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MASTER'S THESIS

Production of Poly-β-Hydroxyalkanate (PHA) from *Cupriavidus necator* Using Toluene as The Sole Carbon Source

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RESUMEN

La creciente preocupación por los contaminantes atmosféricos subraya la necesidad urgente de soluciones sostenibles para reducir las emisiones de compuestos orgánicos volátiles (COV) de los procesos industriales. Este proyecto de investigación examina la biodegradación de un COV modelo, el tolueno, mediante el uso de la bacteria *Cupriavidus necator*. Este estudio investiga la capacidad dual de esta bacteria para degradar tolueno y producir compuestos de alto valor como son los poli-β-hidroxialcanoatos (PHA) en un proceso en continuo. Los resultados demuestran una notable eficiencia de biodegradación del tolueno, con un promedio de 97.9% observado en un biorreactor continuo, y una exitosa producción de PHA, en particular polihidroxibutirato (PHB) y polihidroxivalerato (PHV). Estos hallazgos demuestran la efectividad de *Cupriavidus necator* para abordar la contaminación ambiental y producir materiales valiosos de bioplástico, ofreciendo un enfoque biotecnológico prometedor para aplicaciones industriales sostenibles.

ABSTRACT

The growing concern regarding atmospheric pollutants underscores the urgent need for sustainable solutions to reduce volatile organic compounds (VOCs) emissions from industrial processes. This research project examines the biodegradation of VOCs, focusing specifically on toluene, through the use of *Cupriavidus necator* bacteria. This study investigates the dual capability of this bacterium to degrade toluene and produce high-value poly-β-hydroxyalkanoates (PHA) within continuous bioreactor systems. The results demonstrate a notable biodegradation efficiency of toluene, with an average of 97.9% observed in continuous reactors, and successful PHA production, particularly polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV). These findings demonstrate the effectiveness of *Cupriavidus necator* in addressing environmental contamination and producing valuable bioplastic materials, offering a promising biotechnological approach for sustainable industrial applications.

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1. INTRODUCTION

The increasing global concern about the effects of atmospheric pollutants on climate and public health has led to the development of analytical and abatement methods for the detection and elimination of airborne volatile organic compounds (VOCs). Studies have widely demonstrated that these substances can harm human health and the environment due to their chemical and biological properties (Davidson et al., 2021). Furthermore, air quality assessment is critical for improving future policies as it provides essential information to regulatory authorities in the legal decision-making processes (Lan et al., 2020). In this context, the need to comply with air quality regulations and protect health and the environment from VOC emissions from industrial processes has boosted the development of research on novel air treatment techniques. This research is dedicated to pioneering a method that not only reduces these pollutants but also transforms them into high-value products.

Chemical entities consisting of at least one carbon atom bonded with hydrogen atoms are defined as organic compounds. Volatile organic compounds have relatively high vapor pressure at room temperature (25 °C) being in their gaseous form at a vapor pressure over 0.1 mmHg (Khademi et al., 2022). The boiling point of VOCs ranges between 50 and 260 °C (Alyüz et al., 2006). Within VOCs, benzene, toluene, ethylbenzene, and xylene (i.e. BTEX), are a highly toxic group of contaminants known for their use in various industrial processes, including the manufacture of plastics, resins, and synthetic fibers. BTEX compounds are significant environmental pollutants, often detected in air, water, and soil, primarily due to fuel spills, industrial discharges, and vehicle emissions. Exposure to BTEX can pose serious health risks; benzene, for instance, is a well-known carcinogen, while the others can cause neurological and respiratory issues. Monitoring and managing BTEX level are crucial for protecting public health and the environment (Khademi et al., 2022).

Toluene, a chemical compound with the formula C_7H_8 and a molecular weight of 92.14 g mol^{-1}, is subject to specific regulatory standards concerning workplace exposure (United Nations Environmental Program. et al., 1985). The metabolism of toluene in the human body exhibits a dual half-life, approximately 0.88 hours for the fast phase and 12.9 hours for the slow phase, indicating variable processing and excretion rates (Davidson et al., 2021). Known to be harmful to both the environment and human health, the main health risks caused by toluene include neurological, cardiac, pulmonary, renal and integumentary effects (Holmes & Murray, 2024; *Toluene Toxicity - StatPearls - NCBI Bookshelf*, n.d.).

Toluene is an important intermediary chemical, being produced on a large scale worldwide, ranging from 5 to 10 million tons annually. It is manufactured both as an isolated chemical and as part of various mixtures. In its isolated form, toluene finds a wide range of applications in the synthesis of other chemicals and serves as a solvent carrier in paints, thinners, adhesives, inks, pharmaceuticals, and cosmetics. Thus, one of the main concerns regarding the health risks posed by toluene is its widespread presence in the environment, particularly in the atmosphere, and its persistent activity. Given its extensive use across various industries and applications in modern life

human exposure to toluene is not uncommon. Consequently, the scientific community has developed several methods and systems to effectively treat and mitigate the impact of toluene on public health (Murindababisha et al., 2021).

Physical treatment methods for toluene removal include adsorption, using adsorbents like activated carbon or zeolite to remove toluene from gas or liquid phases, and distillation, which separates toluene based on differences in boiling points. Chemical treatment methods involve thermal or catalytic oxidation, converting toluene into harmless compounds such as carbon dioxide and water, and advanced oxidation processes like ozonation or photocatalytic oxidation that disrupt toluene molecules. Biological treatment methods rely on degrading microorganisms and can be implemented in bioreactors such as biofilters and stirred tank reactors (Barbusinski et al., 2017; Gospodarek et al., 2019). Membrane technologies, including membrane filtration using semi-permeable membranes and pervaporation combining evaporation and permeation processes, are used to separate toluene, especially in gas phase separation processes (Ezugbe & Rathilal, 2020). Advanced oxidation processes utilize ozone as a strong oxidant or the Fenton reaction, which employs an iron catalyst and hydrogen peroxide to oxidize toluene with hydroxyl radicals (Kumari & Kumar, 2023).

Biological systems for toluene abatement offer several advantages compared to their physicochemical counterparts. They are environmentally friendly alternatives, operating without toxic or hazardous chemical reagents, thus reducing the potential for environmental damage. While chemical treatment methods may sometimes result in secondary pollution, biological methods are generally less prone to such risk. Moreover, biological systems are highly economically efficient, consuming less energy and having lower operating costs compared to chemical or physical methods. This lower energy consumption also results in a lower carbon footprint. Additionally, biological methods are effective for treating low concentrations of organic pollutants like toluene. Various bioreactor configurations, such as biofilters and moving bed biofilm reactors (MBBRs), are used for water and air treatment. Biofilters allow microorganisms to grow on packing material and are successful for removing VOCs and H_2S from gas streams. MBBRs combine the benefits of both attached and suspended growth systems. The choice of system depends on specific application needs. In particular, this study emphasizes suspended growth systems for pollutant degradation and high-value product production, aiding in the extraction of target compounds.

Polyhydroxyalkanoates (PHA) are biopolymers used by various bacteria for carbon and energy storage. First observed in 1888 by Beijerinck, their function and composition were identified later. In 1926, Lemoigne isolated poly-3-hydroxybutyric acid (P(3HB)) from *Bacillus megaterium*, advancing PHA research. In 1958, Macrae and Wilkinson confirmed that PHA serve as reserve materials for carbon and energy in bacteria, accumulating under high carbon-to-nitrogen ratios (Możejko-Ciesielska & Kiewisz, 2016).

Polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV), two significant members of the PHA family, have a diverse range of applications in various industrial and biomedical contexts. PHB is a polymer with the chemical formula $(C_4H_6O_2)_n$ and

exhibits a crystalline structure. PHB is synthesised from carbon sources (e.g. glucose, starch) by bacteria such as *Bacillus, C. necator* and *Alcaligenes* (Sudesh et al., 2000). It has a melting point of approximately 180 °C and is used as a bioplastic. PHB is a biodegradable and biocompatible polymer that finds applications in areas such as packaging materials, medical devices and biological sewing threads (Sudesh et al., 2000).

Polyhydroxyvalerate (PHV) is another member of the PHA family and is a polymer with the chemical formula $(C_5H_8O_2)_n$, exhibiting lower crystallinity and high flexibility. PHV is produced by bacteria from valeric acid or related carbon sources and is typically found in the form of PHBV (polyhydroxybutyrate-co-valerate), which is a copolymer with PHB. PHBV was developed with the objective of reducing the brittleness of PHB and increasing the flexibility of the material. Copolymers with different PHV contents offer a range of mechanical properties and melting points, allowing the material to be optimised for different applications (Sudesh et al., 2000).

PHB and PHV polymers are preferred in biomedical and environmental applications due to their biodegradable and biocompatible properties. However, high production costs and mechanical limitations hinder their widespread use. Future advances in production technologies and greater environmental awareness are expected to promote the adoption of PHB and PHV, which are anticipated to play a significant role in the search for sustainable materials (Chen & Patel, 2012; Sudesh et al., 2000).

Cupriavidus necator is a bacterium extensively studied for its remarkable ability to accumulate PHA, specifically poly-β-hydroxybutyrate (PHB), making it a valuable organism in biotechnology. This Gram-negative, rod-shaped bacterium thrives in diverse environments, including soil and water, and is characterized by its metabolic versatility. *Cupriavidus necator* can utilize a wide range of carbon sources, including organic acids, sugars, and even industrial waste streams, which positions it as an efficient microbial cell factory for biopolymer production (Berezina et al., 2015).

The bacterium was first identified for its PHA-producing capabilities in the early 20th century, with significant advancements in understanding its metabolic pathways occurring later. *Cupriavidus necator*'s robust metabolic network allows it to efficiently convert carbon substrates into PHB, especially under nutrient-limited conditions, as nitrogen starvation, with an excess of carbon. This ability is regulated by complex genetic and enzymatic mechanisms based on the transformation of toluene to benzoic acid to subsequently generate catechol. Catechol is metabolized to Acetyl-CoA which is used in the synthesis of PHB or PHV(Berezina et al., 2015). In industrial applications, *Cupriavidus necator* has been leveraged for the large-scale production of biodegradable plastics. Advances in genetic engineering have further enhanced the productivity and versatility of *Cupriavidus necator*, enabling the biosynthesis of diverse PHA copolymers with tailored properties for specific applications (Berezina et al., 2015; Sudesh et al., 2000).

Despite its potential, the commercialization of polyhydroxyalkanoates (PHA) synthesized by *Cupriavidus necator* is impeded by challenges such as high production costs and competition with conventional plastics. Nonetheless, ongoing research

endeavours to optimize fermentation processes, enhance strain performance, and reduce costs, thereby augmenting the economic feasibility of PHA production. A noteworthy advancement in this domain is the valorisation of BTEX (benzene, toluene, ethylbenzene, and xylene) compounds, with particular emphasis on toluene, into valuable products such as PHA. Preliminary studies have demonstrated that *Cupriavidus necator* can metabolize toluene, thereby transforming this hazardous pollutant into bioplastics. This dual benefit underscores the necessity of our research, as optimizing the bioconversion of toluene into PHA could substantially propel the development of sustainable bioplastics while concomitantly mitigating environmental pollution caused by BTEX compounds. Thus, *Cupriavidus necator* remains pivotal in microbial biotechnology, with its applications significantly advancing the progress of sustainable bioplastic production (Sohn et al., 2021; Zhang et al., 2022)

2. OBJECTIVES

This study is guided by two primary objectives carefully designed to advance the understanding and implementation of biotechnological solutions for the removal of VOCs and the synthesis of high-value chemical compounds:

- Evaluate the biodegradation of toluene by the *Cupriavidus necator* bacterial culture.
- Optimise the operating conditions to simultaneously degrade toluene and synthesise a high-value chemical compound, poly-β-hydroxyalkanate (PHA).

3. HYPOTHESIS

This study aims to identify and evaluate the optimal conditions for the effective removal of gaseous toluene and its subsequent conversion to biopolymer. The hypothesis supporting this objective is based on previous research demonstrating that the *Cupriavidus necator* bacterium can reduce environmental pollution through the biodegradation of various pollutants, including VOCs (Możejko-Ciesielska & Kiewisz, 2016; Ronďošová et al., 2022). PHA production is integrated into the metabolic mechanisms of *Cupriavidus necator* cells and can be harvested as valuable by-product during the bioremediation process. Consequently, leveraging the metabolic capabilities of *Cupriavidus necator* not only facilitates the biodegradation of harmful VOCs but also enables the simultaneous production of commercially valuable biopolymers, highlighting the dual benefits of this biotechnological approach.

4. MATERIALS & METHODS

4.1. Chemicals and mineral salt medium

M9 mineral medium, often utilized for bacterial growth and studies involving nutrient-limited conditions, was prepared as follows (per L of distilled water): 100 mL of a 10× M9 salt solution (containing 33.7 mM Na_2HPO_4, 22.0 mM KH_2PO_4, 8.55 mM NaCl, and

9.35 mM NH_4Cl), 1 mL of 1 M $MgSO_4$, 0.3 mL of 1 M $CaCl_2$, 1 mL of biotin solution (1 mg mL^{-1}), 1 mL of thiamin solution (1 mg mL^{-1}), and 10 mL of a 100× trace elements solution. The nitrogen proportion in the medium was adjusted to 0×, 1×, and 2× depending on the experimental requirements.

The *M9 salt solution* was prepared by dissolving 75.2 g L^{-1} $Na_2HPO_4 \cdot 2H_2O$, 30 g L^{-1} KH_2PO_4, 5 g L^{-1} NaCl, and 5 g L^{-1} NH_4Cl in water, adjusting the pH to 7.2 with NaOH, making up the volume to 1 liter, and autoclaving at 121 °C for 15 minutes. The 20% *glucose stock solution* was prepared by dissolving 200 g of glucose in 500 mL water and autoclaving. Glucose was only used to activate the strains. During the assays, the only carbon source supplied was toluene. The 1 M *$MgSO_4$ stock solution* required 24.65 g of $MgSO_4 \cdot 7H_2O$ in 100 mL water, and the 1 M *$CaCl_2$ stock solution* involved dissolving 14.70 g of $CaCl_2 \cdot 2H_2O$ in 100 mL water, both followed by autoclaving.

For the *biotin solution*, 50 mg of biotin was dissolved in 45 mL of water, small aliquots of 1 N NaOH were added until the biotin dissolved, then water was added to a final volume of 50 mL. The solution was sterilized over a 0.22-µm filter, 1 mL aliquots were prepared, and stored at -20 °C. For the *thiamin solution*, 50 mg of thiamin-HCl was dissolved in 45 mL of water, water was added to a final volume of 50 mL. The solution was sterilized over a 0.22-µm filter, 1 mL aliquots were prepared, and stored at -20 °C.

The 100× *trace elements solution* was prepared by dissolving 5 g of EDTA in 800 mL of water, adjusting the pH to 7.5 with NaOH, then adding $FeCl_3 \cdot 6H_2O$ (415 mg), $ZnCl_2$ (42 mg), $CuCl_2 \cdot 2H_2O$ (6.5 mg), $CoCl_2 \cdot 2H_2O$ (5 mg), H_3BO_3 (5 mg), and $MnCl_2 \cdot 4H_2O$ (0.8 mg). The volume was adjusted to 1 L, the solution was sterilized over a 0.22-µm filter and stored appropriately.

4.2. Microorganisms and inoculate preparation

The experiments were conducted using a pure strain of *Cupriavidus necator*, obtained from DSMZ (Leibniz Institute for Natural Product Research and Infection Biology, German Collection of Microorganisms and Cell Cultures) according to the specifications provided in their catalogue. The inoculum was cultivated for 14 days at 25 °C and 200 rpm in an orbital shaker, through 120 mL sterile glass bottles containing 40 mL of M9 medium with glucose at 4 g L^{-1}. The inoculated bottles were sealed with gas-tight butyl septa and aluminium caps to ensure optimal growth conditions.

4.3. Continuous bioreactor for toluene conversion into Poly-β-hydroxyalkanoate (PHA)

The bioreactor system comprised a 2.5-liter reactor with 2-liter working volume (R1) and a 0.5-liter secondary reactor with 0.4-liter working volume (R2) (Figure 1). The R1 reactor was controlled by an advanced Sartorius Biostat® B system (Göttingen, Germany), which allowed continuous monitoring of temperature and pH values. This system was developed by Sartorius and is a versatile benchtop bioreactor controller that is employed in a variety of applications, including cell culture and microbial fermentation. The Biostat® B system is capable of controlling both reusable and

disposable culture vessels, offering both stirred and oscillating motion (*Biostat® B - Benchtop Bioreactor Controller* | *Sartorius*, n.d.).

Reactor 1 (R1) was devoted to biomass growth using toluene as the only carbon source. The temperature and pH were maintained at a constant value of 25 °C and 7, respectively, by the control system, and measured by a Hamilton EasyFerm Bio HB MS 225 probe (Reno, USA). The daily medium exchange varied between 100 to 350 mL, resulting in a liquid dilution rate ranging from 0.05 to 0.175 d^{-1}.The dilution rate was adjusted based on the biomass growth phases: during the lag phase, the dilution rate was kept at the lower end to allow for initial adaptation (100 mL M9 1×N, 0.05 d^{-1}); in the exponential phase, the dilution rate increased to accommodate the rapid growth of biomass (200 mL M9 1×N, 0.1 d^{-1}); and in the stationary phase, it was adjusted to stabilize the biomass concentration (350 mL M9 1×N or 2×N, 0.175 d^{-1}).

In contrast, reactor 2 (R2) was maintained with a consistent liquid dilution rate (20 mL M9 0×N, 0.05 d^{-1}) throughout the experiment, ensuring steady conditions for comparison. The secondary reactor was operated with nitrogen-free M9 medium (0×N) to encourage PHA synthesis. Given that PHA extraction processes necessitate cell lysis, and that nitrogen restriction impedes the ability of cells to resume normal growth, employing a secondary reactor such as R2 to perform N-limitation is imperative to maintain constant cell growth in the main reactor R1. Thus, it is feasible to repeat N limitation and PHA synthesis uninterruptedly without losing the starting inoculum.

Toluene was introduced into the bioreactor by evaporating liquid toluene into an air stream using a Fisherbrand™ Single Syringe Pump (Hampton, USA) at a constant rate of v=0.04 mL h^{-1}. The pressure of the inlet gaseous stream was monitored manually on a daily basis through a Ifm Electronic PN709s7 sensor (Essen, Germany), while the flow rate was calculated by an inverted test tube.

The concentration of total suspended solids (TSS), total nitrogen (TN), total organic carbon (TOC), inorganic carbon (IC) and optical density (OD) was determined on a daily basis throughout the period of media exchange. Reactor R1 was operated for a total of 34 days, while Reactor R2 was operated for a total of 11 days, with the objective of producing samples for PHA analysis.

Toluene was supplied to both reactors in the gaseous phase. Initially, the gas flow rate in Reactor R1 was maintained at an average value of 314 mL min^{-1} with an empty bed residence time (EBRT) of ~6.4 minutes. In contrast, when R2 was operated, the air flow was divided by two. Approximately 60 mL min^{-1} were provided to R2, which means a EBRT of ~6.7 min, while the rest of the air flow, ~254 mL min^{-1}, were transferred to R1, changing the EBTR to ~7.9 min. The concentration of toluene in the inlet gas stream was maintained at an average value of 4 g m^{-3} for both reactors.

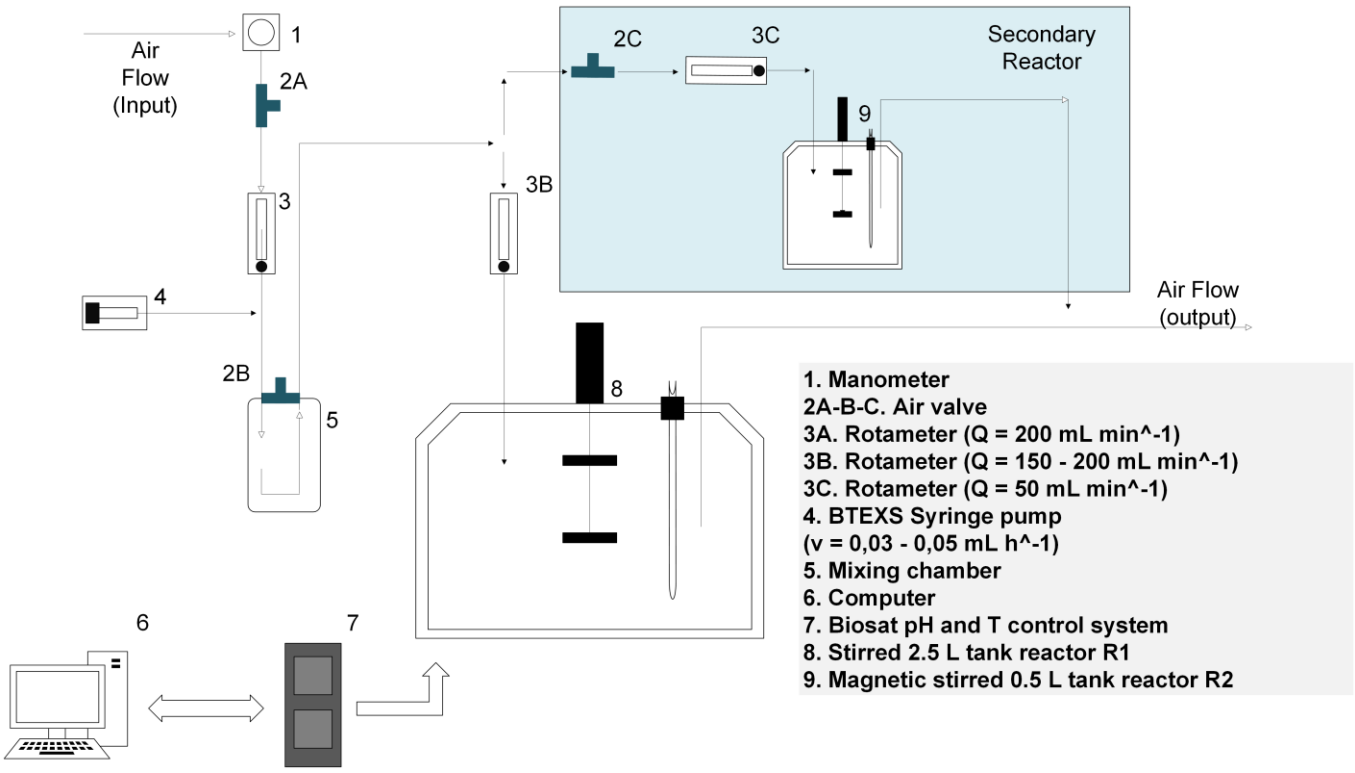

Figure 1. Diagram of a continuous bioreactor

Table 1. Controlled parameters and control methods of continuous bioreactor

CONTROLLED PARAMETERS	ANALYTICAL METHOD
Toluene Concentration	Agilent Gas Chromatography with Flame Ionization Detector (GC-FID)
CO_2 Level	Bruker Gas Chromatograph with Thermal Conductivity Detector (GC-TCD)
Total Nitrogen (TN), Total Organic Carbon (TOC), and Inorganic Carbon (IC) analyses	Catalytic Oxidation in Shimadzu TOC-L TNM-L
Optical Density (OD)	Spectrophotometer (650 nm)
pH	Biostat pH and T control system
Temperature	Biostat pH and T control system
PHA Analysis	5977E Agilent Mass Spectrometer
Effluent Flux	Manual Measurement
Total Suspended Solids (TSS)	TSS Procedure

4.4. Analytical procedures

Toluene analysis was conducted utilizing an Agilent 8860 GC System. This system featured a Split/Splitless (SSL) inlet maintained at 150 °C and 10.7 psi. Separation

occurred via an HP-5 column (30 m × 320 µm × 0.25 µm) with helium as the carrier gas at a flow rate of 2 mL min^{-1}. The column oven was initially set at 80 °C, and detection was performed using a Flame Ionization Detector (FID) maintained at 250 °C. This configuration ensured precise quantification of toluene in the samples. Samples were collected using a 250 µL Hamilton glass syringe and injected into the GC device.

For O_2 and CO_2 analysis, a Bruker 430 GC gas chromatograph was employed, featuring an injector set at 150 °C. Separation was accomplished using a CP-Molsieve 5A column (15 m × 0.53 µm × 15 µm) for CO_2, and a CP-PoraBOND Q column (25 m × 0.53 µm × 10 µm) for O_2, with helium as the carrier gas at a flow rate of 24 mL min^{-1}. The column oven was initially set at 45 °C, and detection was carried out using a Thermal Conductivity Detector (TCD) maintained at 175 °C. Samples were collected similarly to the above method but with a 100 µL Hamilton glass syringe.

Analyses for Total Nitrogen (TN), Total Organic Carbon (TOC), and Inorganic Carbon (IC) were performed using a TOC-L TNM-L Shimadzu system. Optical Density (OD) measurements, conducted to determine cell density, were performed with a Shimadzu UV mini-1240 spectrophotometer at a wavelength of 650 nm. Samples were placed in cuvettes for measurement. Total Suspended Solids (TSS) analysis involved filtering a known volume of sample through a 0.45 µm filter. Biomass concentration was determined by weighing the filter before and after filtration and drying at 100 °C.

For the qualitative and quantitative analysis of poly-β-hydroxyalkanate (PHA) composition, a 7820A GC coupled to a 5977E Agilent mass spectrometer (GC-MS, Santa Clara, USA) equipped with a DB-WAX column (30 m × 250 µm × 0.25 µm) was utilized. The injector and column temperatures were set at 250 °C and 40 °C, respectively, with an oven ramp temperature of 8 °C min^{-1} until 200 °C. MS source and MS quadrupole temperatures were 230 °C and 150 °C, respectively. Helium served as the carrier gas at 1 mL min^{-1}. The PHA extraction followed an adapted methodology from (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 to glass tubes containing freeze-dried biomass, followed by digestion in a Thermoblock Digital Dry Bath Thermo Scientific (Waltham, USA) at 100 °C for 4 hours. After cooling, 1 mL of distilled water was added to separate the organic and inorganic phases. Approximately 1.5 mL of the organic phase was collected, filtered through a 0.22 µm filter, and transferred to a chromatography vial. Calibration curves for PHB and PHV were also prepared.

The pH and temperature were continuously monitored using sensors integrated into the bioreactor's control system. Daily pressure measurements were taken using a calibrated pressure gauge at the reactor's inlet. The effluent flow rate from the reactor was manually measured using an inverted test tube technique and recorded in millilitres per minute (mL min^{-1}).

5. RESULTS & DISCUSSION

5.1. Performance of continuous bioreactor R1

The bioreactor was successfully operated for a 34-day period, daily monitoring the operating and performance parameters. The average toluene inlet concentration during the stationary phase was 3.7 ± 0.4 g m^{-3}, while the output toluene concentration remained at 0.1 ± 0.05 g m^{-3} since the stationary phase was reached. This means that R1 was operated with an average toluene load of 32.1 ± 6.3 g m^{-3} h^{-1}. These results correspond to an average toluene removal efficiency of 97.9 ± 1.5% (Figure 2). Considering the biomass production, the specific toluene consumption in the stationary phase was of 22.9 ± 0.8 $mg_{toluene}$ g_{DCW}^{-1} h^{-1}.

Table 2. Average and standard deviation of input and output toluene concentrations and removal efficiency in R1

AVERAGE INPUT (g m^{-3})	AVERAGE OUTPUT (g m^{-3})	AVERAGE ELIMINATION (%)
3.7 ± 0.4	0.1 ± 0.05	97.9 ± 1.5%

A low standard deviation value indicates that the data points in the set are in close proximity to the mean, with minimal differences among them. This suggests that all values within the data set are concentrated around the mean, thereby demonstrating a high degree of homogeneity and low variance, and therefore a very stable toluene removal performance.

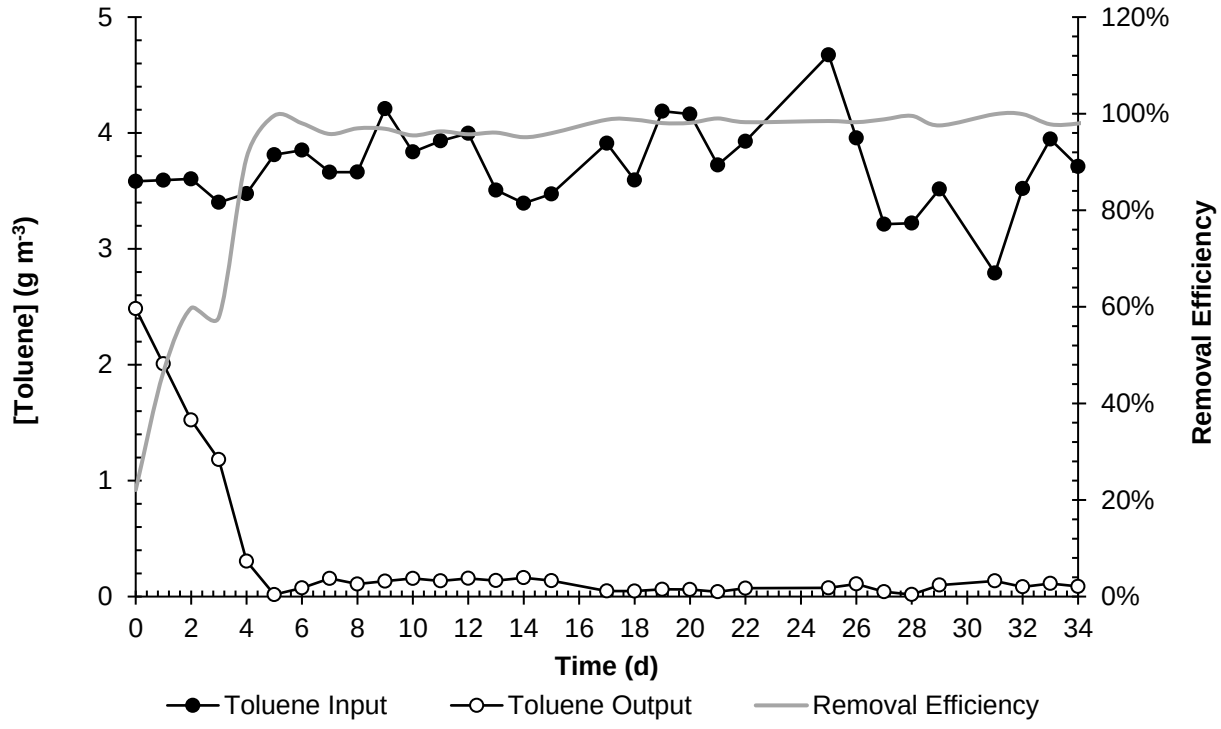

Figure 2. Toluene input (black line with black circles), toluene output (black line with white circles), and toluene removal efficiency (grey line) in R1 over time.

During the initial five-day period of culture growth, the data indicated a relatively low level of efficiency regarding the removal of toluene. This was associated to the acclimation of the inoculum to the new conditions and to cell growth. Then, the efficiency of toluene removal exhibited a gradual increase, reaching an average value of 97.9% from day 5 onwards. Prior to the reactor entering the stationary phase, the volume of media daily exchanged was increased from 100 mL (lag phase) and 200 mL (exponential phase) to 350 mL upon stabilization.

The fluctuations in efficiency observed can be attributed to variations in the inlet concentration of toluene. Based on the data, the bioreactor demonstrates effective toluene removal when operated with an empty bed residence time of ~6.4 to ~7.9 minutes. These operational parameters underscore the system's capability in efficiently managing toluene removal under the given conditions.

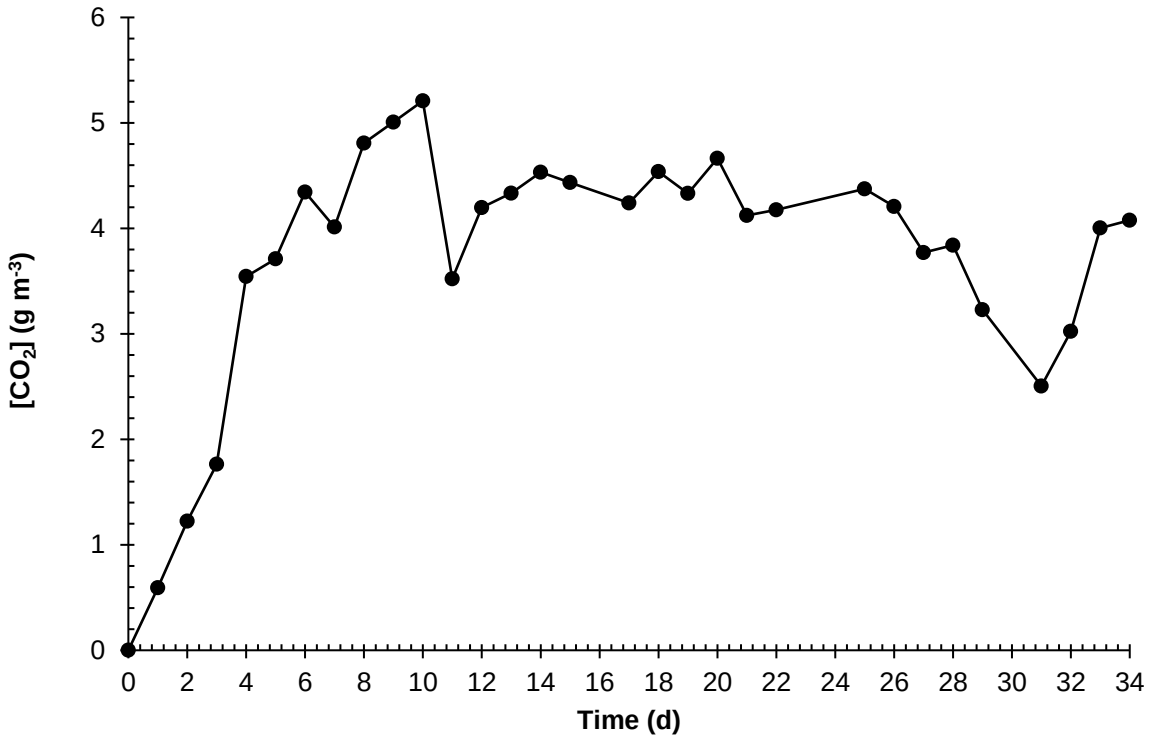

Figure 3. CO_2 production (black line with black circles) in R1 over time.

The CO_2 concentration measured at the outlet of the bioreactor offers a dynamic analysis of the metabolic activities of the microorganisms. The CO_2 outlet concentration steadily increased from the inoculation due to the adaptation process of the microorganisms to the bioreactor and the continuous supply of toluene. This considerable increase observed during the initial period demonstrates that *C. necator* exhibits a high level of metabolic activity employing toluene as the sole carbon source. During this assay, the average CO_2 production in the stationary phase was 4.1 ± 0.6 g m^{-3} while the maximum concentration reached was of 5.2 g m^{-3}. Considering biomass concentration, the specific CO_2 production was of 25.7 ± 1.9 mg_{CO_2} g_{DCW}^{-1} h^{-1}. Fluctuations observed in CO_2 levels may be attributed to the inherent variability of conditions within the bioreactor, including medium and nutrients exchange, oxygen levels, and pH. From day 4 onwards, a more stable trajectory of CO_2 concentrations is observed, indicating that the microorganisms have reached a certain equilibrium and are operating at a constant metabolic rate.

In conclusion, the time-dependent variation of CO_2 concentrations highlights the dynamic nature of microbial activity in the bioreactor, which is critical for optimizing its operations. The observed average mineralization rate of 30.4 ± 5.1% refers to the proportion of organic carbon, in this case toluene, that was converted into inorganic forms like CO_2 in the stationary phase. The mineralization rate specifically serves as an indicator of microbial activity and degradation efficiency.

When comparing this mineralization rate with studies on biofiltration of toluene, the results show some variation based on system design and microbial species. For example, a study conducted by the Department of Occupational Health Engineering, Faculty of Public Health, Tehran University of Medical Sciences in 2020, compared fungal and bacterial biofiltration systems for the treatment of volatile organic compounds (VOCs), including toluene. Their findings indicated that bacterial biofiltration systems outperformed fungal ones in terms of removal efficiencies and mineralization ratios, with bacterial systems achieving rates of up to 40.2 ± 5.4% compared to 27.7 ± 8.9% for fungal systems (Ghasemi et al., 2020.). Compared to this, our values were within the expected range for toluene degradation by other microorganisms.

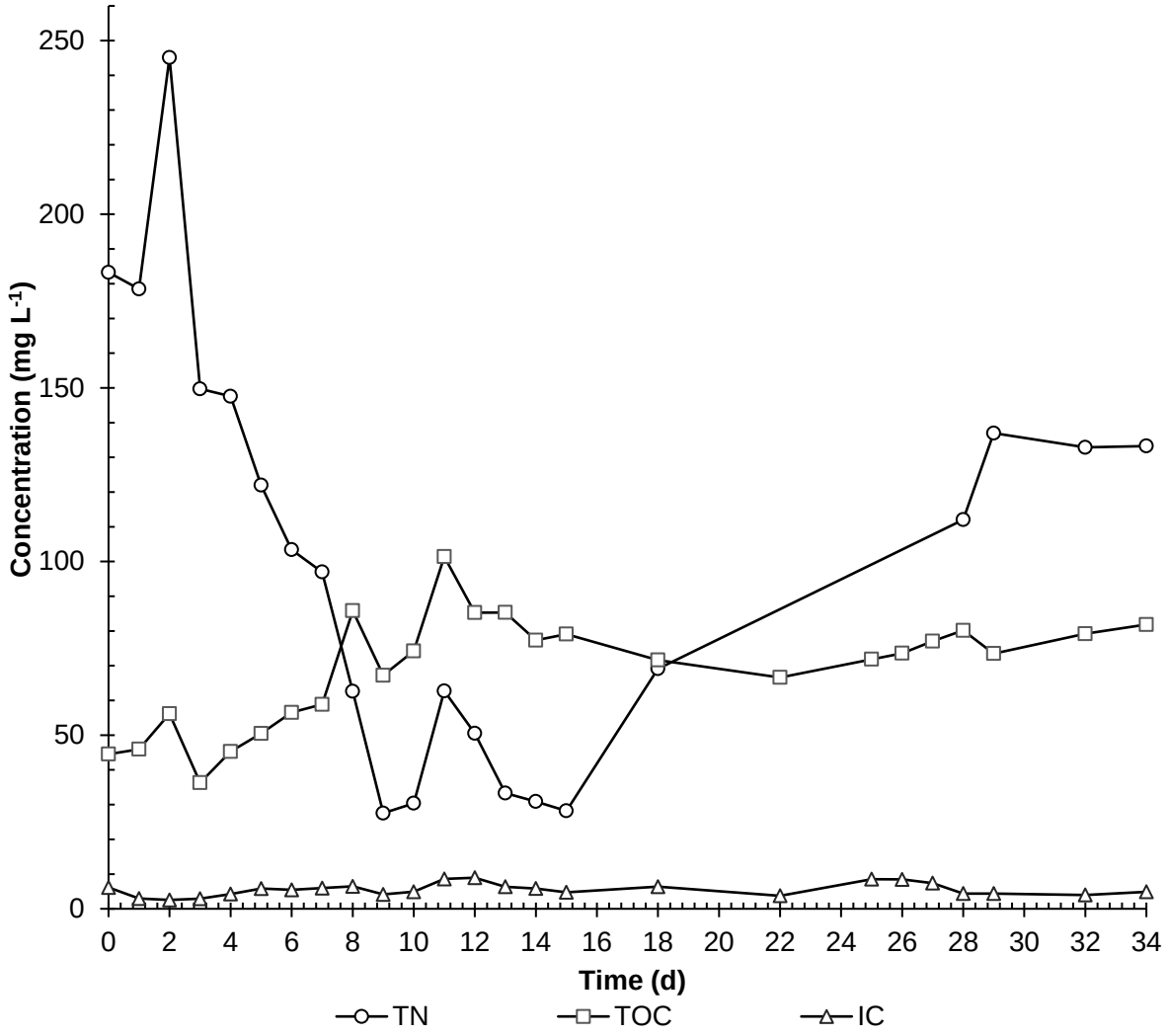

Figure 4. TOC (black line with white circle), TN (black line with white square) and IC (black line with white triangle) in R1 over time

Analyses of total nitrogen (TN), total organic carbon (TOC), and inorganic carbon (IC) conducted within the reactor demonstrate the alterations occurring across the various phases of the bioreactor. During the initial nine-day period, a notable decline in TN

concentration was observed, which can be attributed to the combined influence of biological activity and toluene removal within the bioreactor. However, the TOC concentration showed an increasing trend from day 0 to day 12-14, suggesting an accumulation of intermediate metabolites from the toluene degradation process. These secondary metabolites are usually soluble compounds that increase the dissolved TOC concentration. The low initial medium dilution rate likely resulted in their accumulation in the cultivation broth at the beginning of the experiment.

Table 3. Average and standard deviation of TOC, TC and IC concentration in R1 during the experiment

AVERAGE TOC ($mg\ L^{-1}$)	AVERAGE TC ($mg\ L^{-1}$)	AVERAGE IC ($mg\ L^{-1}$)
78.3 ± 8.4	84.3 ± 9.3	6.0 ± 1.8

The concentration of TOC exhibited fluctuations over the course of the experiment, although it remained relatively stable overall, particularly after the complete degradation of toluene by days 6-8 onwards. The mean concentration of TOC in the stationary phase was 78.3 ± 8.4 mg L^{-1}, while the concentration of IC remained low and constant at 6.0 ± 1.8 mg L^{-1}. The data demonstrate that the bioreactor was effective and that the system exhibited stability. Furthermore, total carbon (TC) analyses corroborate these findings, with a mean TC value of 84.3 ± 9.3 mg L^{-1}.

Nitrogen consumption played a critical role in cell growth and toluene removal within the bioreactor. Total nitrogen levels were carefully monitored, and in instances where nitrogen levels were insufficient, supplementation was provided through the utilization of a 2×N M9 medium during medium exchange (from day 15 onwards). This approach ensured uninterrupted biological activity and maintained toluene removal efficiency and biomass growth. The effective nitrogen consumption in conjunction with toluene removal and CO_2 production highlights the bioreactor's capability in sustaining microbial growth and activity. These findings suggest that the bioreactor is an effective method for toluene removal, with significant degradation of organic carbon and nitrogen contributing to the system's overall efficiency.

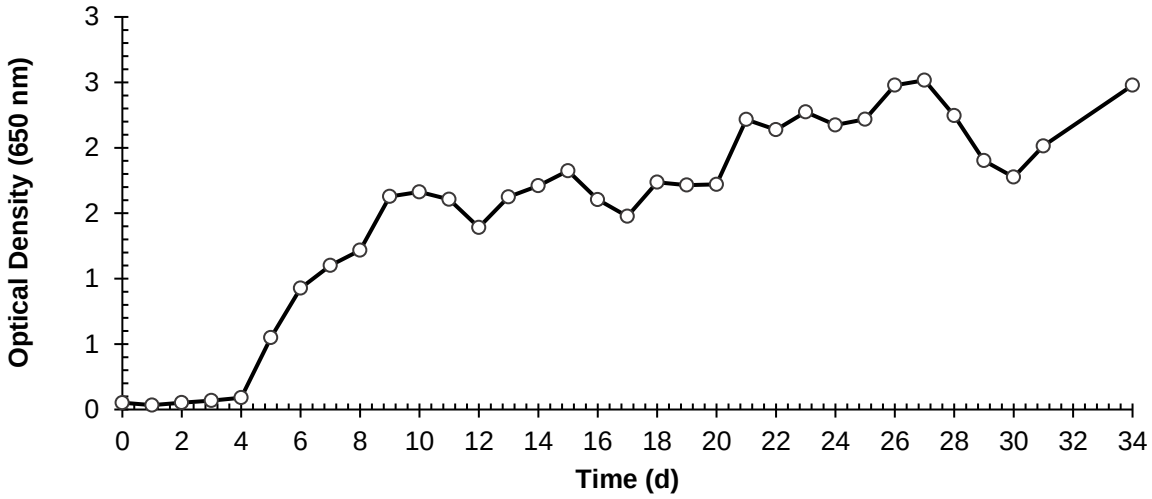

Figure 5. Optical density (black line with white circle) in R1 over time

The optical density (OD) plot, measured at a wavelength of 650 nm, demonstrates the growth of the microorganism within the bioreactor (Figure 5). During the initial five-day period, there was minimal increase in optical density, suggesting that the microorganisms were in the process of adaptation and that a notable increase in biomass had yet to occur. Following the fifth day, a notable rise in optical density was recorded until day 8, which refers to the exponential phase and the increasing of biomass within the reactor. From day 9 to the end of the experiment, the microorganisms moved to the stationary phase, but still with continuous growth as the system evolved. This increasing trend indicates that the microorganisms have reached optimal growth conditions and are undergoing active multiplication. The average OD value observed during the experiment was 1.9 ± 0.3 ABS, with the maximum OD reaching 2.5 ABS.

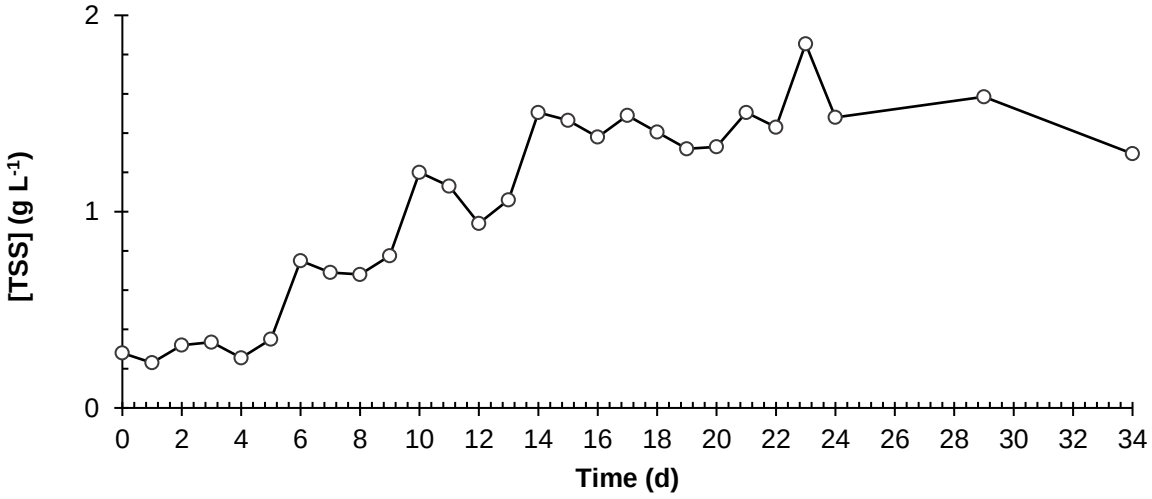

Figure 6. Total suspended solids (black line with white circle) in R1 over time

Figure 6 depicts the biomass concentration within the bioreactor, measured as TSS, which should exhibit similar behaviour to that of OD. During the first five days, no significant increase in TSS values was observed, indicating that the microbial population was likely adapting to the new environment. From the fifth day onwards, there was a steady rise in TSS values, reaching an average of 1.4 ± 0.2 g L^{-1} in the stationary phase throughout the experiment. A maximum TSS value of 1.8 g L^{-1} was recorded around day 24. However, from day 14 onwards, the values became more stable, suggesting that the biomass had reached a steady-state phase.

The following table presents the means and standard deviations of the daily measured temperature (T), pH, pressure (P) and flow rate values of the bioreactor.

Table 4. Average and standard deviation of T, pH, P and flow rate

AVERAGE T (°C)	AVERAGE pH (-)	AVERAGE P (mbar)	AVERAGE FLOW RATE (mL min^{-1})
25.1 ± 0.5	7.0 ± 0.1	429.1 ± 65.2	314.0 ± 24.8

The data demonstrate that the environmental conditions within the bioreactor remained relatively constant and were effectively maintained. The temperature values were maintained within the optimal growth temperature range for the microorganisms, exhibiting minimal deviation with an average of 25.1 ± 0.5 °C. This suggests that the bioreactor was functioning optimally, and that temperature control was conducted with efficacy. The pH value was also maintained at a neutral level, with an average of 7.0 ± 0.1. Therefore, microorganisms operated within the pH range in which they are most effective. The pressure values exhibited a somewhat broader range of variation, with an average of 429.1 ± 65.2 mbar. However, these pressure values were not at levels that would have a detrimental impact on reactor performance. The pressure control was adjusted in accordance with the internal dynamics of the bioreactor. The gas flow rate was maintained at 314.0 ± 24.8 mL min^{-1}, corresponding to a gas residence time of ~6.4 min. This suggests that the feeding and product extraction processes in the bioreactor were conducted correctly and that the nutritional requirements of the microorganisms were satisfied.

5.2. Performance of the PHA synthesis reactor R2

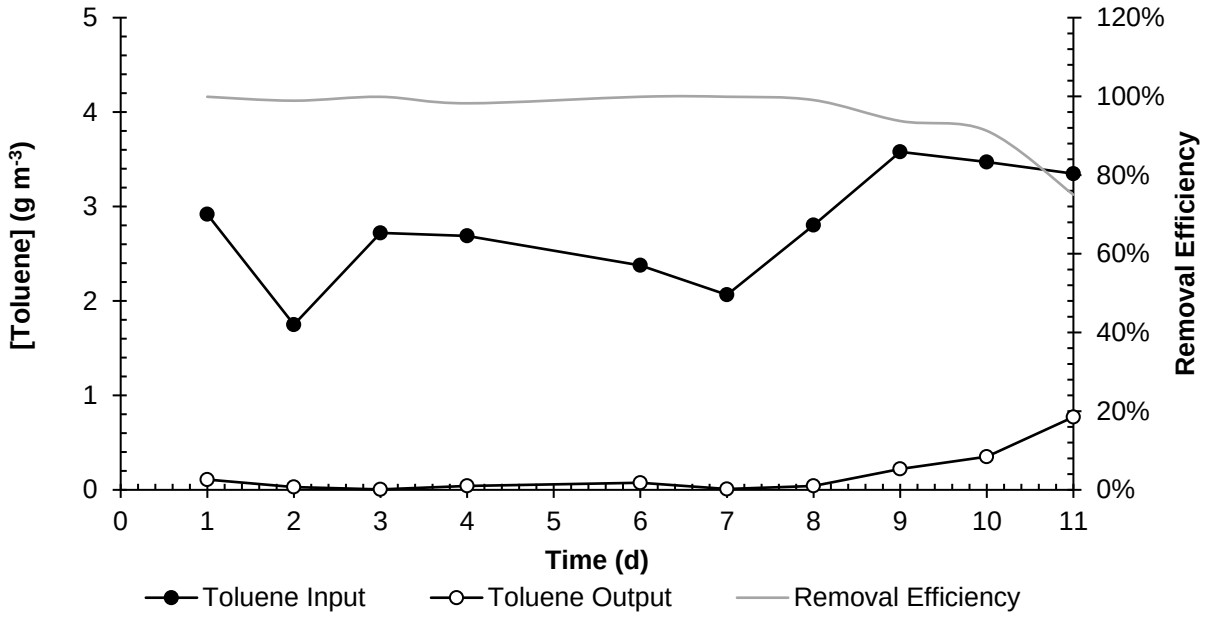

Figure 7. Toluene input (black line with black circle), toluene output (black line with white circle), and removal efficiency in R2 over time

The graph demonstrates the efficacy of the reactor in removing toluene over an 11-day period. Throughout the experiment, the average inlet concentration of toluene was 2.8 ± 0.6 g m^{-3}, while the average outlet concentration was 0.2 ± 0.2 g m^{-3}, resulting in an average removal efficiency of 95.6 ± 7.8 %. Notably, despite the use of the M9-NitrogenFree medium, which lacks nitrogen and is designed to promote PHA accumulation rather than biomass growth, the system maintained a high removal efficiency. The reactor exhibited over 90% removal efficiency for several days, even in the absence of nitrogen. By day 9, a slight decline in performance was observed, with removal efficiency dropping to 90%. These findings highlight the ability of the reactor to maintain high toluene removal efficiency in nitrogen-free conditions, where toluene serves solely as the carbon source.

In conclusion, the reactor consistently demonstrated high performance throughout the 11-day period, maintaining >90% removal efficiency despite the absence of nitrogen in the system.

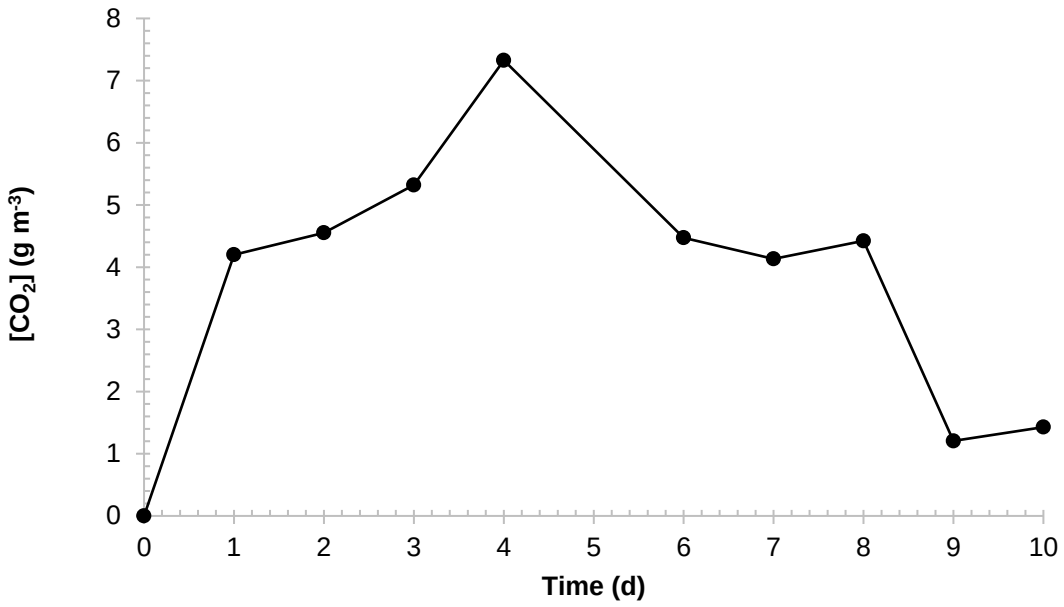

Figure 8. CO_2 production (black line with black circle) in R2 over time

The graph demonstrates the fluctuations in CO_2 concentration in the outlet stream of the R2, which was operated in a nitrogen-limited environment over a 11-day period. A rapid increase in CO_2 production was observed during the initial stages of the experiment, with the maximum value (~7.3 g m^{-3}) being reached on day 4. This increase indicates that the cells initially undergo a phase of elevated metabolic activity and rapid growth, using the remaining N from the liquid media of R1. Following day 4, a steady decline in CO_2 concentration was observed due to nitrogen absence, reaching the lowest value of ~1 g m^{-3} by day 9.

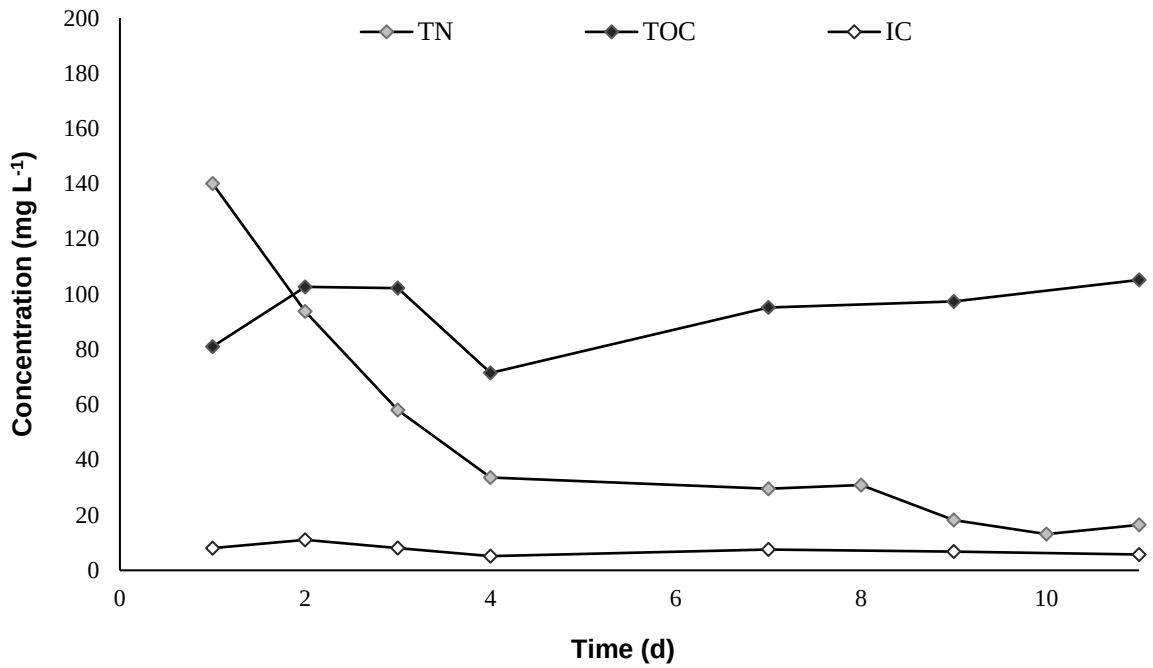

Figure 9. TN (black line with white triangle), TOC (black line with white square) and IC (black line with white circle) concentrations in R2 over time

The initial total nitrogen (TN) concentration was approximately 140 mg L^{-1}, which then rapidly decreased to 80 mg L^{-1} over the first two days. This suggests that the cells consumed nitrogen quickly, leading to a nitrogen-limited environment. From day 4 onwards, the system can be considered nitrogen-limited, with concentrations dropping below 30 mg L^{-1}. The initial total organic carbon (TOC) concentration was approximately 100 mg L^{-1} and remained relatively stable throughout the 11-day period, indicating that organic carbon was available but cellular growth was constrained due to nitrogen limitation. This further suggests that no significant production or accumulation of secondary metabolites occurred during this time.

In contrast, the concentration of inorganic carbon (IC) remained relatively constant, starting at around 10 mg L^{-1}, with only a slight increase observed after day 10. This indicates that the transformation of inorganic carbon was limited, likely because CO_2 production was a minor process, as organic carbon was the primary carbon source for the cells, and the pH remained stable throughout the experiment.

The primary aim of this experiment was to investigate the simultaneous degradation of toluene and the synthesis of a high-value chemical compound, poly-β-hydroxyalkanoate (PHA). A calibration curve was constructed using GC-MS to determine the concentrations of PHA subtypes, polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV), present in the samples. The concentrations of PHB and PHV were quantified based on the areas under the calibration curve (Figures 10 and 11).

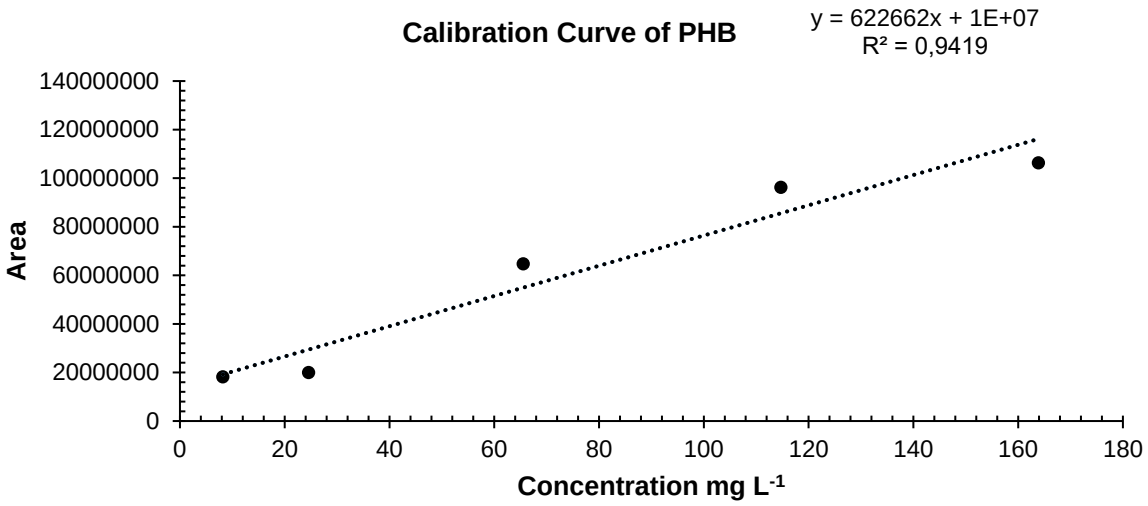

Figure 10. Calibration curve for PHB

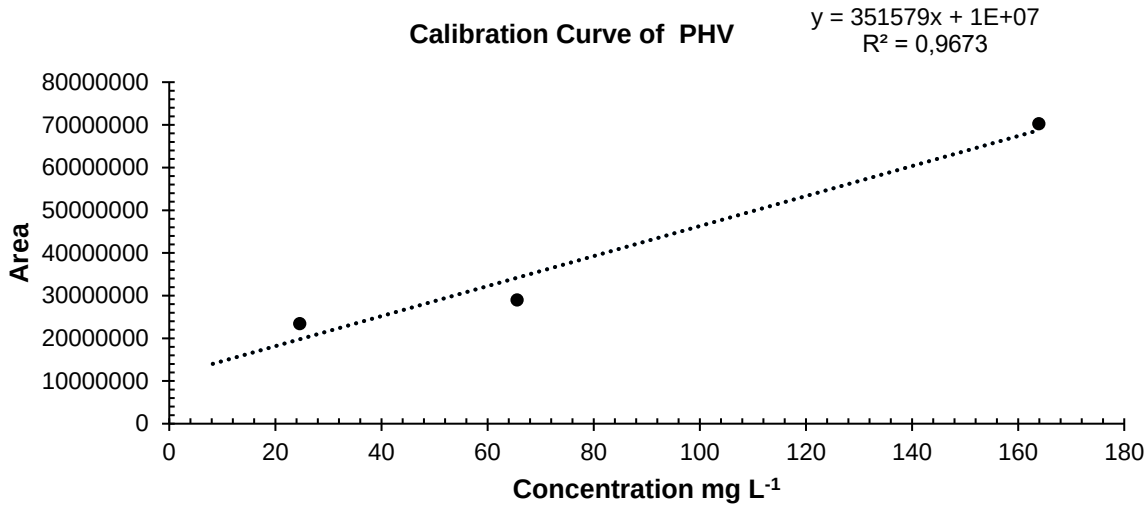

Figure 11. Calibration curve for PHV

The quantity of PHB and PHV produced by the bioreactor over the 11-day period was determined in terms of area. The concentration in mg L^{-1} was calculated using the area data obtained from the analysis and the calculated calibration curves.

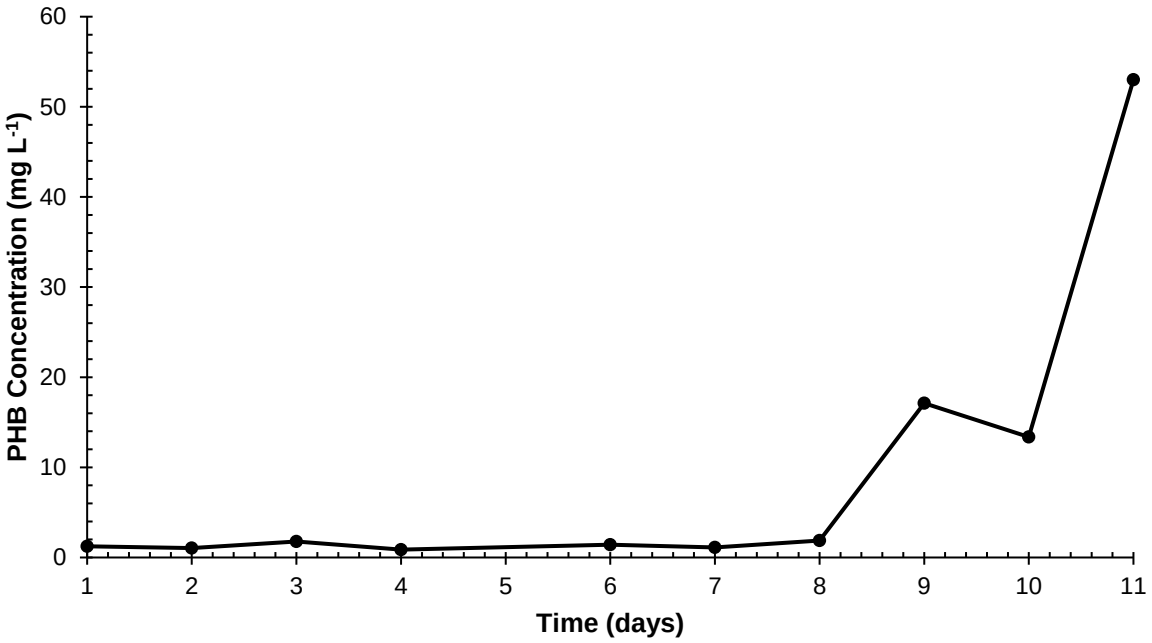

Figure 12. PHB concentration (mg L^{-1}) (black line with white circle) over time

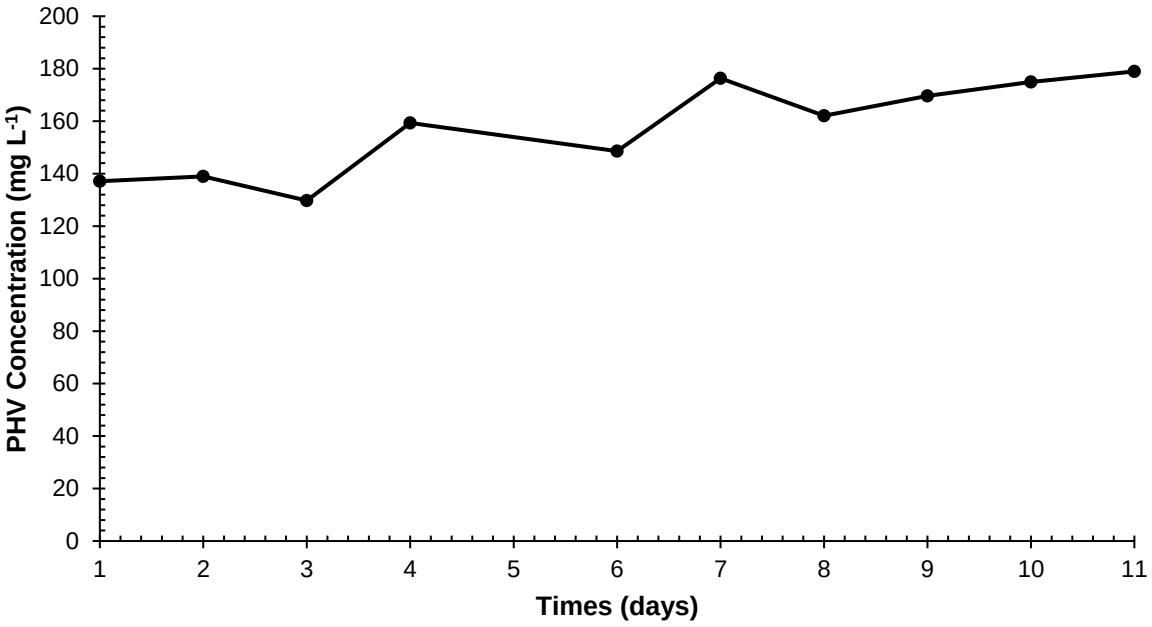

Figure 13. PHV concentration (mg L^{-1}) (black line with white circle) in R2 over time

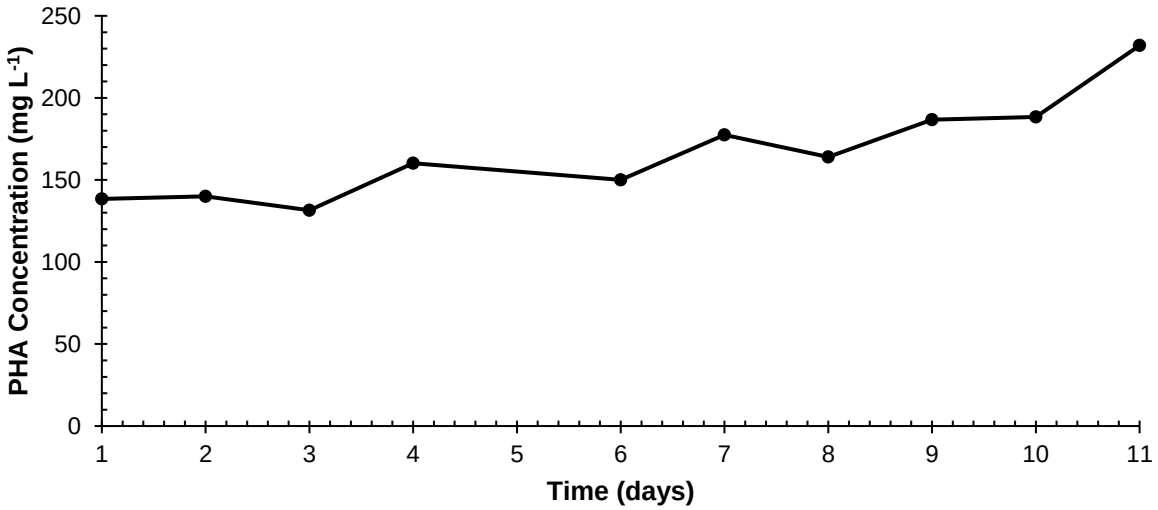

Figure 14. PHA concentration (mg L-1) (black line with white circle) over time

Table 5. Average and standard deviation of concentration of PHB, PHV and PHA

Average PHB concentration (mg L^{-1})	Average PHV concentration (mg L^{-1})	Average PHA concentration (mg L^{-1})
9.3 ± 16.4	157.6 ± 18.0	166.9 ± 30.4

The concentration of PHB increased significantly during the experiment, peaking at 53.0 mg L^{-1} by day 11. This is probably because high PHB production takes several days once nitrogen limitation is reached. In contrast, PHV production remained relatively stable throughout the experiment, with concentrations consistently higher than those of PHB, fluctuating between 137.14 mg L^{-1} and 178.96 mg L^{-1}, with the highest concentration observed on day 11. This suggests that the conditions favored a more efficient PHV production process. Additionally, it was observed that the total concentration of PHA increased over time under nitrogen-limited conditions, and the composition of the synthesized PHA shifted, leading to a higher PHB/PHV ratio. This variation in composition is significant, as different PHA ratios result in distinct mechanical properties. Extending the experimental period could have provided more insights, especially regarding whether the increased PHB production could be sustained over a longer duration.

Specific PHA concentrations are represented in Figures 15-17. Higher specific concentrations indicate a more efficient conversion of substrates to the desired bioproduct, in this case PHB and PHV. This is a crucial factor in optimising production processes. By focusing on specific concentrations, researchers can gain a deeper understanding of the intrinsic productivity of the microbial culture, which in turn allows them to make informed decisions that will lead to an increase in yield. Further research is required to ascertain whether these trends can be maintained or optimised over an extended period of time.

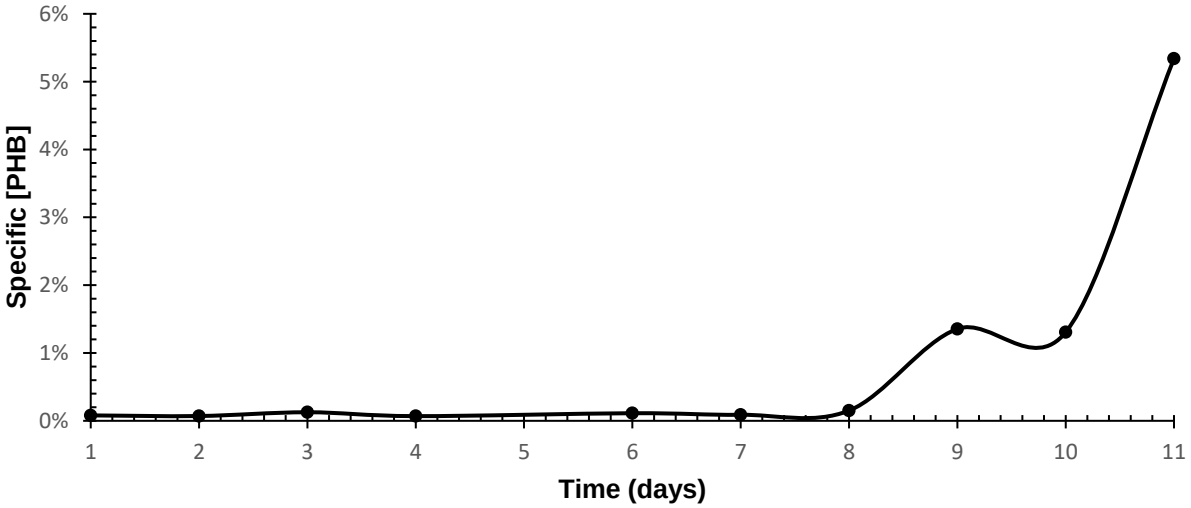

Figure 15. Specific PHB value (%) (black line with white circle) over time

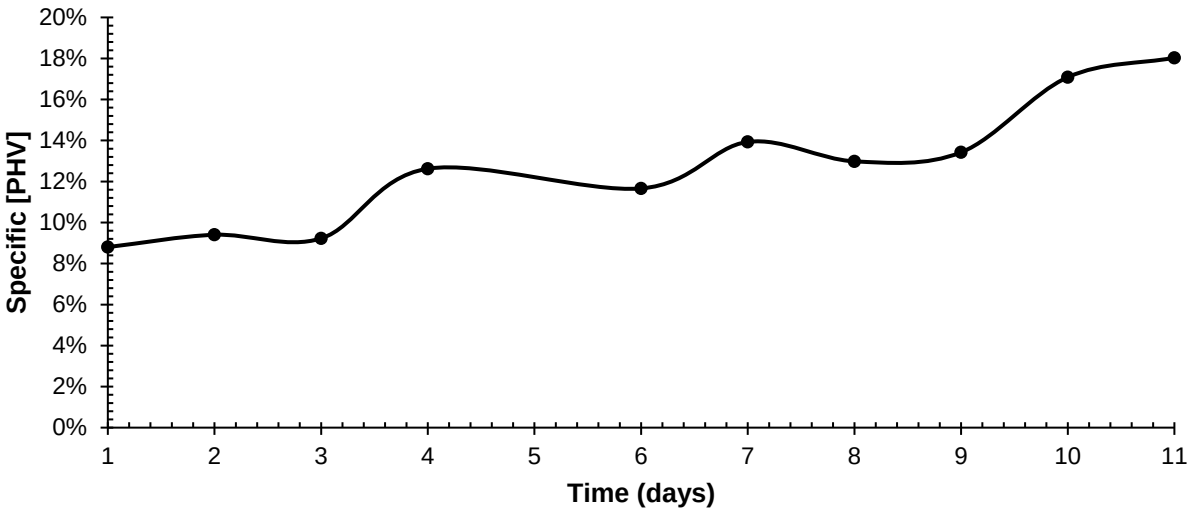

Figure 16. Specific PHV value (%) (black line with white circle) over time

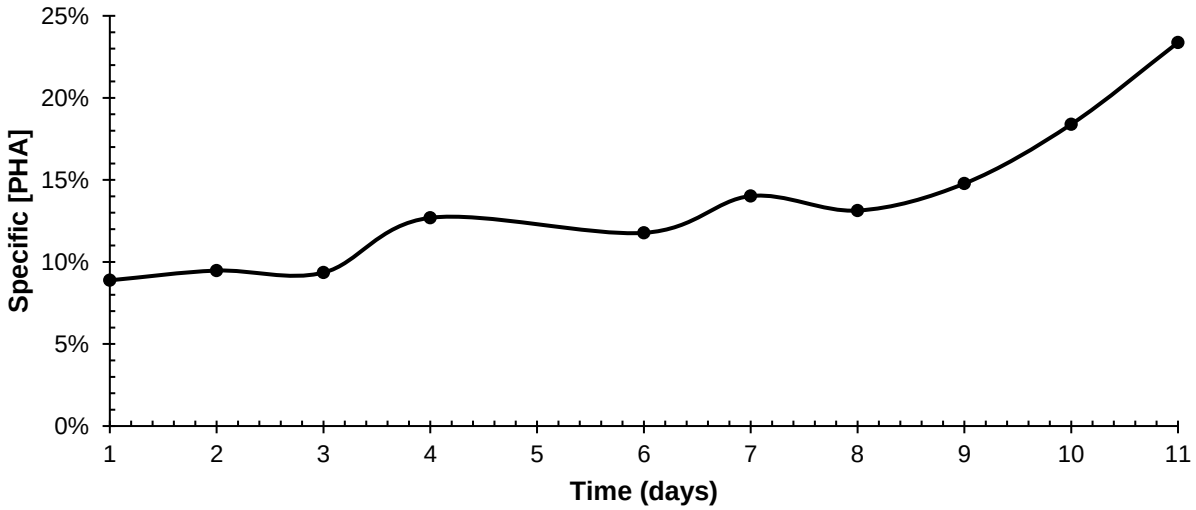

Figure 17. Specific PHA value (%) (black line with white circle) over time

Table 6. Average and standard deviation of specific PHB, PHV and PHA

Average specific PHB (%)	Average specific PHV (%)	Average specific PHA (%)
0.9 ± 1.7	12.7 ± 3.1	13.6 ± 4.5

Table 7. PHB:PHV final proportion

	PHB:PHV final proportion (%)
PHB	22.8
PHV	77.2

The final proportion analysis demonstrated that PHB constituted 22.8% and PHV 77.2% of the total PHA at the end of the experiment, thereby substantiating the experimental conditions' greater suitability for PHV production.

In order to facilitate a comparison of this study with the existing literature, I selected the study conducted by Berezina et al. (2015), which investigated the capacity of *Cupriavidus necator* to biodegrade aromatic compounds such as benzoic acid and produce bioplastics. This study shares certain similarities with previous research, while also introducing new insights that are crucial for assessing the biotechnological potential of the bacterium.

In my study, toluene was employed as the sole carbon source, and the production of PHA (particularly PHB and PHV) resulting from the biodegradation of this VOC was investigated. The biodegradation of toluene was observed to occur at a rate of 97.9% in the experiments conducted with the R1 bioreactor system. In the experiments conducted under nitrogen-limited conditions, it was observed that PHV was produced at a higher rate (77.2% vs. 22.8%) than PHB.

In 2015, the capacity of *Cupriavidus necator* to biodegrade monosubstituted aromatic compounds, such as benzoic acid, was investigated (Berezina et al., 2015). In this study, the optimisation of PHA production during the biodegradation of benzoic acid was achieved, with 43.7 g L^{-1} of benzoic acid successfully converted to PHB in batch and fed-batch experiments. The study demonstrated that a benzoic acid concentration of 2.5 g L^{-1} was not toxic to bacteria, with up to 35% PHA accumulation occurring under these conditions.

The results of both studies demonstrated that *Cupriavidus necator* exhibits a high capacity for the biodegradation of environmental pollutants. It should be noted, however, that the pollutants used differed between the two studies. In my own study, toluene was used, whereas Berezina et al. (2015) employed benzoic acid. This discrepancy has a direct impact on the bioreactor design, experimental conditions and the types of PHAs obtained. Given that toluene is a more volatile compound, it is notable that PHV production is the dominant process during its biodegradation. Conversely, in Berezina's study, the greater stability of benzoic acid resulted in the dominance of PHB production.

A comparison of the two studies demonstrates that the utilisation of *Cupriavidus necator* in biotechnological applications presents a substantial opportunity for the biodegradation of both volatile organic compounds (VOCs) and aromatic compounds. However, the type of biopolymer obtained may vary depending on the carbon source used, and this should be considered in the context of targeted bioplastic production strategies.

6. CONCLUSION

The conducted study successfully demonstrated the efficacy of a two-stage continuous bioreactor for the removal of toluene and the production of poli-β-hydroxyalkanoates (PHA) over a 34-day operational period. The first reactor, devoted to the maintenance of a *Cupriavidus necator* inoculum using toluene as the only C source, consistently maintained high toluene removal efficiencies, averaging 97.9 ± 1.5%, with a notable stability in output concentrations after the initial adaptation period. This stability underscores the reactor's robustness in handling organic pollutant degradation under controlled conditions. The microbial activity within the bioreactor was validated by the CO_2 production rates, which provided insight into the metabolic processes of *C. necator*. The CO_2 production initially showed a dynamic pattern, stabilizing as the microorganisms adapted and reached a steady metabolic state. The observed average mineralization rate was 30.4 ± 5.1%, indicating the proportion of organic carbon from toluene converted into inorganic forms such as CO_2. This observation was corroborated by the consistent total organic carbon (TOC) measurements, further confirming effective carbon mineralization. Nutrient management, particularly nitrogen availability, was critical in maintaining microbial growth and reactor efficiency. The study highlighted the importance of nitrogen supplementation in preventing performance decline, especially noted in the secondary reactor devoted to PHA synthesis under N deprivation conditions. In this second stage, nitrogen limitations led to reduced toluene degradation efficiency over time, although the system was able to

maintain >90% removal efficiency. In terms of PHA production, the bioreactor demonstrated the potential for synthesizing valuable biopolymers, with PHV being produced more efficiently than PHB, reaching concentrations of 179.0 mg L^{-1} and 53.0 mg L^{-1} after 11 days of N limitation. The observed concentrations and the specific yields of PHB and PHV indicated a favourable environment for PHV synthesis, making up 77.2% of the total PHA produced. The study's results emphasize the bioreactor's capability not only for effective pollutant removal but also for sustainable biopolymer production. Future research should focus on long-term stability, optimizing nutrient supply, and exploring different operational parameters to maximize both toluene degradation and PHA production. This approach will contribute significantly to the advancement of bioreactor applications in environmental remediation and industrial biotechnology.

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