination capacity was observed, such as *Bradyrhizobium* or *Mesorhizobium* (Fig. 5b). In fact, *Bradyrhizobium* was also identified in other BTEXS specialized consortia as an important community in the degradation of metabolic intermediates (Wu et al., 2023a). This suggests that the culture may behave as a syntrophic microbial community and that there were synergistic interactions among the different members that promoted both toluene and ethylbenzene degradation. However, further omics-based studies are required to definitively demonstrate metabolic cross-feeding and to identify the specific active species involved in each step of BTEXS degradation. Other microorganisms, such as *Sphingopyxis*, constituted an important share of the culture community; however, they lacked the genes associated with BTEXS degradation, despite literature reports indicating their capacity to degrade some aromatic compounds (Huang et al., 2019; Oelschlägel et al., 2015).

4. Conclusions

This study demonstrated for the first time the potential of a two-stage system for the continuous degradation of aromatic hydrocarbons and the simultaneous synthesis of polyhydroxyalkanoates using a specialized mixed microbial culture. The system successfully treated gaseous toluene and ethylbenzene with removal efficiencies of 97 % and 92 %, respectively. Moreover, the nitrogen-limited second stage of the process enabled the production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), with monomer ratios modulated according to nitrogen limitation duration. Although *Pseudonocardia* and *Rhodococcus* were identified as the main degraders, their genomic potential for polyhydroxyalkanoates synthesis was limited, suggesting that poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) accumulation relied on synergistic interactions with other communities.

CRediT authorship contribution statement

Nicolás Díaz-Moreno: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Cecilia Lobos:** Investigation. **Andrea Carvajal:** Writing – review & editing, Supervision. **Ignacio Poblete:** Writing – review & editing, Methodology. **Sara Cantera:** Writing – review & editing, Validation, Supervision, Conceptualization. **Raquel Lebrero:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Raquel Lebrero reports financial support was provided by Spanish Ministry of Science and Innovation. Nicolas Diaz reports financial support was provided by Regional Government of Castilla y Leon. Raquel Lebrero reports financial support was provided by European Union. If there are other authors, they 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.2025.132991.

Data availability

Data will be made available on request.

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