high-value products from agricultural residues through sustainable chains

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1. Executive Summary

This deliverable reports the progress achieved, from month M1 to month M36. toward the sustainable valorization of agro-industrial residues through the integrated production of high-value bioproducts, namely medium-chain carboxylic acids (MCCAs), microbial protein (MP), and polyhydroxyalkanoates (PHAs).

Each partner contributed to the development of complementary technological routes:

- UGent successfully optimized an MCCA production process using an expanded granular sludge bed (EGSB) reactor fed with ethanol-rich wine lees. The process achieved high productivity (up to 46.5 gCOD L^{-1} d^{-1}) and selectivity (76–78% caproate), demonstrating robustness under low hydraulic retention times (12 h). The resulting MCCA-rich effluent served as a feedstock for subsequent PHA production

- AVECOM developed and optimized the enzymatic hydrolysis of grey starch (GS), a byproduct of potato processing, to generate fermentable hydrolysates for microbial protein synthesis. Process optimization resulted in concentrated hydrolysates (COD = 122.5 g L^{-1}) suitable for fed-batch cultivation by UGENT of *Cupriavidus necator* H1 G+3, achieving biomass levels up to 9.6 ± 2.5 g L^{-1} and protein contents of 50%–73%

- NID demonstrated the conversion of lipidic fractions of tomato pomace and brewer's spent grain into PHAs using both single strains (*Burkholderia thailandensis* E264, *Cupriavidus necator* DSM 428) and mixed microbial cultures (MMCs). Under optimized conditions, PHB contents reached up to 87.6%, while using MMC-based process for PHA production from carboxylic acid-rich streams (e.g., hexanoic acid mixtures) yield a total of 106 g of dried biomass with approximately 55 g of PHA. The polymers exhibited tunable monomer compositions (3HB: 78–83%, 3HV: 7–15%, 3HHx: 3–10%).

- UNIROMA developed and operated a continuous-flow multi-reactor process that allowed to obtain a poly(hydroxybutyrate-hydroxyvalerate) (PHBV) copolymer with an intracellular biopolymer content of ca. 55 (%, wt/wt) in the accumulation reactor, and an HV monomer content ranging between 20 and 57 (%, wt/wt). The role of sc-CO_2 was assessed as an extraction agent (firstly alone and then in combination with green solvents) on different PHA-enriched biomasses. Ethyl acetate was used to perform green solvent extraction on UNIROMA PHA-enriched biomass and NID PHA-enriched biomass. High values of PHA purity of the recovered biopolymer (over 80 %, wt/wt) were obtained using Ethyl Acetate as green solvent. The recovery of extracellular polymeric substances (i.e., proteins and carbohydrates) from the residual biomass collected during PHA recovery from the continuous-flow process was also assessed, to maximize the overall recovery yield.

- CSIC pioneered an innovative biological extraction method using the predatory bacterium *Bdellovibrio bacteriovorus* HD100. The bacterium effectively lysed PHA-producing cells without solvents, enabling polymer release while avoiding chemical degradation. Three novel depolymerases (Bd0282, Bd0873, Bd2637) were identified and expressed in *E. coli*, confirming their activity against amorphous PHB and enabling the engineering of predator strains optimized for polymer recovery.

- ENTO shows that biological digestion by *Hermetia illucens* larvae may significantly simplify PHA recovery by breaking down the biomass matrix, enabling efficient polymer extraction and clean purification through a mild solvent-based process.

2. Introduction

Globally, one third of food produced is lost as waste before even reaching the consumer. Beyond the substantial economic cost of approximately 936 million USD annually, this also contributes significantly to environmental pressures, including greenhouse gas emissions, water depletion, and land-use impacts (European Commission, 2019).

The transition toward a circular and sustainable bioeconomy requires the development of innovative bioprocesses capable of transforming agro-industrial residues into high-value bioproducts. This deliverable presents the collective progress achieved within the project toward establishing integrated and sustainable valorization routes for such residues, focusing on the production of medium-chain carboxylic acids (MCCAs), microbial protein (MP), and polyhydroxyalkanoates (PHAs). These three product streams represent complementary and interlinked pathways that together exemplify a resource-efficient biorefinery approach—closing material loops, minimizing waste, and enhancing overall process sustainability.

The work reported herein demonstrates how distinct technological developments, implemented by the project partners, converge into a cohesive circular system where carbon-rich agro-waste streams are sequentially or synergistically converted into multiple value-added outputs. Process engineering and microbial biotechnology innovations were leveraged to overcome challenges associated with substrate variability, product selectivity, and downstream recovery.

The production of MCCAs (Task 3.1) was optimized using ethanol-rich residues such as wine lees, enabling selective chain elongation and high productivity through anaerobic bioconversion. The resulting MCCA-rich effluents not only represent marketable chemical intermediates but also serve as carbon sources for PHA synthesis, demonstrating efficient resource coupling. In parallel, microbial protein (Task 3.2) production was established through the enzymatic hydrolysis of starch-rich side streams from the potato-processing industry (Task 3.1), generating nutrient-rich hydrolysates suitable for microbial growth and protein accumulation, with applications in sustainable feed formulation.

Complementarily, PHA production (Task 3.3) was advanced through both single-strain and mixed microbial culture (MMC) processes, utilizing lipidic and carboxylic acid-rich fractions from tomato pomace, brewer's spent grain, and fermentation-derived effluents. These processes yielded high polymer contents and tunable copolymer compositions, confirming their flexibility toward diverse waste streams. Furthermore, innovative green extraction and biological recovery methods (Task 3.4) were developed—ranging from solvent-free enzymatic and microbial approaches to the use of ethyl acetate and alkaline–oxidative treatments—reinforcing the project's commitment to environmentally compatible biopolymer purification.

I. Precursor production

I.I Acidogenic fermentation (UGENT)

Aim

The main objective was to develop a highly selective medium-chain carboxylic acid (MCCA) production process from aqueous agri-waste streams, targeting productivities of 10–15 gCOD/L·d and selectivity above 75%.

Materials and methods

Experiments were conducted in an expanded granular sludge bed (EGSB) reactor, to decouple hydraulic retention time (HRT) from solids retention time (SRT), allowing operation at low HRTs without biomass washout. A 2.5 L glass EGSB reactor was operated under mesophilic conditions (37 °C) at a controlled pH of 6.8 ± 0.1. The system was inoculated with 50 mL of a mesophilic chain-elongating community available in-house. An internal recirculation rate of approximately 1.5 m/h was maintained. The reactor was fed with an ethanol-rich waste stream (wine lees) obtained from a winery in Spain. This substrate was used as an alternative to grey starch due to the issues encountered with its hydrolysis. The composition of the wine lees is detailed in Table 1. Ethanol present in the waste stream served as the electron donor, promoting efficient chain elongation and high carboxylic acid production. The feed consisted of fivefold diluted wine lees supplemented with 0.1 M acetate, corresponding to 6.4 gCOD L^{-1}. The influent was supplied from the bottom of the reactor without further pretreatment. The effect of HRT on productivity, selectivity, and granulation toward MCCA was evaluated at 4 d, 2 d, 1 d, and 12 h HRT.

TABLE 1: UNDILUTED WINE LEES CHARACTERIZATION (DESSÌ ET AL., 2024).

Parameter	Unit	Values
pH	-	3.56
Conductivity	mS/cm	2.09
Total solids (TS)	g/L	28.08 ± 2.72
Volatile solids (VS)	g/L	23.98 ± 2.11
COD	g/L	284.0 ± 5.2
sCOD	g/L	274.5 ± 3.3
sTOC	g/L	98.16 ± 1.03
Acetic acid	g/l	1.99 ± 0.10
Ethanol	g/l	105.70 ± 8.14
Cl^-	mg/L	24.78 ± 0.53
NO_2^-	mg/L	0.23 ± 0.01
NO_3^-	mg/L	0.09 ± 0.00
PO_4^{3-}	mg/L	3.29 ± 0.04
SO_4^{2-}	mg/L	193.12 ± 0.92
Ca^{2+}	mg/L	66.69 ± 0.83
K^+	mg/L	946.05 ± 13.73
Na^+	mg/L	20.97 ± 0.42
NH_4^+	mg/L	20.48 ± 0.23

Results and Discussion

Operation of the expanded granular sludge bed (EGSB) reactor under progressively shorter HRT demonstrated high MCCA productivities and selectivities. After the start-up phase and once the reactor performance had stabilized, the caproate concentration in the fermentation broth reached concentrations around 25-30 gCOD L^{-1} , with selectivities close to 80%. By decreasing the HRT the total concentration of caproate dropped to about 15 gCOD L^{-1}, with an increased accumulation of unconsumed ethanol in the fermentation broth. At the shortest HRT of 12h, the system reached a peak caproate production rate of 19.3 g L^{-1} d^{-1}, with total MCCA production reaching 46.5 gCOD L^{-1} d^{-1}. These values place the process among the highest reported for real substrates, outperforming processes fed with thin stillage (12.4 g caproate L^{-1} d^{-1}; Carvajal-Arroyo et al. (2019)) and liquor-making wastewater (14.7 g caproate L^{-1} d^{-1}; Wu et al. (2020)), and approaching the productivity reported by Grootscholten et al. (2014) for acidified OFMSW with external ethanol supply (30.3 g L^{-1} d^{-1}). The product profiles of individual carboxylic acids across the tested HRTs, combined with the residual ethanol concentration are shown in Figure 1A.

Selectivity toward caproate remained stable across all HRT conditions, averaging 76–78%, which is significantly higher than previous studies using wine lees (e.g., 36%, Kucek et al. (2016)). The selectivity profiles for individual carboxylic acids are presented in Figure 1B. Besides high selectivity, the system also maintained high substrate conversion efficiency, with caproate yields reaching 0.69 gCOD $gCOD^{-1}$.

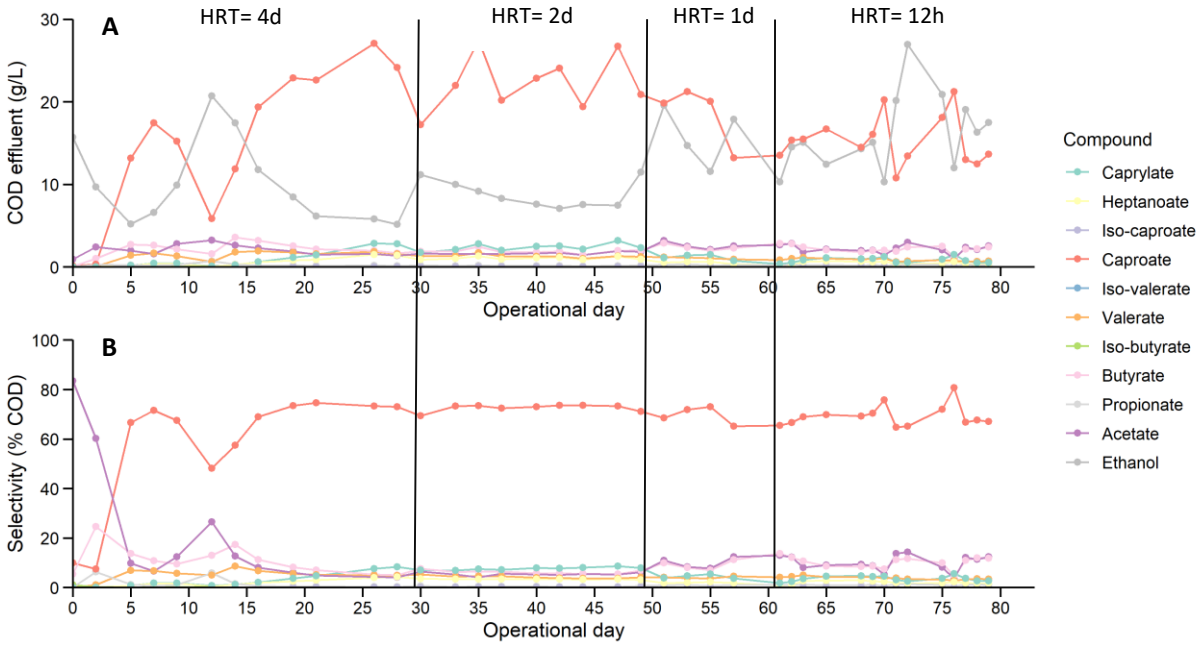


FIGURE 1: PERFORMANCE OF THE EGSB REACTOR UNDER VARYING HYDRAULIC RETENTION TIMES (HRTS): (A) CONCENTRATION PROFILES OF ETHANOL AND INDIVIDUAL CARBOXYLIC ACIDS (C2–C6) IN THE REACTOR EFFLUENT, EXPRESSED IN GCOD/L. (B) SELECTIVITY OF INDIVIDUAL CARBOXYLIC ACIDS, EXPRESSED AS PERCENTAGE OF TOTAL CARBOXYLIC ACID CONTENT BASED ON COD EQUIVALENTS.

Reactor effluent was collected during operation at an HRT of 12 and 24 hours and stored at 5 °C prior to shipment to NID and UNIROMA for downstream mcl-PHA production. The composition of the stored effluent prior to shipping is provided in Table 2.

TABLE 2 : CHARACTERIZATION OF EGSB REACTOR EFFLUENT COLLECTED AT AN HRT OF 12 AND 24 HOURS, PRIOR TO SHIPMENT FOR MCL-PHA PRODUCTION.

Parameter	Unit	Values
Total suspended solids (TSS)	g/L	0.7 ± 0.2
Volatile suspended solids (VSS)	g/L	0.6 ± 0.2
sCOD	gCOD/L	36.0 ± 1.0
Acetic acid	g/L	1.1 ± 0.10
Propionic acid	g/L	0.1 ± 0.1
Butyric acid	g/L	1.4 ± 0.0
Valeric acid	g/L	0.6 ± 0.0
Caproic acid	g/L	10.5 ±0.3
Octanoic acid	g/L	1.0 ±0.1
Ethanol	g/L	2.2 ± 0.0
Lactic acid	g/L	0.0 ± 0.0
NO_2^-	mg/L	0.0 ± 0.0
NO_3^-	mg/L	0.0 ± 0.0
PO_4^{3-}	mg/L	0.1 ± 0.0
NH_4^+	mg/L	0.0 ± 0.0

Conclusions

Using a mesophilic expanded granular sludge blanket (EGSB), this study demonstrated the conversion of ethanol-rich wine lees into a highly selective medium-chain carboxylic acids (MCCAs) stream. The reactor achieved MCCAs productivities of 46.5 $gCOD \cdot L^{-1} \cdot d^{-1}$, with caproate concentrations up to 42.6 $gCOD \cdot L^{-1} \cdot d^{-1}$ and overall caproate selectivity above 75%. These results indicate that wine lees can be effectively transformed into a caproate-rich effluent, providing a promising precursor for mcl-PHA synthesis

I.II. Enzymatic hydrolysis of grey starch for microbial protein production (AVECOM)

Introduction

Within the Agriloop project, the potential of using grey starch, a residual stream from the potato processing industry, as a feedstock for the production of single cell protein (SCP) is explored. The residual stream has a high content of starch, which consists of long chains of glucose molecules, entangled as linear and branched chains. Due to the complexity and large size of the molecule it cannot be transported into microbial cells directly. Thus, a pretreatment step, breaking down starch into fermentable sugars (mono- and disaccharides), is required to make it bioavailable for MP production. Initially, the potential of acidogenic fermentation to solubilize the grey starch was evaluated. However, the results of this were not sufficient for an efficient large-scale process, as only 33% of the initial volatile solids (VS) were solubilized (see D3.1). Enzymatic treatment was therefore explored as an alternative pretreatment step targeting a concentrated stream of sugars with at least 60 g_{COD}/L for a fed-batch MP production process.

Materials and methods

Grey starches were obtained between February and August 2025 from three local potato processing companies, later referred to as GS1 – 3. The enzyme cocktail was supplied by C-source Renewables.

Chemical oxygen demand (COD) and phosphor were measured using Hach Lange kits LCK014 and LCK350, respectively (Hach Lange, Germany), and read on a Hach-Lange DR 3900 spectrophotometer. The glucose concentration was determined by REFLECTOQUANT® Glucose Test Strips measured in a RQflex® 20 (Merck, Germany). Dry solids (DS) were determined gravimetrically by weighing a representative aliquot before and after drying overnight at 105°C. The volatile solids content was then found by subsequent incineration in a muffle furnace at 550°C for at least two hours (APHA, 1998). Nitrogen was determined via Kjeldahl analysis, as described in ISO 5663:1984.

Preliminary tests were conducted using 120 g of grey starch mixed with tap water at dilutions ranging from undiluted to a 1:4 ratio (grey starch:water) to determine the optimal loading rate. The mixtures were placed on a shaker at 100 rpm inside an incubator controlled at 50°C. Two types of heat treatment were also evaluated, autoclaving at 121°C for 20 minutes and incubation at 90°C for approximately 16 hours.

For the process development and optimization of the microbial protein process by UGent, the production of concentrated solubilized starch by Avecom was performed at 50°C in 5 and 10 L stirred bioreactors (Sartorius BIOSTAT Aplus and Eppendorf BioFlo 320). These reactors were equipped with a dual Rushton turbine stirrer and operated at an agitation rate of 100–200 rpm. The hydrolysis pH was set to 4.5 at the start using 5 M NaOH. Unless otherwise specified, all experiments were conducted with an enzyme loading rate of 1 µL/g wet weight.

Results

Characterisation of different grey starches

Grey starch is a solid substance characterized by a high moisture content and is rich in organic substances (Figures 2 and Table 3). It also contains nitrogen and a limited amount of phosphor. The sample of grey starch, originating from GS3, had the highest dry solids content, followed by the sample of grey starch originating from GS2. The grey starch sample from GS1 had the lowest dry solids content. The grey starch

samples originating from GS1 and GS2 were grey, while the grey starch sample from GS3 was white.

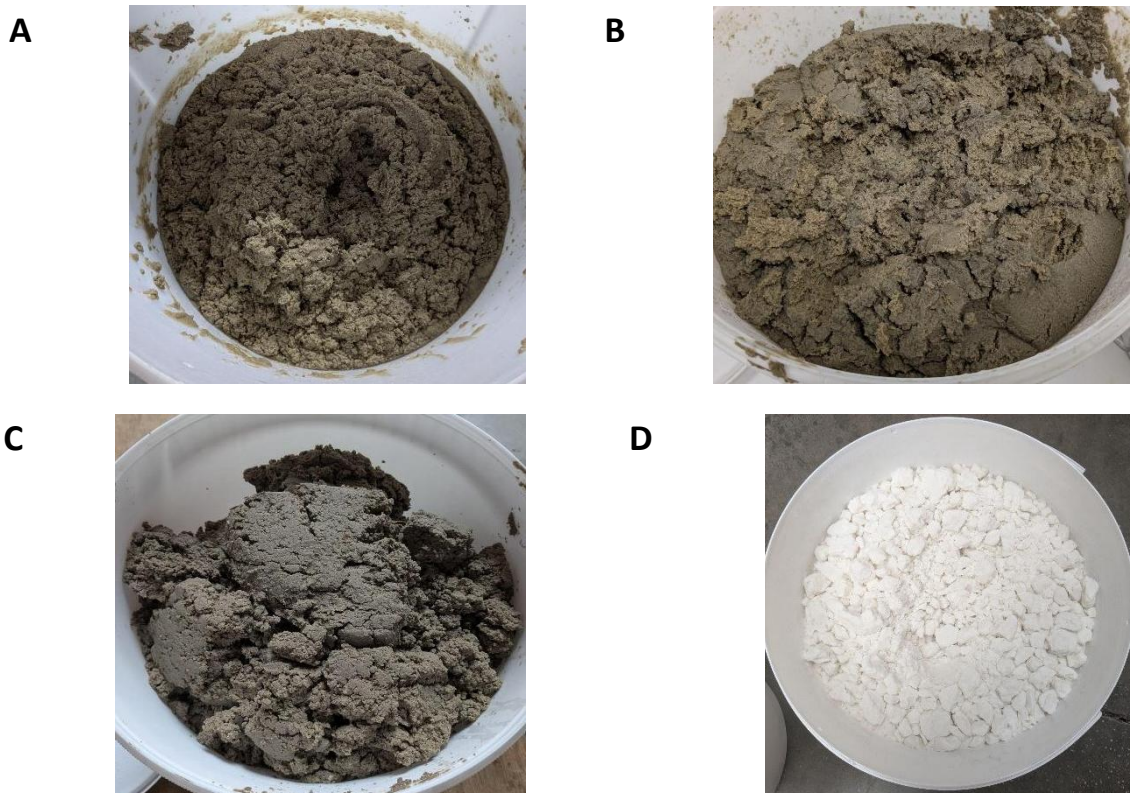


FIGURE 2: RAW GREY STARCH FROM GS1 (A AND B; LEFT: BATCH 19/02/2025, RIGHT: BATCH 28/04/2025), GS2 (C; BATCH 28/05/25) AND GS3 (D; BATCH 28/08/25).

TABLE 3 : CHARACTERISTICS OF GREY STARCH, CHEMICAL OXYGEN DEMAND (COD), KJELDAHL NITROGEN (KJ-N) AND PHOSPHORUS ARE EXPRESSED AS WEIGHT PER WEIGHT OF WET GREY STARCH (WW).

Parameter	Unit	GS1 batch 19/02/25	GS1 batch 28/04/25	GS2 Batch 28/05/25	GS3 Batch 06/06/25	GS3 Batch 26/06/25	GS3 Batch 27/08/25
Dry solids (DS)	%	11.0	12.5	32.1	62.0	55.5	57.6
Volatile solids (VS) /Dry solids	%	89.4	80.1	89.4	99.6	99.4	99.6
COD/WW	g/ gWW	0.267	0.177	0.410	0.601	0.792	0.656
	g/g DS	2.430	1.419	1.279	0.970	1.428	1.139
Kj-N/WW	mg/ gWW	3.891	4.714	4.715	0.867	0.754	0.36
P/WW	mg/ gWW	0.315	0.068	0.275	0.643	0.524	0.537
N/COD	%	1.45	2.66	1.15	0.14	0.10	0.05
P/COD	%	0.04	0.39	0.07	0.11	0.07	0.08
Mass density	kg/L	0.991	0.999	1.006	-	-	0.697

Optimisation of the process conditions

Different loading rates (Table 3), two heat treatments and an increased enzyme loading rate were evaluated to determine the optimal process conditions (Table 4). The % COD conversion and the COD concentration in the centrate of the undiluted grey starch was higher compared to the percentage of COD conversion of the negative control. The COD concentration in the centrate was higher in less diluted mixtures of grey starch The % COD conversion of enzyme-treated grey starch ranged between 12.5% and 16.6%. The %wt centrate remained constant. By the end of the experiment, between 67.0 and 84.1% of the TSS remained in the mixture of enzyme-treated grey starch.

TABLE 4 : EFFECT OF ENZYMES ON DIFFERENT GREY STARCH DILUTIONS.

Condition	Dry solids [%]*	Total COD [g/L]*	Time [h]	g_{COD}/ $L_{centrate}$	COD conversion [%]**	TSS [g/L]	wt% TSS remaining
Negative control: no enzymes	11.0	267	0	25.3	5.0	95.7	-
			73	31.7	6.4	90.4	94.5
Undiluted	11.0	267	0	26.5	5.3	89.9	-
			73	56.4	12.5	73.1	81.3
Dilution 3/4	8.3	201	0	19.8	6.3	67.2	-
			73	39.9	13.3	56.6	84.1
Dilution 1/2	5.5	134	0	12.7	7.3	40.1	-
			73	27.9	14.6	28.4	70.6
Dilution 2/5	4.4	107	0	9.1	6.7	35.7	-
			73	22.2	16.6	23.9	67.0
Dilution 1/4	2.8	67	0	6.1	7.8	20.5	-
			73	12.7	14.9	15.1	73.6

*Estimated based on analysis of raw grey starch. **$g_{COD}/L_{centrate}$ times $v\%_{centrate}$/total COD.

Since the previous experiments did not result in an efficient solubilization of grey starch,an additional heat treatment prior to the enzymatic hydrolysis and an increased enzyme loading rate was considered. Autoclaving and an overnight (O/N) heat treatment at 90°C were tested. This resulted in an increase of the COD conversion from 7.2% to 14.8% and from 7.5% to 14.6%, respectively. The addition of enzymes further increases the COD conversion from 14.8% to 20.7% (autoclaving) and from 14.6% to 20.6% (O/N at 90°C). When comparing the ⅖ dilution (Table 4) to this (Table 5), it can be concluded the COD conversion is increased from 16.6% to 20.6-20.7% and the COD concentration from 22.2 $g_{COD}/L_{centrate}$ to 28.8 - 29.0 $g_{COD}/L_{centrate}$ by using a heat treatment combined with a 10-fold higher enzyme dosage.

Table 5: Effect of a higher enzyme dosage combined with heat treatment on the COD solubilisation.

Condition	Dry solids [%]*	Total COD [g/L]*	Time [h]	g_{COD}/ $L_{centrate}$	COD conversion [%]**	TSS [g/L]	wt% TSS remaining
Dilution ⅗ autoclaving	4.4	107	Before heat treatment	9.3	7.2	34.2	-
			After heat treatment	20.1	14.8	26.6	77.9
			0	-	-	-	-
			65	29.0	20.7	21.9	64.0
Dilution ⅗ O/N at 90°C	4.4	107	Before heat treatment	9.7	7.5	34.3	-
			After heat treatment	19.8	14.6	27.2	79.4
			0	-	-	-	-
			65	28.8	20.6	23.2	67.6

*Estimated based on analysis of raw grey starch. **$g_{COD}/L_{centrate}$ times $v\%_{centrate}$/total COD.

Selection of the most suitable grey starch

Enzymatic hydrolysation of the different grey starch batches were compared (Table 6), the grey starch mixture of GS3 contained the highest higher dry solids content and total COD concentration, followed by the grey starch mixture from GS2. The grey starch mixture of GS1 was characterised by the lowest dry solids content. After enzyme and heat treatment, the COD concentration in the centrate was for both the GS1 and GS2 samples around 34 to 36 g_{COD}/L. Drying and grinding the grey starch mixture from GS2 prior to enzyme treatment, increased the COD concentration to 48.0 g_{COD}/L. Enzymatic treatment of the grey starch mixture from GS3 resulted in the highest COD concentration with 134.2 g_{COD}/L.

The COD conversion was lower for GS2 samples compared to the GS1 sample. The COD concentration and COD conversion of heat-treated and non-heat treated GS2 samples were similar. It was noted that it was not possible to obtain supernatant of the GS2 sample after heat treatment. Drying and grinding the GS2 sample prior to enzyme treatment resulted in a higher COD conversion. The COD conversion of the grey starch mixture from GS3 treated by enzymes was 35.2%, which was the highest conversion without prior pretreatment.

TABLE 6 : EFFECT OF ENZYMES ON DIFFERENT BATCHES OF GREY STARCH.

Condition	Dry solids [%]*	Total COD [g/L]*	Time [h]	g_{COD}/ $L_{centrate}$	COD conversion [%]**	TSS [g/L]	wt% TSS remaining
GS1 28/04/25 2 h @ 90 °C	5.0	70.9	Before heat	10.1	-	49.9	-
			After heat	23.4	22.7	-	-
			0	19.1	19.0	-	-
			67	33.7	38.0	22.2	44.5
GS2 28/05/25 2h 25 min @ 90 °C	12.8	163.9	Before heat	7.3	3.3	110.9	-
			After heat	-	0	131.8	118.9
			0	14.7	5	76.6	68.7
			66	35.5	12.6	62.5	56.3
GS2 28/05/25	12.8	163.9	0	-	-	110.1	-
			66	33.1	13.6	72.0	65.4
GS2 28/05/25 Dry @ 105 °C, ground	17.6	225.6	0	10.4	5.3	43.3	-
			47	48.0	20.9	28.2	65.2
GS3 06/06/25	24.8	240.6	0	3.8	0.8	266.2	-
			19	84.9	20.5	203.4	76.5
			91	134.2	35.2	155.4	54.4

*Estimated based on analysis of raw grey starch. **$g_{COD}/L_{centrate}$ times $v\%_{centrate}$/total COD.

Scale-up of enzymatic hydrolysis for lab scale MP production experiments

The enzymatic hydrolysis was scaled up by Avecom to provide sufficient feed stream for UGent to develop a Fed-batch process for MP production on grey starch hydrolysate (Table 7). The conditions were based on the preliminary tests, using the GS3 grey starch with a grey starch loading rate of 40% (2/5 dilution). In total, 8 batches were performed at 5 L and 10 L scale (Figure 3), resulting in 40.6 L concentrated hydrolysate with an average COD concentration of 122.5 g/L of which 90% was in the form of Glucose. The average COD solubilization was 45%, which was already an improvement compared to the 35.2% obtained during the preliminary tests. Mixing was observed to play a crucial role during the hydrolysation, which might explain the improvement compared to the preliminary tests. Since grey starch does not dissolve in water, the starch is prone to settle and accumulate in zones with poor mixing. This reduces the overall enzymatic reaction

rate due to mass transfer limitations. A stirred-tank reactor equipped with a dual Rushton turbine impeller operating at 200 rpm proved effective at preventing settling. This configuration represented a significant increase in mixing power compared to the orbital shaker set to 100 rpm, which was used during the preliminary tests. Moreover, it was noticed that the pH dropped during the hydrolysation, which can be explained by the freed aldehyde groups when glucose is released from the starch chain. The aldehyde groups are likely to be oxidized to carboxylic acid groups under the given conditions (high temperature and low pH), which results in a pH drop. This can reduce the enzyme activity. Thus, manual pH control was applied during the last two batches. Controlling the pH proved to be essential and resulted in the highest COD solubilization of up to 78.1%.

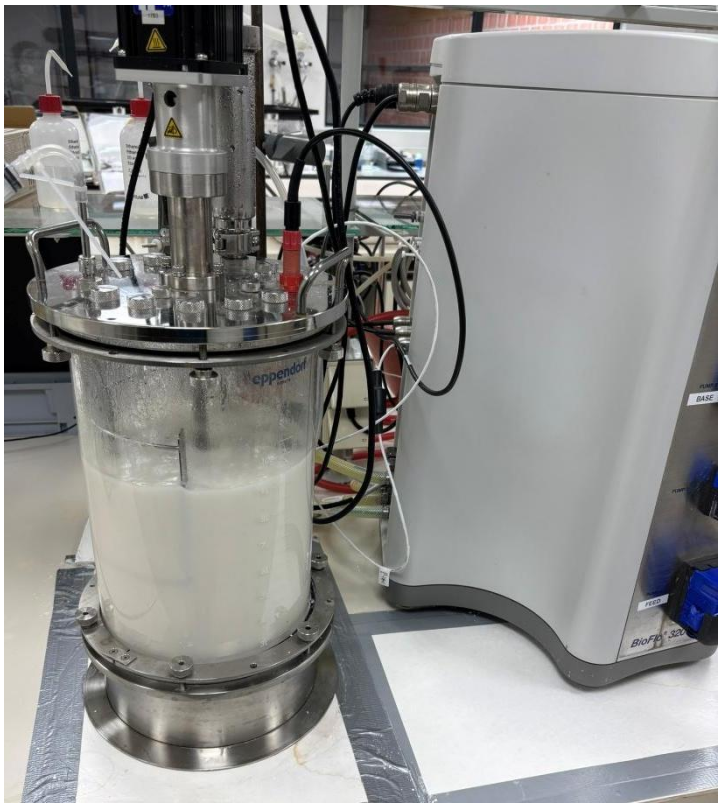


FIGURE 3: ENZYMATIC HYDROLYSIS OF GREY STARCH IN A 10 L STIRRED TANK REACTOR.

TABLE 7 : PROCESS OUTCOMES OF THE SCALED-UP ENZYMATIC HYDROLYSATION.

Date	Vessel size [L]	Duration [h]	Harvest [L]	COD soluble start [g/L]	COD soluble end [g/L]	Glucose end [g/L]	COD solubilization [%]
27/06/25	10	71	3.95	5.9	119	n.d.	41.6
27/08/25	10	42	6	5.7	118	n.d.	41.3
27/08/25	5	42	2.75	5.2	126	n.d.	46.7
29/08/25	10	67	6	6.6	44.2	17	15.8
29/08/25	5	67	2.4	6.7	50.3	39	17.2

Date	Vessel size [L]	Duration [h]	Harvest [L]	COD soluble start [g/L]	COD soluble end [g/L]	Glucose end [g/L]	COD solubilization [%]
01/09/25	10	72	5.1	n.d.	37.6	43	n.d.
01/09/25 *	5	48	3.18	n.d.	211	270	74.6
10/09/25 *	10	48	6.7	n.d.	246	185	78.1
Total (T) / Average (A)		57.1 (A)	36.1 (T)	6.02 (A)	122.5 (A)	110.8 (A)	45.0 (A)

*pH was adjusted during the hydrolysation using 5 M NaOH.

Conclusions

Enzymatic hydrolysis of grey starch was evaluated as an alternative to acidogenic fermentation to release fermentable sugars for the production of microbial proteins. During preliminary experiments, the GS3 grey starch was most suitable and an optimal grey starch loading rate of 40% was determined. Heat treatment and an increased enzyme loading rate, showed minor improvements on the COD solubilization (increase from 15 to 21%). However, the benefits of this additional step were considered too low to justify the increased effort and resources required. Initial trials were not satisfactory which resulted in several meetings with C-Source Renewables (provider of the enzymes) to resolve the occurring problems.

Nonetheless, the enzymatic hydrolysis was scaled up to provide 40.6 L of concentrated hydrolysate with an average COD concentration of 122.5 g/L to UGent to develop a MP production process. Sufficient mixing and pH control were crucial for a good solubilization. The last batches indicated that the process can still be further improved, especially through automated pH control. Hence, a COD conversion of more than 80% resulting in feed streams with more than 250 g_{COD}/L should be achievable and targeted in the following pilot-scale process.

II. Microbial protein production (UGENT)

Agri-residues such as grey starch (GS), a byproduct of potato processing, are often used as low-grade animal feed. To enhance their value and convert them into high-value bioproducts, pretreatment is often required to generate more bioavailable compounds, such as carboxylic acids or glucose. Task 3.2 of the Agriloop project focuses on utilizing intermediates from T3.1 and from a hydrolytic pretreatment process done by AVECOM. UGENT employed the fermented and hydrolyzed grey starch as a feedstock for microbial protein (MP) production. The organism of choice is *Cupriavidus necator* and, while it is well studied and characterized for polyhydroxyalkanoate (PHA) synthesis, limited data on its protein production capabilities has left a gap in understanding the key parameters necessary to achieve high protein yields.

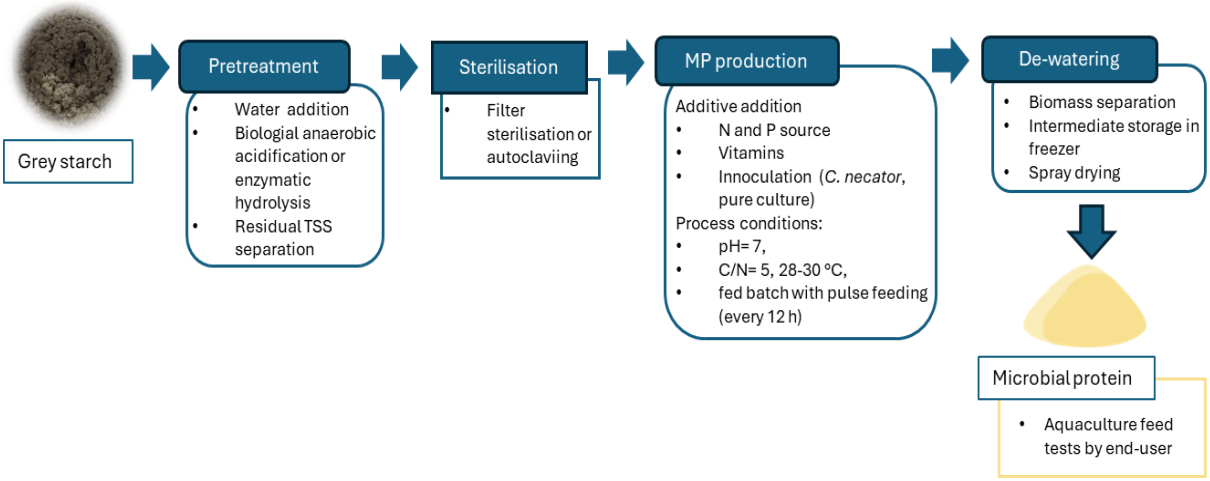


FIGURE 4: CRITICAL STEPS INVOLVED IN MP PRODUCTION

Fermented grey starch

Fermented grey starch was available in limited amounts and therefore we also used synthetic mixture of organic acids mimicking its composition. In series of batch tests, we have determined optimal conditions for protein production by *C. necator* LMG 1199 such as neutral pH or carbon to nitrogen (C/N) ratio 5-10. Results from the batch experiment using fermented grey starch demonstrated its ability to support a biomass concentration of 1.98 gCDW/L with a protein content of 37%. This outcome was attributed to the low concentration of nutrients in the fermented GS effluent, as the total concentration of organic acids reached 6.6 g/L. In addition, tests conducted with synthetic medium yielded an average biomass concentration of 2.07 ± 0.04 gCDW/L and a protein content of 30%.

Hydrolyzed grey starch

Since the strain *C. necator* H16, originally used in experiments with fermented GS, was not able to grow on glucose, the strain *C. necator* H1 G+3 (DSM 545) was purchased. Well-plate tests confirmed that *C. necator* H1 G+3 was able to grow in the synthetic medium supplemented with glucose and in follow up tests on the hydrolyzed grey starch as well. Fed batch reactor tests with *C. necator* H1 G+3 (DSM 545) resulted in average biomass concentration of 9.6±2.5 gCDW/L with protein content of 50-73% and average yield 0.38±0.02 gCDW/gCOD (roughly 0.52±0.02 g CDW-COD/gCOD). This confirms the viability of valorization of grey starch as substrate for protein production.

Characterization of microbial protein

Data from previous tests with *Cupriavidus necator*, courtesy of Dr Sakarika (Kerckhof et al., 2021), were utilized to inform our expectations on protein characteristics. While the exact concentrations of amino acids may vary depending on the substrate used as reported by Lee et al., (2006) and Sakarika et al., (2020), we anticipate that the amino acid composition will be similar to that observed in prior analyses. This assumption is based on the consistent performance of *Cupriavidus necator* in similar conditions and substrates. *C. necator* protein content on formate was 52 %CDW, while it was 46 %CDW on acetate (Sakarika et al., 2020).

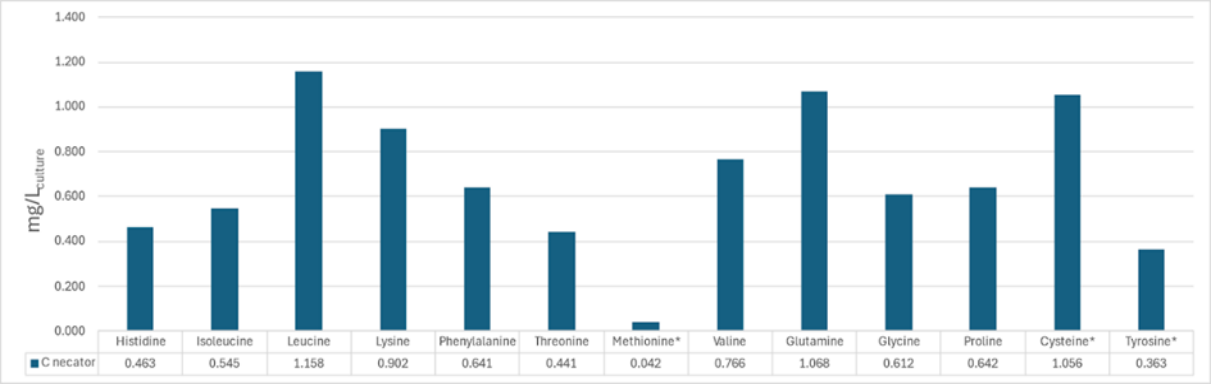


FIGURE 5: AMINO ACID COMPOSITION (DSMZ MEDIUM 81; 46% H2, 12% CO2, 30% O2, AND 12% CH4 IN HEADSPACE) (ADAPTED FROM (KERCKHOF ET AL., 2021)). *PARTIALLY DESTROYED DURING ACID HYDROLYSIS

Conditions for the MP production

Process conditions are based on work done in lab-scale and information requested by scale-up partner AVECOM. *C. necator* H1 G+3 (DSM 545) inoculum for reactor was cultivated in modified AMS medium (Table 8) with 3.5 g/L of acetate as carbon source and following conditions: 30 °C, pH= 7, medium volume = 100 mL, final OD 600= 1. Microbial protein was produced under sterile fed-batch conditions in a 2L bioreactor. Optimal process parameters and preparation of feedstock (hydrolyzed GS) are in Table 9.

TABLE 8: COMPOSITION OF MODIFIED AMMONIUM MINERAL SALTS (AMS) MEDIUM FOR THE CULTIVATION OF *CUPRIAVIDUS NECATOR*

Compound	**Value**	**Unit**
MgSO4.7H2O	1	g/L
NH4Cl	0.5	g/L
CaCl2.2H2O	0.15	g/L
FeNaEDTA	5	mg/L
Buffer		
Na2HPO4.12H2O	0.717	g/L
KH2PO4	0.272	g/L
Trace elements		
Na2EDTA × 2 H2O*	*0.5*	mg/L
FeSO4 × 7 H2O	*0.2*	mg/L
H3BO3	*0.03*	mg/L
CoCl2 × 6 H2O	*0.02*	mg/L
ZnSO4 × 7 H2O	*0.01*	mg/L
MnCl2 × 4 H2O	*0.003*	mg/L
Na2MoO4 × 2 H2O	*0.003*	mg/L
NiCl2 × 6 H2O	*0.002*	mg/L
CuSO4 × 5 H2O	*2.5*	mg/L
Vitamins		
Riboflavin	*0.5*	mg/L
Thiamine-HCl x 2 H2O	*2.5*	mg/L
Nicotinic acid	*2.5*	mg/L
Pyridoxine-HCl	*2.5*	mg/L
Ca-pantothenate	*2.5*	mg/L
Biotin	*0.005*	mg/L
Folic acid	*0.01*	mg/L
Vitamin B12	*0.05*	mg/L

Table 9: Process conditions for MP production based on work in 2L lab-scale bioreactor

Preparation of the influent of MP fermentor	Value	Notes
Extra additions, dilutions, pH adjustments of nutrients needed or sterilization needed		
Sterilization	Autoclaved (121 °C, 40 min)	N and P source autoclaved separately and added later in sterile manner
Volume (l) or weight treated grey starch (from Task 3.1)	0.9-1 L	Hydrolyzed grey starch
Water (kg or l)	-	
Phosphorus (Na2HPO4*2H2O) (g)	17	
Nitrogen (NH4Cl) (g)	40	
2N HCl (ml)	10	
2N NaOH (ml)	60-90	
pH	6.5-7	
Process conditions		
Procedure	sterile fed batch	
Working volume	1.3-1.6 L	
Temperature (°C)	28	
pH	7	
DO control	HAMILTON VisiFerm DO ECS	DO measured as % saturation
Stirring speed (rpm)	400 base, up to 800 rpm	Controlled by dO sensor
Inoculant	100 ml pure culture C. necator	OD600= 1; cultivated on AMS medium with C source (3.5/ g/L acetate) or nutrient broth; 30 °C, pH= 7
Estimation Q acid (ml/h)	-	
Estimation Q base (ml/h)	1-1.4	
HRT	-	
Describe how you feed	Pulse feeding every 12H	100 ml 1st day, 200 ml from 2nd day
Concentration COD influent (g/l)	100-108	
Volumentric loading rate (g COD/ (l reactor * day)	14-18	

Conclusions

As part of Task 3.2, UGent evaluated grey starch (GS) as a substrate for microbial protein (MP) production. Two pretreatment methods were applied to GS, resulting in fermented GS and hydrolysed GS.

A series of batch and fed-batch experiments were conducted using *Cupriavidus necator* to assess MP production. Based on the results, we identified the optimal pretreatment method and established key parameters for MP synthesis.

These optimizations led to an average biomass concentration of 9.6 ± 2.5 gCDW/L, with a protein content ranging from 50% to 73%.

The findings confirm the feasibility of using grey starch as a substrate for protein production and support its potential application in future feed trials.

III. Microbial Polyhydroxyalkanoates Production

III.I PHA PRODUCTION USING BOTH SINGLE AND MIXED MICROBIAL CULTURES (NID)

III.I.I. Valorisation of lipidic fraction of tomato Pomace and Brewer´s Spent grain waste by single cultures

Agro-industrial residues such as tomato pomace (TP) and brewers' spent grain (BSG) are abundant and low-cost feedstocks that can be valorized through microbial conversion into polyhydroxyalkanoates (PHAs). TP, a byproduct of tomato processing consisting mainly of peels, seeds, and residual pulp, is produced globally in large quantities, estimated between 5.4 and 9.0 million tons per year (Chabi et al., 2024). Its composition, rich in lipids, fibers, and nutrients, makes it a promising substrate for biotechnological processes. Several studies have demonstrated the conversion of tomato residues and tomato-processing effluents into PHA by mixed microbial cultures (Rodrigues et al., 2024).

Similarly, Brewer´s Spent Grain (BSG), the main solid residue generated during brewing, is produced at approximately 20 kg per 100 L of beer and contains cellulose, hemicellulose, proteins, and residual lipids (Mussatto et al., 2014). Recent studies have highlighted the potential of BSG as a substrate for microbial PHA synthesis (Carvalheira et al., 2022; Terfa et al., 2024). Since the carbon source can account for up to 30–50% of total production costs, the use of such residues represents a crucial step toward the economic viability of biopolymer production (Zytner et al., 2023; de Melo et al., 2023). Although the direct use of extracted oils from TP and BSG as PHA substrates is still underexplored, the well-documented use of waste frying or cooking oils as feedstocks supports the feasibility of valorizing the lipid fractions of these residues for sustainable PHA production (Srendran et al., 2020). Therefore, in Task 3.3 NID investigates the use of both oils as feedstock by different bacterial strains to produce both scl-PHA (short chain length -PHA) and mcl-PHA (medium chain length-PHA).

Material and Methods

Tomato Pomace and Brewer´s Spent Grain Oil extraction and characterization

Tomato Pomace (TP) was supplied by TomaPaint S.r.l. (Italy) and stored at -20 ºC until further use, while Brewer´s spent grain (BSG) was supplied by UGENT from a local Belgium brewery. TP was freeze-dried to remove moisture, then both TP and BSG were ground in a mill. The oil from both wastes was extracted by Soxhlet extraction using a ratio of 10 g of TP to 250 mL *n*-hexane/ ethyl acetate, at 70-80 ºC, for 12 h. *n*-hexane was evaporated using a Rotavapor.

The oils fatty acid composition was determined through transesterification of the lipids to the corresponding fatty acid methyl esters (FAMEs). Briefly, TP oil samples (2-3 mg) were mixed with 1:1 mixture of chloroform (containing the internal standard methyl benzoate), and methanol:sulfuric acid (80:20, v/v), and heated at 100 ºC for 4 h. The samples were analyzed by gas chromatography (GC) (TRACE 1300, ThermoScientific, USA) with a flame ionization detector (FID) and a Restek column (Crossbond, Stabilwax, USA). The calibration curve was created using known standards (mixture of FAMEs composed of C14-C22 (Sigma-Aldrich)), between 0.1 and 1 g/L (Rebocho et al., 2025).

PHA PRODUCTION

To evaluate the ability of TP and BSG oils as feedstock for bacterial growth and PHA production different strains were tested. *Burkholderia thailandensis* E264, *Cupriavidus necator* DSM 428, *Pseudomonas chlororaphis* subsp. *aurantiaca* DSM 19603, *Pseudomonas citronellolis* NRRL B-2504, *Pseudomonas oleovorans* NRRL B-14682, *Pseudomonas oleovorans* NRRL B-14683, *Pseudomonas oleovorans* NRRL B-3429, *Pseudomonas putida* NRRL B-14875, *Pseudomonas putida* NRRL B-14887, and *Pseudomonas resinovorans* NRRL B-2649 were used in this study. The inoculum used for the shake flask assays were prepared in Luria–Bertani (LB) broth (Bacto tryptone 10 g/L; yeast extract, 5 g/L; sodium chloride, 10 g/L). The cultivation assays were performed in mineral media (Medium E) supplemented with 10 g/L TP oil. The composition (per litre) of the Medium E* was as follows: $(NH_4)_2HPO_4$, 1.1 g; K_2HPO_4, 5.8 g; KH_2PO_4, 3.7 g; 10 mL of a 100 mM $MgSO_4$ solution; and 1 mL of a micronutrient solution (Brandl et al., 1988). The composition of the micronutrient solution (per litre of 1 N HCl) was as follows: $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich, St. Louis, MO, USA), 2.78 g; $MnCl_2 \cdot 4H_2O$, 1.98 g; $CoSO_4 \cdot 7H_2O$, 2.81 g; $CaCl_2 \cdot 2H_2O$, 1.67 g; $CuCl_2 \cdot 2H_2O$, 0.17 g; and $ZnSO_4 \cdot 7H_2O$, 0.29 g.

A screening was performed in 500 mL shake-flasks with a working volume of 200 mL. The ten strains were cultivated in the TP/ BSG oil supplemented medium for PHA production. Cultures grew for 72 h, at 30 °C and 200 rpm. Daily samples (3 mL) were collected for the quantification of the cellular dry weight (CDW), PHA content and composition in the biomass. At the end of the experiments, the cultivation broth was collected for residual oil quantification.

Further, the best strains were cultivated in a 3 L bioreactor (F1, Bionet, Spain) with a working volume of 2 L. Cultivations were carried out in Medium E (Brandl et al. 1988) supplemented with TP/ BSG oil (10 g/L) as the main carbon source and inoculated with 10 % (v/v) of inoculum prepared in LB medium. Temperature was controlled at 30 ºC and pH at 7.00 ± 0.02 by the automatic addition of 5 M NaOH and 5 M HCl. The aeration rate (1 vvm, volume of air per volume of reactor per minute) was kept constant during the experiments. The dissolved oxygen concentration was controlled at 30% saturation by the automatic variation of the stirring rate between 300 and 1000 rpm given by the two six-blade impellers.

PHA EXTRACTION and CHARACTERIZATION

The PHA was recovered from the dried biomass by Soxhlet extraction with chloroform at 80 ºC for 48 h. The polymer was then precipitated in ice-cold ethanol (1:10, v/v) under stirring, and dried at room temperature until constant weight, following the method described in Alfano et al. (2021). The extracted PHA was characterized in terms of its composition by GC using the same method for the fatty acid composition. Calibration curves for 3-hydroxyalkanoate methyl esters were established using two reference materials: the co-polymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV, 12 mol% 3HV, Sigma-Aldrich) and an in-house-produced medium-chain-length PHA (mcl-PHA) containing 3 mol% 3-hydroxyhexanoate (3HHx), 17 mol% 3-hydroxyoctanoate (3HO), 57 mol% 3-hydroxydecanoate (3HD), 11 mol% 3-hydroxydodecanoate (3HDd), and 12 mol% 3-hydroxytetradecanoate (3HTd), validated by GC-MS. Calibration was performed at concentrations ranging from 0.05 to 1 g/L for the most abundant monomers.

Results

TP and BSG OIL CHARACTERIZATION

TP and particularly the seeds are considered a utilizable source of oil given the significant amount of oil they contain. The TP used was determined to contain 11.5 ± 0.3 wt. % oil, which is within the range reported for TP (between 8.4 – 16.2 %). BSG contains 10.42 ± 1.30 wt. % oil when extracted with hexane and 11.64± 1.85 wt.% oil when extracted with ethyl acetate.

The fatty acid composition of the TP and BSG oil determined that both oils are mainly composed of linoleic acid and oleic acid, and palmitic acid with minor contents of stearic acid. In TP oil was also detected low contents of arachidic acid, myristic acid, and linolenic acid. TP oil presents a significant amount of unsaturated fatty acids, representing over 75 % of total lipid content. Given its fatty acid profile, TP oil is considered to belong to the linolenic-oleic acid group of oils, alongside cottonseed and grapeseed oil, presenting a high content in polyunsaturated fatty acids, especially in linoleic acid (up to 57%), followed by monounsaturated fatty acids, such as oleic acid (up to 30%), and saturated fatty acids (palmitic, stearic, myristic and arachidic acids) (Liadakis et al., 2022). Moreover, BSG oil also belongs to the linoleic–oleic acid group of oils, presenting a high content of polyunsaturated fatty acids (linoleic acid > 45 %), followed by monounsaturated fatty acids (oleic acid > 29 %), and smaller amounts of saturated fatty acids (Del Rio et al., 2013).

PHA PRODUCTION

TP oil - Screening tests

Considering the nature of the substrate, different PHA producing strains, known for their ability to utilize fatty acids-enriched substrates, were chosen to evaluate the potential of TP oil to be used for PHA production. The scl-PHA (short chain length-PHA) producers selected were *B. thailandensis* E264, *C. necator* DSM 428, and *P. oleovorans* NRRL B-14682, and the mcl-PHA (medium chain length-PHA) producers were *P. chlororaphis* subsp. *aurantiaca* DSM 19603, *P. citronellolis* NRRL B-2504, *P. oleovorans* NRRL B-14683, *P. oleovorans* NRRL B-3429, *P. putida* NRRL B-14875, *P. putida* NRRL B-14887, and *P. resinovorans* NRRL B-2649. PHA production of these strains was evaluated through batch cultivation experiments in shake flasks, over 72 h, using 10 g/L of TP oil as the sole carbon source. The results of the bacterial growth and PHA accumulation for each strain are reported in Table 10.
Among the strains tested, *B. thailandensis* showed the highest performance in terms of bacterial growth and PHA production. After 72 h, this strain achieved a CDW of 9.9 ± 0.44 g/L, with a polymer content in the biomass of 71.6 ± 1.91 wt. %, corresponding to a PHA production of 7.05 ± 0.38 g/L. These values are higher than those reported for non-oil based feedstocks, such as glucose, xylose, and oleic acid.

C. necator was the strain which achieved the highest polymer accumulation, corresponding to 72.6 ± 1.41 wt. % of the CDW. Still, the CDW and PHA production (5.1 ± 0.48 g/L and 3.71 ± 0.44 g/L, respectively) was significantly lower than observed for *B. thailandensis*.

TABLE 10: CELL DRY WEIGHT (CDW) AND PHA % OF DIFFERENT BACTERIAL STRAINS CULTIVATED ON TP OIL AND BSG OIL.

Bacterial Strain	Substrate	CDW (g/L)	PHA (%)
B. thailandensis E264	TP oil	9.9 ± 0.44	71.6 ± 1.9
	BSG oil	2.05	32.9±2.0

C. necator DSM 428	TP oil	5.1 ± 0.48	72.6 ± 1.4
	BSG oil	2.79	15.4±1.0
P. chlororaphis DSM 19603	TP oil	2.3 ± 0.04	6.4 ± 0.4
	BSG oil	3.29	8.6±0.1
P. citronellolis NRRL B-2504	TP oil	2.6 ± 0.01	1.9 ± 0.2
	BSG oil	0.97	8.0±0.1
P. oleovorans NRRL B-14682	TP oil	1.0 ± 0.09	0
P. oleovorans NRRL B-14683	TP oil	0.9 ± 0.01	0
P. oleovorans NRRL B-3429	TP oil	1.5 ± 0.02	0
P. putida NRRL B-14875	TP oil	1.7 ± 0.03	0
	BSG oil	1.86	6.0±0.1
P. putida NRRL B-14887	TP oil	0.7 ± 0.18	0
P. resinovorans NRRL B-2649	TP oil	6.3 ± 0.24	39.6 ± 2.8
	BSG oil	3.67	5.7±0.2

P. resinovorans reached a CDW of 6.3 ± 0.24 g/L, with a PHA content of 39.6 ± 2.76 wt. %. This corresponds to a PHA production 2.76 ± 0.25 g/L.

All the *Pseudomonas* strains tested, aside from *P. resinovorans* NRRL B-2649, showed very low values of CDW and practically did not produce PHA, although these strains have been reported to grow and accumulate PHA using lipid substrates as carbon sources (Cruz et al., 2016; Morais et al, 2014). This is likely due to the complex composition of TP oil, including various bioactive compounds, some of which, i.e. polyphenols, flavonoids, and lycopene, have been reported to possess antibacterial activity (Liadakis et al., 2022).

BSG oil - Screening tests

Considering the results achieved for TP oil, six bacterial strains were tested using BSG oil (Table 10). Among the strains tested, *Burkholderia thailandensis* exhibited the highest PHA accumulation, reaching 32.9 ± 2.0 wt.%, despite a moderate biomass concentration (2.05 g L^{-1}). *Cupriavidus necator* achieved a higher cell density (2.79 g L^{-1}) but accumulated a lower polymer fraction (15.4 ± 1.0 wt.%). In contrast, *Pseudomonas* strains generally produced higher biomass but low PHA contents. *P. chlororaphis* and *P. resinovorans* showed the highest CDW among *Pseudomonas* spp. (3.29 and 3.67 g L^{-1}, respectively), yet their PHA accumulation remained below 9 wt.%. *P. citronellolis* and *P. putida* yielded the lowest polymer levels (8.0 ± 0.1 and 6.0 ± 0.1 wt.%, respectively). These results suggest that *B. thailandensis* and *C. necator* are more efficient in converting the lipidic components of BSG oil into PHAs, whereas *Pseudomonas* strains primarily directed carbon toward cell growth rather than polymer synthesis

When comparing the results obtained using BSG oil and TP oil as feedstocks for PHA production, clear differences in microbial performance were observed. Both oils supported PHA synthesis by strains known to utilize lipid-rich substrates; however, PHA yields were considerably higher with TP oil, which may indicate that TP oil provides a more accessible and nutrient-rich carbon source for PHA accumulation. The

lower polymer yields observed for BSG oil likely result from its more complex composition, containing higher amounts of non-lipid components and recalcitrant residues, which may limit substrate assimilation.

Bioreactor Assays

PHA production on bioreactor were conducted using both TP oil and BSG oil as carbon sources and the bacterial strains that reach the highest PHA accumulation and cellular growth, namely *B. thailandensis* E264 and *C. necator* DSM 428 (Table 11). The assays were performed under controlled conditions of temperature, aeration, and pH.

When TP oil was used, *B. thailandensis* achieved the highest biomass concentration under batch conditions, reaching a cell dry weight (CDW) of 8.82 g/L when cultivated without pH control, with a PHB content of 44.7% (Table 11). Under controlled pH batch operation, the strain produced 7.32 g/L CDW and 45.5% PHB, suggesting that pH regulation did not significantly affect polymer accumulation but slightly influenced biomass formation. In contrast, during fed-batch cultivation with an initial oil concentration of 10 g/L, both biomass and PHB content decreased to 4.05 g/L and 38.7%, respectively, likely due to substrate or oxygen transfer limitations during extended operation. The PHA accumulation achieved was much lower in bioreactor than in the shake-flask assays, which could be related to the increased production of biosurfactants under bioreactor conditions. *B. thailandensis* strains are known to produce surface-active compounds that may interfere with oil availability and carbon flux toward PHA biosynthesis, partially explaining the reduced polymer content observed in controlled fermentations (Gil et al., 2022).

TABLE 11: CELLULAR GROWTH AND PHA ACCUMULATION BY *BURKHOLDERIA THAILANDENSIS* E264 AND *CUPRIAVIDUS NECATOR* DSM 428 DURING BIOREACTOR CULTIVATIONS USING TP OIL BSG.

Oil	Bacterial Strain	Operation mode	CDW (g/L)	% PHA
Tomato Oil	*B. thailandensis E264*	Batch	7.32	45.51
		Batch without pH control	8.82	44.7
		Fed-batch (10 g/L)	4.05	38.68
	C. necator DSM 428	Batch	6.49	79.31
BSG Oil	*C. necator* DSM 428	Batch	11.5	67.6

For the same substrate, *C. necator* displayed a distinct metabolic profile, accumulating a much higher intracellular PHB fraction (79.3%) while reaching 6.49 g/L CDW under batch conditions (Table 11). Despite producing less biomass than *B. thailandensis*, this strain demonstrated superior polymer storage efficiency, confirming its well-known capacity for PHB accumulation from lipid-rich substrates.

When BSG oil was used as feedstock, *C. necator* again exhibited robust growth, achieving the highest CDW of all assays (11.5 g/L) with a PHB content of 87.6%. These results suggest that the composition of BSG oil provided a more balanced carbon and nutrient supply for this strain, supporting both cell growth and PHA synthesis.

Overall, comparing both oils, TP oil supported higher PHB accumulation by *B. thailandensis*, while BSG oil favored biomass formation and polymer productivity by *C. necator*. The results highlight the importance of aligning the microbial strain with the substrate characteristics: *B. thailandensis* showed better adaptability to the fatty acid profile of TP oil, whereas *C. necator* effectively utilized the more complex BSG oil matrix. In both cases, the PHB content obtained in the bioreactor were markedly higher than those achieved in shake flask assays, confirming that controlled cultivation conditions significantly enhanced polymer accumulation efficiency.

PHA characterization

The PHA synthesized by the different bacterial strains was extracted and characterized. Both the scl-PHA producing strains, *B. thailandensis* and *C. necator*, were found to produce the homopolymer poly-3-hydroxybutyrate (PHB). On the assays with TP oil, *P. resinovorans* produced a mcl-PHA mainly composed by 3HD (50 ± 1.9 mol%) and 3HO (37 ± 0.9 mol%) monomers, with lower amounts of 3HDd (6 ± 0.6 mol%), 3HTd (4 ± 1.5 mol%), and 3HHx (2 ± 0.6 mol%). On the other hand, using BSG oil, all *Pseudomonas* strains produced mcl-PHAs with more diverse monomeric compositions, reflecting their ability to metabolize long-chain fatty acids via β-oxidation and de novo fatty acid synthesis. *P. citronellolis* accumulated a PHA consisting primarily of 3-hydroxyhexanoate (HHx, 3.4 ± 0.2%), 3-hydroxyoctanoate (HO, 12.7 ± 0.6%), 3-hydroxydecanoate (HD, 52.2 ± 0.6%), 3-hydroxydodecanoate (HDd, 18.9 ± 0.1%), and 3-hydroxytetradecanoate (HTd, 12.4 ± 0.0%). A similar pattern was observed for *P. chlororaphis*, *P. resinovorans*, and *P. putida*, although with varying monomer distributions. Among them, *P. putida* showed the highest incorporation of longer-chain monomers, namely HD (60.5 ± 0.3%), HDd (24.6 ± 0.1%), and HTd (16.2 ± 0.2%).

Conclusions

Both tomato pomace (TP) and brewer's spent grain (BSG) oils proved to be promising and sustainable feedstocks for the microbial production of polyhydroxyalkanoates (PHAs), contributing to the valorization of abundant agro-industrial residues within a circular bioeconomy framework. The oils were rich in linoleic and oleic acids, making them suitable carbon sources for PHA-producing microorganisms. Among the different bacterial strains tested, *Burkholderia thailandensis* E264 and *Cupriavidus necator* DSM 428 demonstrated the highest polymer yields, confirming their strong ability to metabolize lipid-rich substrates. Using TP oil, *B. thailandensis* achieved up to 71.6% PHB content in shake flasks, while *C. necator* accumulated up to 87.6% PHB with BSG oil under bioreactor conditions, reaching a biomass concentration of 11.5 g/L. These results highlight the distinct substrate–strain compatibilities: NID demonstrated the technical feasibility of converting the lipidic fractions of TP and BSG into high-value PHAs and underscores the importance of aligning substrate composition with microbial metabolism to optimize polymer yield, monomer profile, and process efficiency for future scale-up applications.

III.I.II. mcl-PHA production by Mixed Microbial Cultures (NID)

Introduction

Mixed microbial cultures (MMCs) fed with low-cost organic residues have emerged as a promising route for cost-effective and sustainable polyhydroxyalkanoate (PHA) production. Unlike pure cultures, MMCs can operate under non-sterile conditions and tolerate variable feedstock compositions, thereby significantly reducing operational and sterilization costs (Serafim et al., 2008; Valentino et al., 2019). Moreover, MMC systems can directly process complex organic substrates such as agro-industrial residues, food waste, or wastewater-derived carbon sources, aligning with the principles of circular bioeconomy and waste valorisation.

Traditionally, PHA production with MMCs is implemented in three stages: (i) an initial fermentation step in which complex organic residues are biologically converted into carboxylic acids (CAs), the direct precursors for PHA biosynthesis, (II) a selection reactor, where an aerobic microbial community is enriched in PHA-storing organisms through the application of an external selective pressure, and (iii) a separate accumulation reactor, where the selected aerobic biomass is exposed to nutrient-limiting and carbon-excess conditions to maximize intracellular PHA content (Dias et al., 2006; Bengtsson et al., 2008; Valentino et al., 2015). The cornerstone of this approach is the feast/famine (F/F) regime, in which alternating periods of substrate availability and scarcity promote the enrichment of microorganisms capable of storing carbon intracellularly as PHA during the feast phase, and mobilizing it during famine for growth and maintenance. This dynamic selection strategy has been shown to enrich robust microbial populations with high carbon storage capacity and adaptability to variable feedstock conditions (Johnson et al., 2009; Albuquerque et al., 2010).

Within the framework of the project, NID's work focused on the development and optimization of the bioproduction of medium-chain-length PHAs (mcl-PHAs) using MMCs. The overall goal was to establish an efficient and robust biological process capable of producing polymers with distinct 3HB:3HV:3HHx monomeric compositions, by exploring how different operating conditions influence polymer production performance and PHA composition and properties.

To achieve this goal, a stepwise experimental approach was followed:

Phase I – Process optimization using synthetic substrates:

In parallel with upstream partners activities of agro-industrial residues fermentation, the first phase of NID's activities focused on optimizing operating conditions for mcl-PHA production using synthetic CAs (60 % butyric acid, 20 % valeric acid and 20 % hexanoic acid at a concentration of 25 $gCOD.L^{-1}$). Two operational strategies were tested in a 2 L sequencing batch reactor (SBR) operated under a F/F regime:

- **SBR test I:** The organic loading rate (OLR) was gradually increased (2 -> 4 -> 6 $gCOD{\cdot}L^{-1}{\cdot}d^{-1}$) while maintaining a constant nutrient balance (fixed C:N ratio of 14), and the Sludge Retention Time (SRT) was set at 4 days and controlled through biomass removal at the end of the reaction phase. This strategy aimed to enhance biomass productivity and, consequently, overall PHA productivity.
- **SBR test II:** A progressive increase of both the OLR (4 -> 8 $gCOD{\cdot}L^{-1}{\cdot}d^{-1}$) and the C:N ratio (C:N = 14 -> C:N = 28) was tested, and the 4 days SRT was controlled through biomass removal at the end of the feast phase to evaluate the potential for achieving higher intracellular PHA content under these conditions.

For each operation, accumulation assays were conducted in a 1 L fed-batch reactor to assess overall PHA yield, polymer composition, and quality.

Phase II – Process validation using a model substrate of fermented agro-industrial residues (wine lees).

Building on the knowledge acquired from Phase I, the operational conditions that had demonstrated stable and satisfactory performance were applied to a validation test using a synthetic model feedstock simulating the fermented wine lees supplied by UGent **(SBR test III).** We report the preliminary results obtained for culture selection under model feedstock feeding, aiming subsequent validation with the real feedstock. This validation step allows process feasibility assessment under realistic conditions, evaluating the impact of feedstock complexity on reactor performance, microbial community stability, and the characteristics of the resulting polymer.

Moreover, additional accumulation assays were performed in a 2 L fed-batch reactor specifically to produce larger quantities of biomass containing intracellular PHA. These production-oriented runs generated sufficient material to be distributed to project partners (UNIROMA, CSIC, and ENTO) for the assessment of different extraction and purification methods, supporting the cross-validation of downstream processing strategies across the consortium.

Materials and Methods

Culture selection

Across all three SBR tests, the same 2 L enrichment SBR (Figure 6) was used and was always inoculated with activated sludge collected from the municipal wastewater treatment plant of Mutela (Almada, Portugal).

The SBR was operated under the F/F regime with the uncoupled ammonia feeding strategy to impose additional selective pressure on the microbial community and stimulate the enrichment of PHA-storing microorganisms. For all operations, each SBR cycle lasted 12 h and was divided as follows: 11h of reaction phase, 50 minutes of settling phase, 5 minutes of biomass purge and 5 minutes of supernatant withdrawal. During the first 5 min of the reaction phase, the carbon source was supplied together with the mineral solution (solution according to Serafim et al., 2008), whereas the nitrogen and phosphorus solution was added only after the carbon feast phase had ended.

At the end of the settling phase, half of the reactor volume was withdrawn and replaced with fresh feeding solution at the beginning of the subsequent cycle, resulting in a hydraulic retention time (HRT) of 1 day.

FIGURE 6. 2L-SBR REACTOR OPERATED BY NID FOR MMC ENRICHMENT IN MCL-PHA ACCUMULATING BACTERIA.

The reactor was operated at room temperature (20-25°C) and pH was automatically controlled below 8.5 with 1 M HCl dosing. Dissolved oxygen (DO) levels were maintained above 2 $mg.L^{-1}$ by adjusting the air flow rate. The transition from feast to famine was identified based on the DO profile: low DO indicated the presence of residual carbon due to biological oxidation, whereas a sharp rise in DO signalled substrate depletion and the onset of famine. Stirring was provided at 250 rpm with a stainless steel shaft with two impellers: a 6-bladed Rushton disc turbine and a 3-bladed pitched blade impeller (bbi-biotech GmbH, Berlin, Germany).

For each SBR test, specific adjustments were implemented during culture selection. For **SBR test I e II**, the feeding solution consisted of a synthetic mixture of CAs containing 60% butyric acid, 20% valeric acid, and 20% hexanoic acid (COD basis) (8.3 $g.L^{-1}$ butyric acid, 2.5 $g.L^{-1}$ valeric acid, 2.3 $g.L^{-1}$ hexanoic acid). In **SBR test I**, the OLR was progressively increased at 2, 4, and 6 $gCOD \cdot L^{-1} \cdot d^{-1}$, maintaining a fixed C/N/P ratio of 100/7/1. Biomass was purged at the end of the reaction phase (250 mL of mixed liquor per cycle), resulting in a SRT of approximately 4 days. This operational strategy aimed to enhance biomass productivity and, consequently, the overall PHA productivity of the two-stage process.

In **SBR test II,** the SBR was operated with increasing OLRs of 4 and 8 $gCOD.L^{-1} \cdot d^{-1}$, while the C/N ratio was simultaneously raised from 100/7 to 200/7. In this case, biomass was purged at the end of the carbon feast phase (250 mL of mixed liquor per cycle, maintaining an SRT of 4 days). This strategy aimed to assess the potential to increase intracellular PHA content during the feast phase, thereby possibly reducing the need for a dedicated accumulation step and minimizing carbon losses during the famine phase. Overall, this approach sought to maximize both process productivity and yield.

In **SBR test III,** the reactor was initiated using a synthetic model feedstock designed to simulate the composition of the fermentation effluent supplied by UGent (Table 7).

TABLE 12: COMPOSITION OF THE FERMENTED WINE LEES PROVIDED BY UGENT AND THE SYNTHETIC MODEL FEEDSTOCK USED BY NID TO ENRICH A MMC IN MCL-PHA ACCUMULATING BACTERIA. STANDARD DEVIATION IS UNDER BRACKETS, RESULTING FROM A DUPLICATE ANALYSIS BY HPLC. *DERIVED THEORETICALLY FROM HPLC MEASUREMENTS

	Fermented Whine Lees (sent by UGent)	***Synthetic model feedstock (used by NID)***
	g/L	
Acetic acid	1.5 (0.0)	1.5
Propionic acid	0.2 (0.0)	-
Isobutyric acid	0.1 (0.0)	-
Butyric acid	1.5 (0.0)	1.7
Valeric acid	0.6 (0.0)	0.7
Hexanoic acid	9.7 (0.2)	9.6
Heptanoic acid	0.3 (0.0)	0.3
Octanoic acid	0.7 (0.0)	0.7
Ethanol	2.6 (0.2)	2.6
TOTAL (g/L)	17.2 (0.4)	17.2
*TOTAL (g COD/L)**	35.3 (0.4)	35.3

From Table 12 it can be observed that the majority of the carbon source (COD basis) is hexanoic acid (60%), followed by ethanol (15%) and butyric acid (9%), with the remaining components accounting for less than (5%) each.

The OLR of the SBR was initially set to 2 $gCOD \cdot L^{-1} \cdot d^{-1}$ for the first 14 days of operation and subsequently increased to 4 $gCOD \cdot L^{-1} \cdot d^{-1}$. The C/N/P ratio was maintained at 100/7/1, and biomass purging was carried out at the end of the reaction phase, keeping an SRT of 4 days. Once stable reactor performance was achieved under these conditions, the carbon source was to be switched from the synthetic mixture to the real fermentation effluent to evaluate process stability and performance with real feedstock. The objective of this operation was to validate the feasibility of producing mcl-PHAs directly from a real waste-based feedstock, fermented into a CAs mixture enriched in hexanoic acid.

In general, the SBRs performance was assessed weekly by collecting samples throughout the reaction phase. Samples were analysed for TSS and VSS, CAs, N, P and PHA concentrations.

PHA accumulation assays

Fed-batch assays were conducted to assess the maximum PHA accumulation capacity of the cultures previously selected in each SBR test (for each condition tested). The experiments were carried out in a BioFlo®/CelliGen® 115 system (Eppendorf AG, Germany) equipped with a 1 L glass vessel, operated under controlled aeration (3.0 $L \cdot min^{-1}$) and agitation (250 rpm). Each assay was inoculated with 0.250 L of surplus biomass harvested from the selection SBR (purge timing depending on each SBR test). The pH was maintained at 7.5 by automatic addition of 1 M HCl. A pulse-feeding strategy was applied to maintain the same food-to-microorganism (F/M) ratio as in the selection reactor. The carbon source used in each accumulation assay was identical to that used in the corresponding SBR test. The assays continued until a marked slowdown in microbial activity was evident, as indicated by dissolved oxygen (DO) levels reaching 80% of saturation, which typically occurred after 5–6 h.

In addition, six dedicated accumulation assays were carried out to produce biomass and polymer samples for project partners to evaluate different extraction and purification techniques. These tests were conducted in a 2 L reactor (similar to the SBR) using 2 L of surplus biomass collected from the selection SBR, either at the end of the reaction phase (SBR test I, OLR stage 4 $gCOD \cdot L^{-1} \cdot d^{-1}$) or at the end of the carbon feast phase (SBR test II, OLR stage 8 $gCOD \cdot L^{-1} \cdot d^{-1}$). A pulse-wise feeding strategy was applied, consisting of 5–6 carbon feed pulses per assay. Five assays used the same synthetic CA mixture as the corresponding SBR operation (butyric, valeric and hexanoic acids at a ratio of 60/20/20 (% COD basis)), while one assay used a real fermented glucose stream feedstock (simulating grey-starch streams) provided by UGent. The fermentation effluent exhibited a low concentration of residual unconverted COD and consisted of butyric, valeric and hexanoic acids at a ratio of 15/20/65 (% COD basis), with a total concentration of 24.99 $gCOD \cdot L^{-1}$. Throughout all assays, samples were periodically collected to monitor CA consumption and PHA accumulation kinetics.

Analytical methods

Samples for the determination of TSS and VSS were processed in accordance with standard methods. Samples collected over the duration of the SBR cycles and accumulation assays were immediately centrifuged at 10000 rpm for 3 minutes. The resulting supernatant was filtered through 0.2 µm membrane filters (Whatman®, Cytiva, Marlborough, Massachusetts, USA) while the pellets were lyophilized. VFA concentrations in the filtered samples were quantified by high-performance liquid chromatography (HPLC) as described by (Oliveira et al., 2017). Soluble ammonia and phosphate concentrations were determined using a segmented continuous flow analyzer (Skalar San++ automated system, Skalar Analytical B.V, Breda, The Netherlands). Lyophilized pellets were used to quantify 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHx) by gas chromatography with flame ionization detector (Bruker 430-GC, Bruker, Massachusetts, USA) according to the method described by (Lanham et al., 2013). The

concentrations of monomers 3HB, 3HV and $3HH_x$ were calibrated through standard curves with 3-hydroxybutyric acid, 3-hydroxyvaleric acid, methyl 3-hydroxyhexanoate, and heptadecane (Sigma-Aldrich®, Sigma-Aldrich Co. LLC, St. Louis, Missouri, USA) as internal standard (concentration of approximately 1000 $mg.L^{-1}$), subjected to the same digestion and methylation conditions as the samples.

Calculations

The F/F ratio was calculated as the duration of the feast phase divided by the duration of the famine phase. CAs concentration ($g.L^{-1}$) was defined as sum of the different CAs concentrations measured at a given time. The PHA content in the biomass was expressed as a percentage of VSS (wt. basis) and represented to the sum of 3HB, 3HV and 3HHx. The active biomass concentration (X_A, $g.L^{-1}$) was calculated by subtracting PHA concentration ($g.L^{-1}$) from VSS concentration ($g.L^{-1}$).

Specific substrate consumption rate ($-q_S$, $Cmol\text{-}VFA.Cmol\text{-}X_A^{-1}.h^{-1}$) and specific PHA accumulation rate (q_{PHA}, $Cmol\text{-}PHA.Cmol\text{-}XA^{-1}.h^{-1}$) were calculated by dividing the slope of the linear regression of the total VFA and PHA amount over time, respectively, by the average X_A amount during the feast phase. The volumetric PHA productivity ($Prod_{PHA}$, $g\text{-}PHA.L^{-1}.d^{-1}$) was calculated by multiplying the concentration of PHA produced during the feast phase by the number of SBR cycles performed per day (i.e., 2 $cycles.d^{-1}$).

All concentrations (3HB, 3HV, 3HHx, CAs and X_A) were converted into COD in accordance with the following factors: 1.67 $gCOD.g3HB^{-1}$, 1.92 $gCOD.g3HV^{-1}$, 2.08 $gCOD.g3HHx^{-1}$, 1.07 $gCOD.g\text{-}HLac^{-1}$, 1.07 $g\text{-}COD.gHAce^{-1}$, 1.51 $gCOD.gHPro^{-1}$, 2.08 $gCOD.g\text{-}EtOH^{-1}$, 1.82 $gCOD.gHBut^{-1}$, 2.04 $gCOD.gHVal^{-1}$, 2,20 $gCOD.gHCap^{-1}$ and 1.41 $gCOD.gX_A^{-1}$ (X_A formula of $C_5H_7NO_2$). In alternative, all concentrations (3HB, 3HV, 3HHx, CAs and X_A) were converted into Cmol in accordance with the following factors: 0.046 $Cmol.g3HB^{-1}$, 0.050 $Cmol.g3HV^{-1}$, 0.048 $Cmol.g3HHx^{-1}$, 0.033 $Cmol.gHLac^{-1}$, 0.033 $Cmol.gHAce^{-1}$, 0.040 $Cmol.g\text{-}HPro^{-1}$, 0.043 $Cmol.gEtOH^{-1}$, 0.049 $Cmol.gHBut^{-1}$, 0.049 $Cmol.gHVal^{-1}$, 0.052 $Cmol.gHCap^{-1}$ and 0.044 $Cmol.gX_A^{-1}$.

Results

SBR test I – OLR increase at fixed C:N ratio

Starting with activated sludge from a WWTP, the MMC became enriched in PHA storing bacteria by imposing a Feast and Famine selection strategy, and carefully maintaining the F/F ratio below 0.2 along each operational condition, namely, OLR of 2, 4, and 6 $gCOD{\cdot}L^{-1}{\cdot}d^{-1}$ (designated as Periods 1, 2, and 3, respectively) (Figure 7).

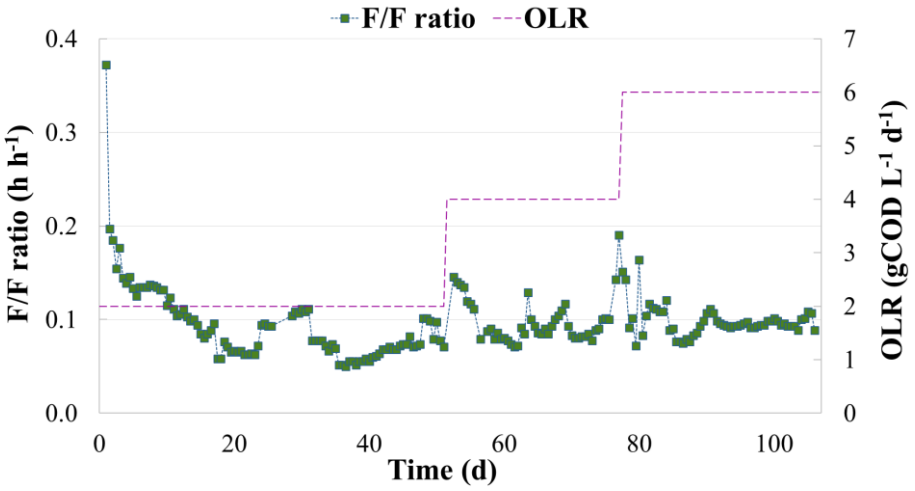


FIGURE 7: FEAST TO FAMINE RATIO AND OLR CHANGE OVER TIME IN SBR TEST I.

During the system start-up, the F/F ratio was 0.37, decreasing to below 0.2 after the first day of operation. Along the three operational Periods, the F/F ratio presented values below 0.1, despite the increase in the OLR parameter. This indicated the implementation in the SBR of a stable and strong selective pressure towards PHA accumulating bacteria, across all conditions.

As an example of the culture performance during an SBR cycle of Period 3, Figure 8 shows that the CA mixture was fed at the beginning of the feast phase in the absence of a nitrogen source. Under these conditions, the supplied carbon was mainly used for PHA synthesis and storage, with no significant biomass growth observed. After approximately two hours, nitrogen and phosphorus were added and were consumed together with the stored PHA, leading to active biomass growth. The supplied nitrogen was completely depleted by the end of the reaction phase. The selected MMCs showed a clear substrate preference, with butyrate and valerate being consumed first, while hexanoic acid was metabolized last (Figure 8B).

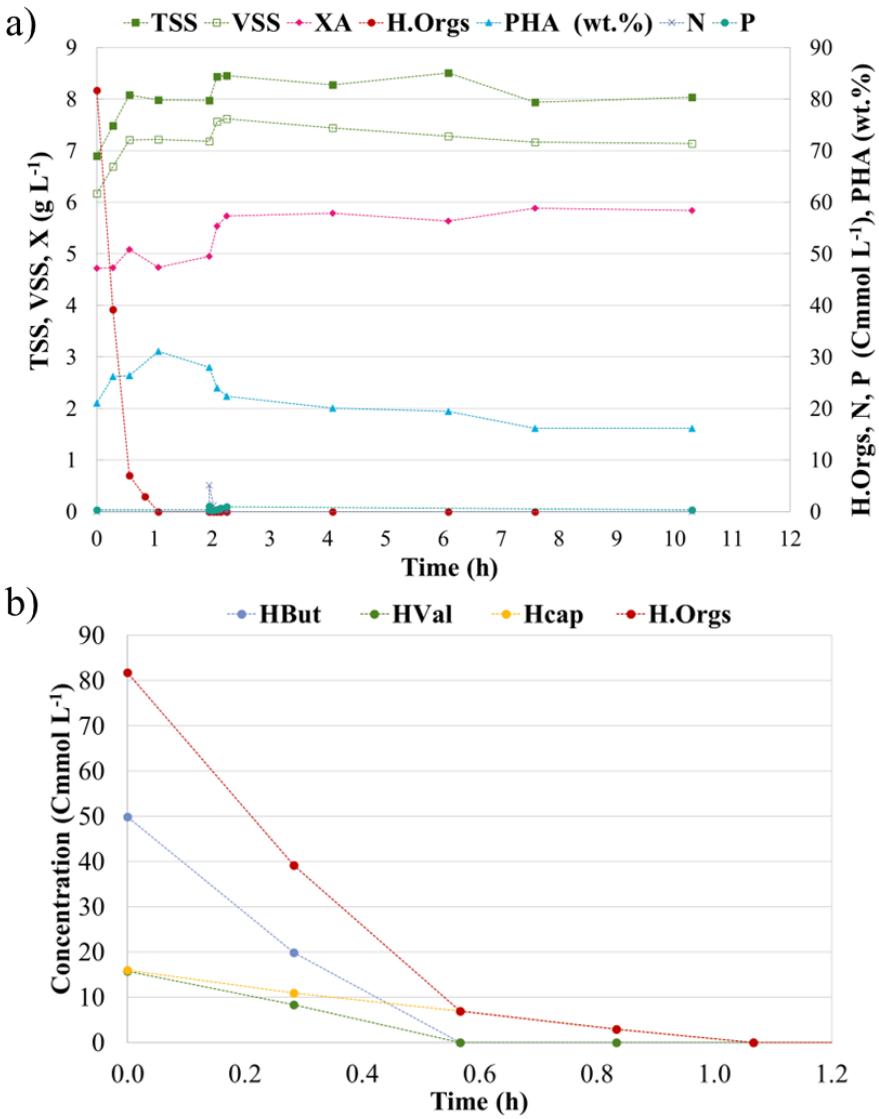


FIGURE 8: SBR TEST I CONCENTRATION PROFILES OF TSS, VSS, X_A, H. ORGS, PHA, N AND P (A) AND CAS CONSUMPTION DURING A TYPICAL CYCLE AT OLR OF 6GCOD.L^{-1}.D^{-1} (B)

Table 13 shows the mean values and standard deviation of the kinetic and stoichiometric parameters of the feast phase and famine phase calculated for all three experimental periods under steady-state conditions. Kinetic parameters were determined based on data collected from at least two complete monitored cycles of the enrichment reactor for each operating condition tested.

TABLE 13: KINETIC AND STOICHIOMETRIC PARAMETERS OF THE MMC PERFORMANCE DURING SBR TEST I OPERATION.

	Period 1	Period 2	Period 3
OLR [gCOD L^{-1} d^{-1}]	2	4	6
OLR [Cmmol CAs L^{-1} d^{-1}]	49	98	147
F/F [h h^{-1}]	0.055 ± 0.003	0.089 ± 0.003	0.085 ± 0.012
F/M [kgCOD $kgSST^{-1}$ d^{-1}]	0.696 ± 0,053	0.829 ± 0,008	0.866 ± 0,004

TSS [g L^{-1}]	3.054 ± 0.228	4.833 ± 0.341	7.881 ± 0.337
VSS [g L^{-1}]	2.745 ± 0.225	4.364 ± 0.322	7.088 ± 0.279
X_A [Cmmol]. average	159.929 ± 20.320	295.912 ± 36.999	459.679 ± 33.246
X_A [g]. average	3.618 ± 0.460	6.694 ± 0.837	20.799 ± 0.877
PHA [wt.%]	39.139 ± 6.119	31.688 ± 2.414	32.038 ± 0.905
ΔPHA feast [%. w/w]	18.057 ± 4.529	19.676 ± 1.841	13.848 ± 3.743
$-q_S$ [CmolS $CmolX_A^{-1}$ h^{-1}]	0.747 ± 0.003	0.440 ± 0.057	0.561 ± 0.038
qPHA [CmolPHA $CmolX_A^{-1}$ h^{-1}]	0.560 ± 0.021	0.449 ± 0.092	0.492 ± 0.080
F/F – feast to famine ratio; F/M – food to microorganism ratio			

Increasing the OLR while maintaining a constant SRT of 4 days resulted in a progressive rise in active biomass (X_A), from 1.8 $gX_A \cdot L^{-1}$ (Period 1) to 3.3 $gX_A \cdot L^{-1}$ (Period 2), and up to 5.0 $gX_A \cdot L^{-1}$ (Period 3) (Table 13).

During Period 1, the maximum specific substrate uptake rate (−qS) and PHA storage rate (qPHA) reached 0.747 ± 0.003 $CmolS \cdot CmolXA^{-1} \cdot h^{-1}$ and 0.560 ± 0.021 $CmolPHA \cdot CmolXA^{-1} \cdot h^{-1}$, respectively. These values decreased during Period 2 before rising again in Period 3, although not reaching Period 1 values (Table 13).

The maximum intracellular PHA content during the feast phase was highest in Period 1 (39.1 ± 6.1 wt.%), decreasing slightly in the following periods (31.7 ± 2.4 wt.% and 32.0 ± 0.9 wt.% for Periods 2 and 3, respectively).

Regarding the accumulation assays performed with the MMCs selected in each period, these were carried out in duplicate to evaluate the maximum PHA accumulation capacity in the accumulation reactor, thereby assessing culture performance and potential changes in the composition and characteristics of the PHAs produced.

As an example from Period 3, the evolution of PHA content (wt %), TSS and VSS concentrations, and CA along a Fed-Batch accumulation assay is shown in Figure 9A, while the variation of CA concentrations is presented in Figure 9B.

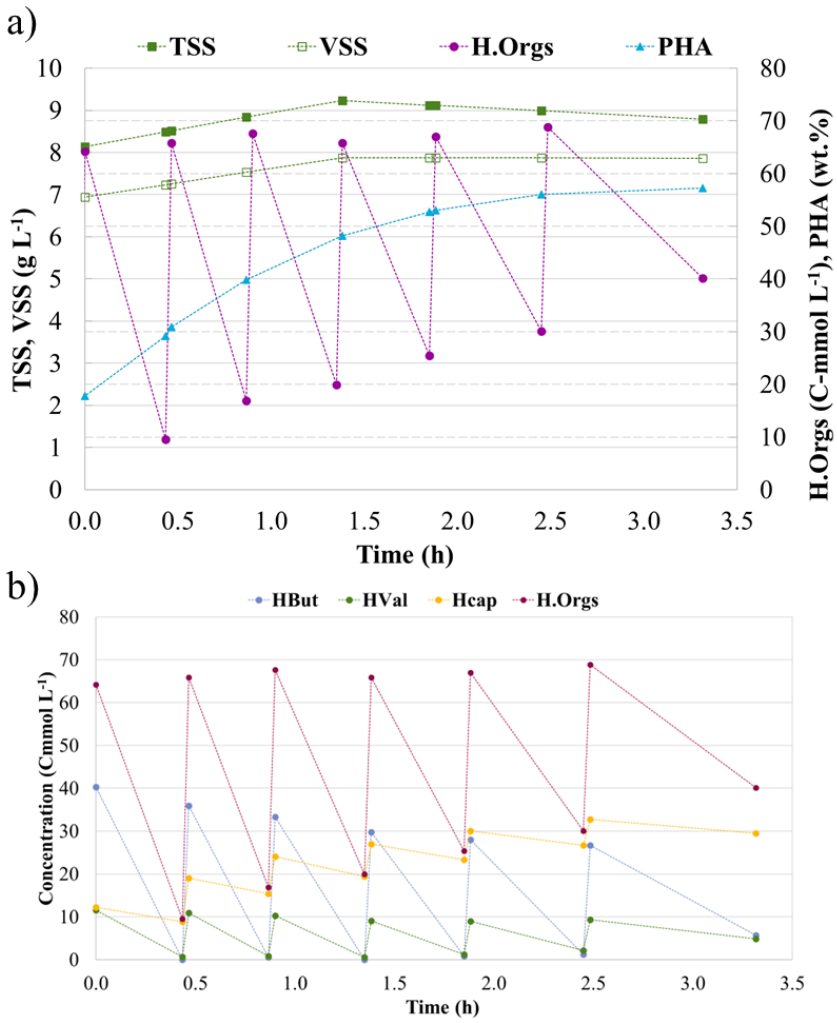


FIGURE 9: EXAMPLE OF THE PHA, TSS, VSS, H.ORG TRENDS (A) AND AMOUNT OF CAS OVER TIME (B) DURING FED-BATCH ACCUMULATION ASSAYS PERFORMED DURING PERIOD 3 OF SBR TEST I. THE MAXIMUM VALUES OF CAS CORRESPOND TO THE ADDITION OF FEED PULSES.

As observed, the PHA content increased progressively until reaching a plateau, indicating that the maximum storage capacity had been achieved. TSS and VSS concentrations remained nearly constant, as bacterial growth was limited by the absence of nitrogen and phosphorus. Similarly to what was observed in the enrichment reactor, the MMCs preferentially consumed butyric acid, while hexanoic acid tended to accumulate during the assay (Figure 9B).

The maximum PHA content (PHAmax) obtained in the batch accumulation assays followed a similar trend, with values of 65.57 ± 4.36 wt.% in period 1, 52.12 ± 1.45 wt.% in period 2, and 58.08 ± 0.83 wt.% in period 3 (Figure 10). These results indicate that, despite the different OLRs applied, the MMCs selected in the three experimental periods exhibited similar specific capacities for PHA accumulation at the cellular level, with only minor variations in overall PHA storage efficiency.

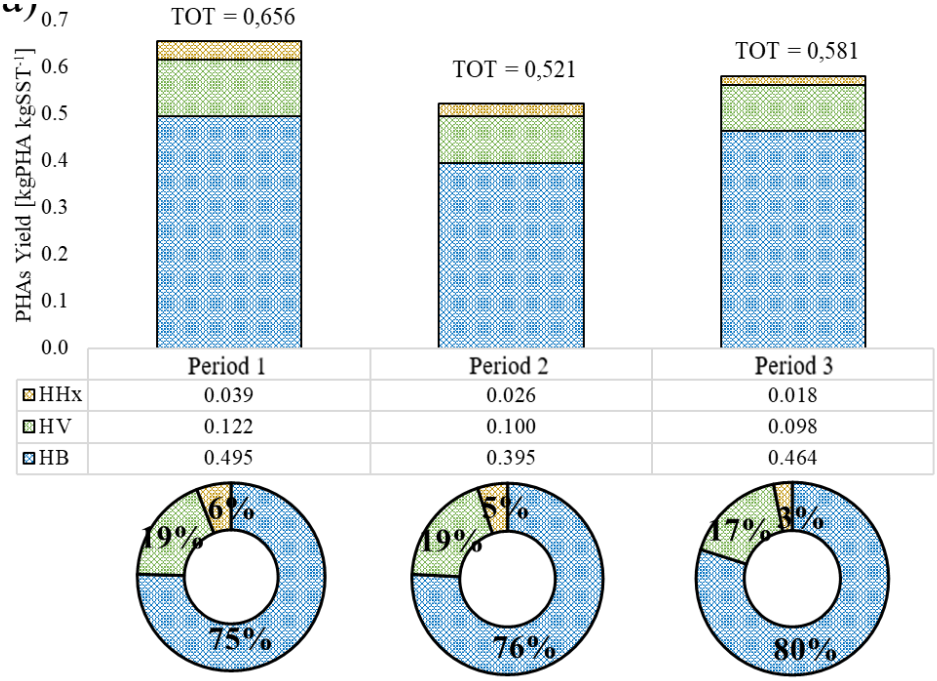


FIGURE 10: POLYMER COMPOSITION OBTAINED IN FED-BATCH ACCUMULATION ASSAYS WITH MMC SELECTED AT DIFFERENT OLR.

As expected, a terpolymer composed of 3HB, 3HV, and 3HHx was obtained. The lower HHx fraction of just ~5% wt. in comparison to the 20% COD hexanoic acid composition, indicates a low culture capability of channelling a C6 precursor into 3HHx, unlike previously reported studies that attained a more close correlation when the culture was selected at higher hexanoic acid fractions (~80% of total COD) (Silva et al., 2022). As shown in Figure 10, 3HB was consistently the predominant monomer, accounting for 75%, 76%, and 80% of the total composition in periods 1, 2, and 3, respectively. This dominance reflects the high proportion of butyric acid (60%) in the substrate, which serves as its metabolic precursor. Meanwhile, the proportions of 3HV and 3HHx showed a slight decrease across the periods, 3HV decreased from 19% in periods 1 and 2 to 17% in period 3, while 3HHx decreased from 6% and 5% in periods 1 and 2, respectively, to 3% in period 3. The variations in 3HV and 3HHx across the three periods were relatively small, indicating that the terpolymer composition remained fairly consistent, although minor differences reflect the influence of the operational conditions on monomer incorporation.

In conclusion, the study demonstrated that mixed microbial cultures can reliably produce mcl-PHA terpolymers with a predominance of 3HB, and that feast–famine selection strategies effectively enriched PHA-storing microorganisms, yielding polymers with slightly variable monomeric compositions depending on the applied conditions.

SBR test II– OLR increase in parallel with increasing C:N ratio

The performance of the SBR test II culture was evaluated along two periods: Period 1 with an OLR of 4 $gCOD \cdot L^{-1} \cdot d^{-1}$ and C/N ratio = 14 and Period 2 with an OLR of 8 $gCOD \cdot L^{-1} \cdot d^{-1}$ and C/N = 28. Along these two periods, the F/F ratio observed in the SBR presented values consistently below 0.2 (Figure 11).

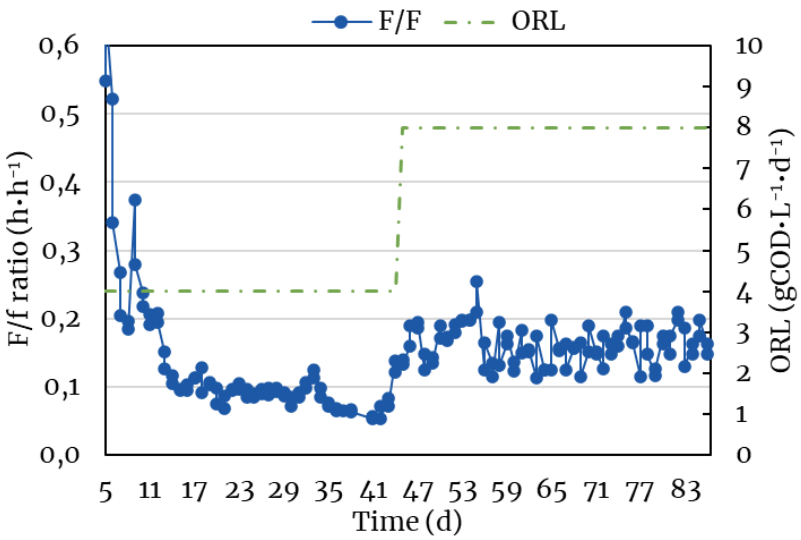


FIGURE 11: VARIATION OF THE FEAST-TO-FAMINE RATIO (F/F) AND APPLIED ORGANIC LOADING RATE (OLR) ALONG SBR TEST II.

The F/F ratio remained approximately 0.1 throughout the first period. A slight increase in the F/F ratio was observed as the OLR was raised. Overall, after the OLR increase to 8 $gCOD \cdot L^{-1} \cdot d^{-1}$ and C/N = 28, the F/F ratio exhibited greater variability compared to the previous period, although it remained below 0.2.

Figure 12 illustrates the behavior of the selected MMC during one SBR cycle of both periods under stable operational conditions, with the profiles of VFA, PHA, X_A and ammonia presented for period 1 and period 2.

Overall, a typical feast to famine profile with uncoupled ammonia addition was observed, similarly to what was reported for SBR test I. Differences are mostly related to the need to provide a second carbon pulse feeding to accommodate the OLR doubling from 4 up to 8 $gCOD \cdot L^{-1} \cdot d^{-1}$, and an extension of the feast phase length that required the delay in N addition to ensure full carbon consumption (Figure 12b).

Despite the OLR increase, by keeping the SRT at 4 days and the same nitrogen loading rate (thus doubling the C:N ratio in period 2 compared to period 1), the active biomass concentration only rose slightly from 3.28 ± 0.29 $gX_A.L^{-1}$ during period 1 to 3.77 ± 0.25 $gX_A.L^{-1}$ during period 2.

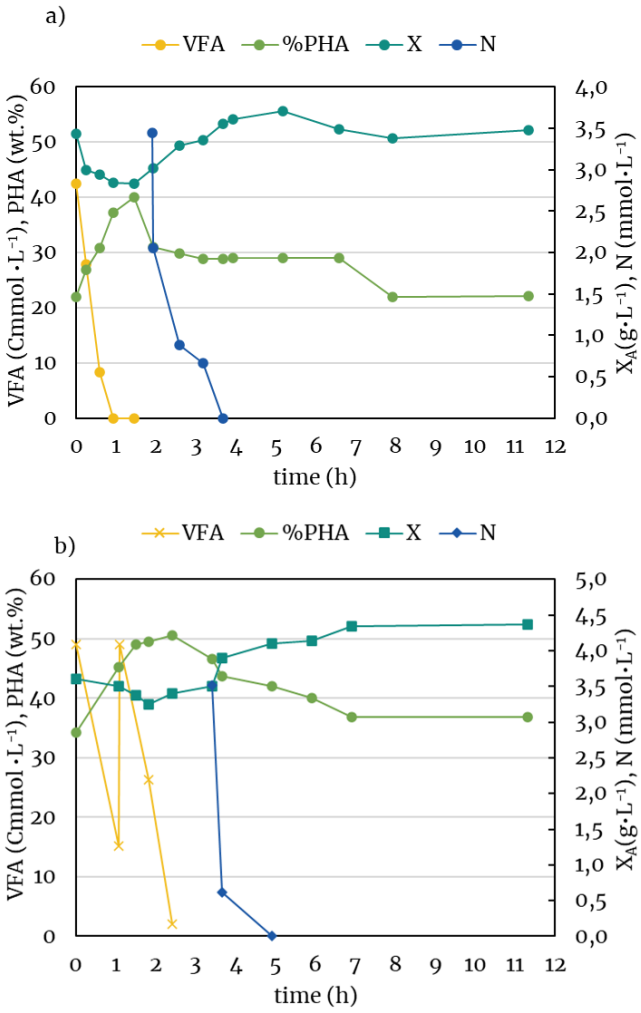


FIGURE 12: CONCENTRATION PROFILES OF CAS, % PHA, ACTIVE BIOMASS (X) AND AMMONIA (N) DURING A TYPICAL CYCLE OF DAY 34 FOR PERIOD 1 (A), AND DAY 75 FOR PERIOD 2 (B).

TABLE 14: PRESENTS THE AVERAGE VALUES AND STANDARD DEVIATION OF THE KINETIC AND STOICHIOMETRIC PARAMETERS DETERMINED FOR THE FEAST AND FAMINE PHASES IN BOTH PERIODS.

	Period 1	***Period 2***
ORL ($gCOD.L^{-1}.d^{-1}$)	4	8
ORL ($Cmmol.L^{-1}.d^{-1}$)	98	196
F/F ($h.h^{-1}$)	0.12 ± 0.02	0.16 ± 0.03
X_A ($g.L^{-1}$)	3.28 ± 0.29	3.77 ± 0.25
PHA^{max}(wt. %)	39.2 ± 3.4	47.3 ± 4.2
ΔPHA (wt. %)	18.2 ± 4.2	14.6 ± 2.8
$-q_S$ ($Cmol\text{-}S.Cmol\text{-}X_A^{-1}.h^{-1}$)	0.38 ± 0.02	0.33 ± 0.03
q_{PHA} ($Cmol\text{-}PHA.Cmol\text{-}X_A^{-1}.h^{-1}$)	0.20 ± 0.02	0.12 ± 0.03
$Prod_{PHA}$ ($g\text{-}PHA \bullet L^{-1} \bullet d^{-1}$)	1.79 ± 0.04	2.25 ± 0.22

The increase in OLR from 4 to 8 gCOD.L^{-1}.d^{-1} induced clear changes in the metabolic dynamics of the enriched MMC. With regard to storage metabolism, the maximum intracellular PHA content rose substantially from 39.2% of TSS in period 1 to 47.3% of TSS in period 2, indicating that cells accumulated more carbon reserves under the higher OLR.

From a kinetic perspective, the specific substrate uptake rate (–qS) decreased from 0.38 Cmol-S.Cmol-XA^{-1}.h^{-1} in period 1 to 0.33 Cmol-S.Cmol-XA^{-1}.h^{-1} in period 2, indicating that the consumption of the available carbon substrate proceeded more slowly under higher ORL conditions. Similarly, the specific PHA production rate (q_{PHA}) declined from 0.2 Cmol-PHA.Cmol-XA^{-1}.h^{-1} in period 1 to 0.12 Cmol-PHA.Cmol-XA^{-1}.h^{-1} in period 2, which indicated a lower efficiency in converting the assimilated carbon into intracellular storage polymers.

Finally, the volumetric PHA productivity ($Prod_{PHA}$) increased from 1.79 g-PHA.L^{-1}.d^{-1} in period 1 to 2.25 g-PHA.L^{-1}.d^{-1} in period 2, demonstrating that the reactor was able to convert the additional substrate into higher PHA production. Although the productivity did not exactly double in proportion to the two-fold increase in OLR, this improvement highlights the system's capacity to handle higher carbon loads and achieve greater overall polymer accumulation.

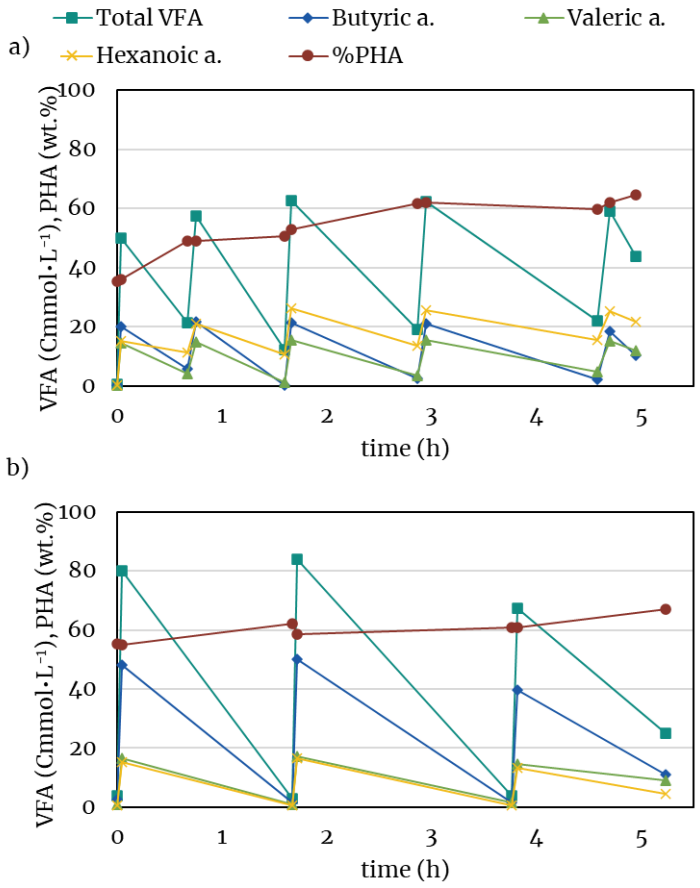


FIGURE 13: CONCENTRATION PROFILES OF TOTAL CAS, BUTYRIC ACID, VALERIC ACID, HEXANOIC ACID AND PHA CONTENT (WT.%) DURING AN ACCUMULATION ASSAY CONDUCTED ON DAY 35 FOR PERIOD 1 (A) AND DAY 76 FOR PERIOD 2 (B).

Accumulation assays were conducted to assess the maximum PHA accumulation capacity of the microbial cultures selected during the two experimental periods. The evolution of PHA content (wt.%) along with the corresponding VFA concentration profiles is presented in Figure 13.

The maximum PHA content achieved was comparable between the two periods, with 63.7 ± 1.3 wt.% in period 1 and 68.1 ± 1.5 wt.% in period 2, indicating that in both cases the intrinsic storage capacity of the biomass was reached. These values were within the storage capacity range observed in other studies where hexanoic acid-rich substrates were used for PHA production with MMC (Silva et al., 2022).

The net PHA accumulation (ΔPHA) differed markedly between the two periods, with assays conducted during period 1 exhibiting a larger increase (28.3 ± 2.9 wt.%) compared to those in period 2 (17.0 ± 5.9 wt.%). This difference reflects the higher initial PHA content in the biomass from period 2, which had already accumulated a substantial fraction of storage prior to the assays.

Differences were also observed in CAs consumption patterns. In the accumulation assays with the biomass from period 1, butyric and valeric acids were preferentially and fully consumed, whereas hexanoic acid tended to accumulate in the medium (Figure 13). In the accumulation assays with biomass from period 2, this substrate selectivity was no longer evident and all VFAs were metabolized in a more uniform manner (Figure 13).

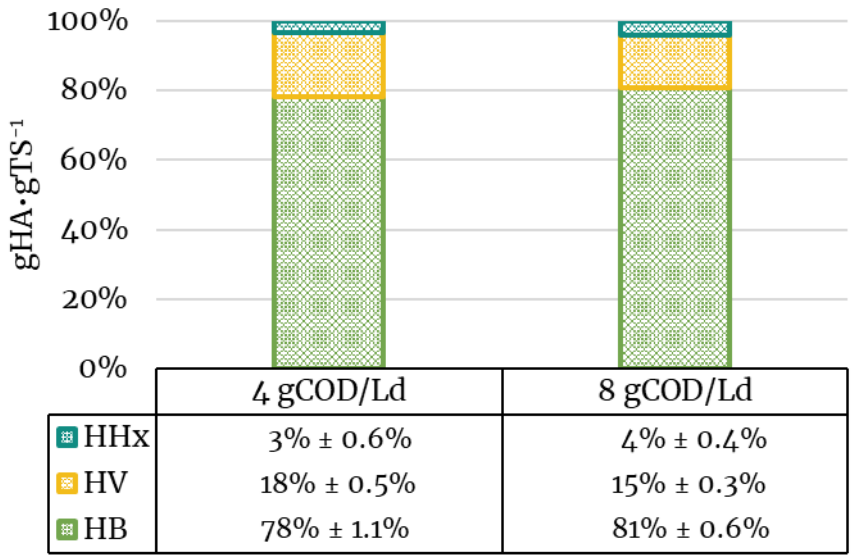


FIGURE 14: PHA MONOMER COMPOSITION (3HB, 3HV, 3HHX) AT THE END OF THE ACCUMULATION ASSAYS OPERATED WITH THE BIOMASS PREVIOUS SELECTED AT TWO DIFFERENT OLRS (4 AND 8 GCOD.L^{-1}.D^{-1}). VALUES REPRESENT AVERAGES WITH STANDARD DEVIATIONS (N =2).

In both accumulation assays, the PHA polymer was predominantly composed of 3HB, which consistently represented ~78–81% of the total monomers, regardless of the applied OLR (Figure 14). The fraction of 3HV remained stable at ~15–18%, while the contribution of 3HHx was minor but detectable, ranging between 3–4%. Similar PHA monomeric composition was obtained in SBR test I when using the same feedstock at different operational conditions.

Overall, the polymer composition was robust across both operational conditions, showing only minor variations in 3HV and 3HHx fractions. The stability of 3HB as the main constituent highlights a strong metabolic preference of the microbial community for short-chain monomers, while the limited incorporation of 3HHx points to a restricted ability to channel medium-chain precursors into the polymer.

These results suggest that changes in OLR primarily affected the kinetics of accumulation rather than substantially altering the qualitative monomer composition of the PHA.

SBR test III – Validation with model feedstock of fermented wine lees

Similarly to the approach followed for SBR test I and II, activated sludge was used as the inoculum for SBR test III. Also, since the model feedstock presented a considerable high content of hexanoic acid and ethanol (COD-basis, 60% hexanoic acid, 15% ethanol), and previous SBR tests had shown a low preference for the C6 substrate, it was decided to follow the operational conditions of SBR test I. Namely, a gradual increase of the OLR from 2 to 4 $gCOD.L^{-1}.d^{-1}$ was implemented (Period 1 and Period 2, respectively), at fixed C:N = 14, in order to firstly evaluate the culture capability of consuming the organic carbon. As anticipated, and unlike the previous SBR tests, the biomass did not show an aptitude to quickly consume all the carbon source, and for the first days of operation, F/F ratio was substantially higher than the desired values of an F/F < 0.2 (Figure 15).

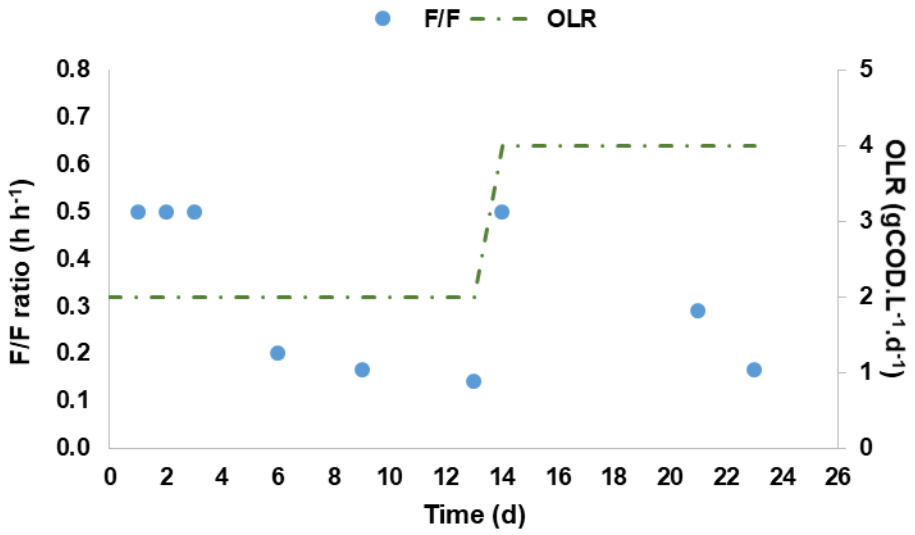


FIGURE 15: FEAST TO FAMINE RATIO AND OLR CHANGE OVER TIME IN SBR TEST III DETERMINED BY HPLC ANALYSIS.

It is important to mention, that F/F data could not be calculated by following the DO profile, but instead, had to be followed by real HPLC measurements of the organic content to confirm the end of the Feast phase. Indeed, the culture showed an interesting behavior where both the pH variation and DO profile gave a misleading indication that all carbon had been consumed (e.g. no pH increase, saturated DO values). This resulted from a quick consumption of the less abundant, but more preferable carbon sources (e.g. acetate, butyrate, valerate), followed by a very slow consumption of hexanoic acid that could not be perceived using the pH and DO sensors.

Nevertheless, the culture adapted and increased the uptake rate of hexanoic acid, leading to F/F ratios below 0.2 at the end of Period 1. On the last operational day at OLR 2 $gCOD.L^{-1}.d^{-1}$, the MMC presented the typical feast and famine profile with uncoupled ammonia feeding (Figure 16).

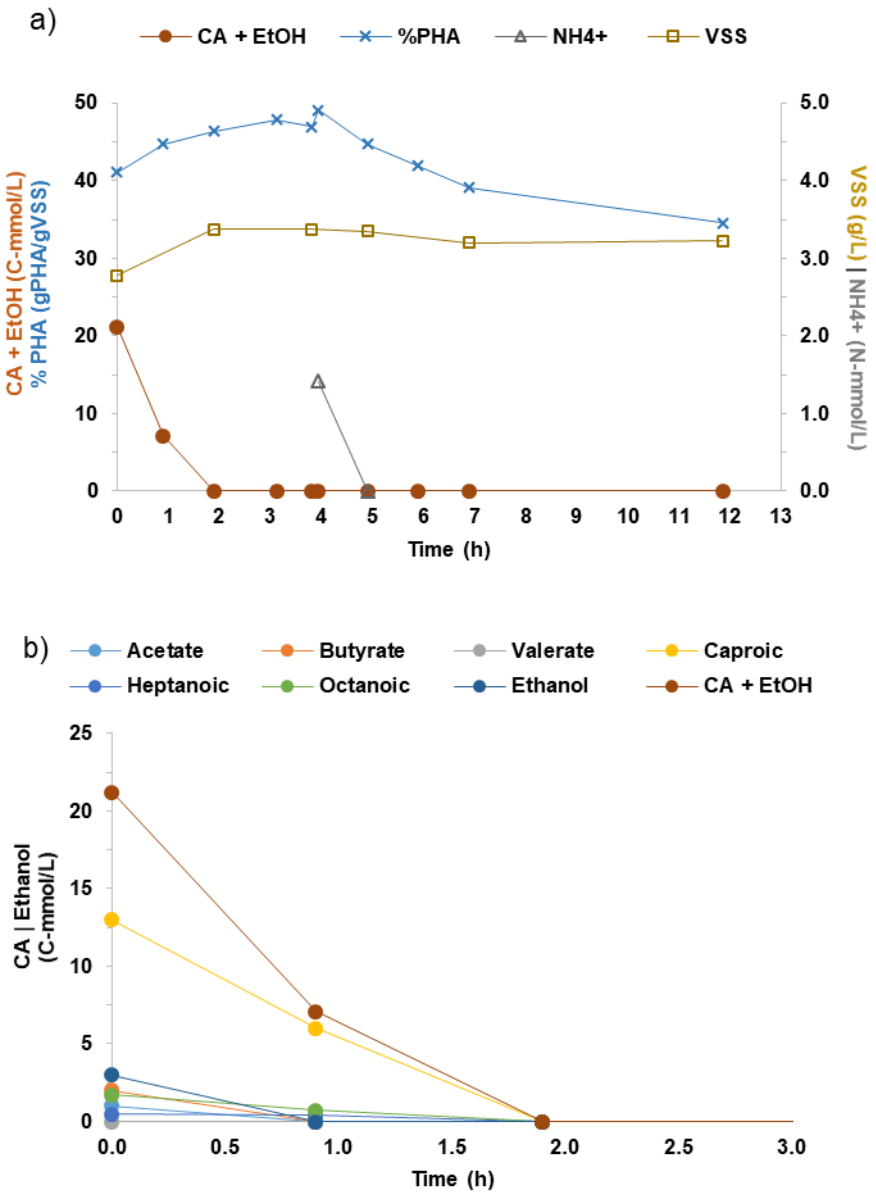


FIGURE 16: SBR TEST III CONCENTRATION PROFILES AT DAY 13 AT AN OLR OF 2 $gCOD.L^{-1}.D^{-1}$. A) VSS, CARBOXYLIC ACIDS (CA), % PHA AND $NH4^{+}$; B) DETAIL OF CA CONSUMPTION DURING THE FEAST PHASE.

The culture stored PHA during the feast phase and only upon ammonia addition was PHA consumed for growth. PHA achieved a maximum content of 48.0 ± 0.9 % gPHA/gVSS prior to N addition, a value substantially higher in comparison to the values registered during SBR test I at the same C:N ratio and across all tested OLR (see Table 2). It can be speculated that the higher hexanoic acid content in the present feedstock may enable higher storage yields due to the increased energetic efficiency of assimilating higher-carbon substrates.

Regarding the PHA monomer composition, the culture presented a consistent composition along the cycle with 3HB:3HV:3HHx values of 80:4:16 (% wt) (76:4:20 in %COD) (Figure 17A). It seems that despite the very high content of hexanoic acid in the feedstock (60% of COD), the culture is unable to store it as 3HHx, possible metabolizing it to C4 and C2 carbons that proceed to storage *via* 3-hydroxybutyryl-CoA pathway.

Furthermore, at the first cycle of OLR increase to 4 gCOD.L^{-1}.d^{-1}, the food to microorganism ratio was temporarily doubled, and this similarity to an accumulation assay, allowed to observe the culture capability of further increasing its PHA content, which rose from 39% up to 54% along the feast phase. Again, the polymer composition remained unaltered (Figure 17B).

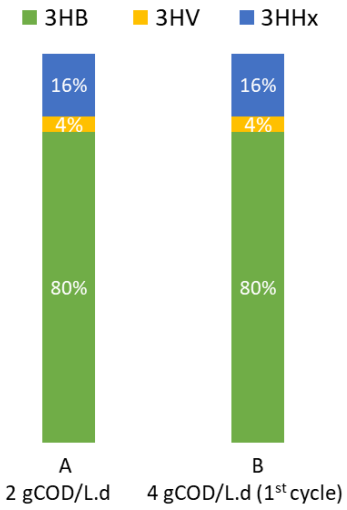


FIGURE 17. PHA MONOMER COMPOSITION (% WT. 3HB, 3HV, 3HHX) AT THE END OF THE FEAST PHASE OF A) CYCLE AT OLR 2 GCOD.L^{-1}.D^{-1} AND B) 1ST CYCLE UNDER OLR 4 GCOD.L^{-1}.D^{-1}.

A similar behavior of mismatch between the hexanoic acid and the HHx content was observed in the previous SBR test I and II. With hexanoic acid being 20% of the feedstock COD, the cultures did not store higher than 3-4% 3HHx. Although there seems to be a correlation between incrementing the hexanoic acid concentration and obtaining higher 3HHx content, and despite current results still being preliminary, other operational conditions may be tested in the future to steer the microbial population towards organisms capable of storing higher 3HHx levels. A more strict selective strategy could be implemented, covering for example operation at limiting DO levels that could pressure for high efficient and quick 3HHX storers.

With the culture thus far achieving 3HHx values up to 16% wt., the next step of culture operation using the real fermented wine lees will provide valuable information on the feedstock impact in the population and polymer composition. A smooth transition from the model to the real feed is expected due to the high similarity, yet, this validation will be crucial to support MMC as robust systems for the production of terpolymers comprising scl and mcl-PHA.

Material Exchange

Throughout NID activities, PHA-rich biomass was produced both for evaluating culture performance and for obtaining material to supply project partners.

Regarding MMC biomass, 5 accumulation assays were conducted using synthetic CAs and 1 using a real fermented glucose stream feedstock (simulating grey-starch streams) provided by UGent. The differing composition of carboxylic acids compared to the synthetic mixture affected the behavior of the mixed microbial cultures during the accumulation assays. Figure 18 illustrates an example of the accumulation profile obtained with the real fermented feedstock.

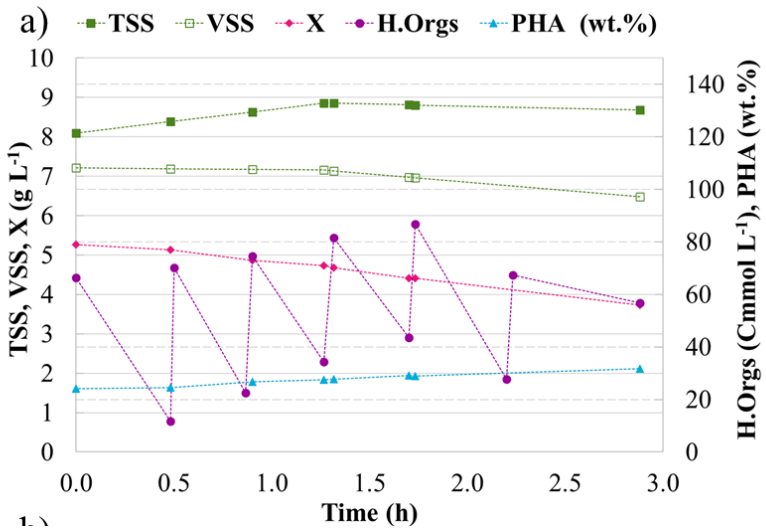


FIGURE 18: EXAMPLE OF THE PHA, TSS, VSS, H.ORG TRENDS DURING FED-BATCH ACCUMULATION ASSAYS PERFORMED USING BIOMASS FROM THE SBR TEST 1 AND REAL FERMENTATE GLUCOSE STREAM FEEDSTOCK FROM UGENT.

Indeed, in comparison to accumulation assays with synthetic feedstock, real fermented feedstock led to a lower final PHA content (32.5%) and a strong mismatch between the CAs composition of the feed and the polymer monomer composition (only 10% 3HHx for a feedstock with 65% hexanoic acid, see Table 16). It is possible that the higher concentration of hexanoic acid in the fermented feedstock could have inhibited the MMC since this had been enriched at lower hexanoic acid concentrations. Figure 19 shows representative biomass samples sent to partners, while Table 10 summarizes the results of mcl-PHA production obtained at the accumulation assays and the material exchange to the partners.

Overall, the accumulation assays produced a total of **106 g of dried biomass**, yielding **approximately 55 g of PHA**, sufficient to supply several project partners for further tests. Synthetic CA feedstock was used in most cases, resulting in PHA contents ranging from ~50 to 62 wt% and monomeric compositions consistently around 77–80% 3HB, 17–18% 3HV, and 3–6% 3HHx. The accumulation assay using the real fermented glucose stream from UGent yielded lower PHA content (~33 wt%) with a slightly different composition (83:7:10, 3HB:3HV:3HHx), reflecting the impact of the feedstock on polymer characteristics. Delivered biomass and PHA amounts varied depending on the partner and assay (as indicated in Table 15), with portions sent to UNIROMA, CSIC, and ENTO for downstream testing.

FIGURE 19: REPRESENTATIVE BIOMASS SAMPLES SENT TO PARTNERS

TABLE 15: SUMMARY OF MMC MCL-PHA ACCUMULATION ASSAYS RESULTS AND OVERVIEW OF BIOMASS MATERIAL EXCHANGE BETWEEN PARTNERS. SYNTHETIC FEEDSTOCK COMPOSITION: BUTYRIC, VALERIC AND HEXANOIC ACIDS AT A RATIO OF 60/20/20 (% COD BASIS)). REAL FERMENTATE COMPOSITION: BUTYRIC, VALERIC AND HEXANOIC ACIDS AT A RATIO OF 15/20/65 (% COD BASIS).

Feedstock	*Biomass condition*	*Dried biomass (g)*	*PHA content (%, wt/wt)*	*PHA Amount (g)*	*HB:HV:HHx (%, wt/wt)*	*Delivered to (Partner)*	*Date of Delivery*
Synthetic	Acidified with H_2SO_4, centrifuged, frozen (part lyophilised for UNIROMA tests)	13.3	56.9	7.5	80:17:3	UNIROMA (19.7g) and CSIC (5g)	Feb 2024
Synthetic		11.5	62.3	7.2	80:17:3		
Real fermentate	Acidified with H_2SO_4, centrifuged, frozen	11.2	32.5	3.7	83:7:10	Not delivered	n.a.
Synthetic	Half acidified with H_2SO_4, Half acidified with HCl	32.2	50.4	16.3	77:18:6	ENTO	May 2024
Synthetic	Acidified with H_2SO_4, centrifuged, frozen	22.0	50.5	11.1	77:17:5	UNIROMA (5.3g)	May 2025
Synthetic	Acidified with H_2SO_4, centrifuged, frozen	15.9	56.7	9.0	79:17:4	CSIC	May 2024

Conclusions

NID's work focused on developing and evaluating an MMC-based process for producing mcl-PHAs from carboxylic-acid-rich substrates enriched in hexanoic acid, using a two-phase experimental strategy. In Phase I, several operational conditions were tested in a sequencing batch reactor fed with a synthetic mixture of carboxylic acids to investigate how organic loading rate, nutrient balance, and sludge retention time influence microbial enrichment, culture stability, and PHA-accumulating behaviour. In Phase II, a set of operational conditions that had shown stable and satisfactory performance in Phase I was applied to a model feedstock representative of fermented wine lees, serving as a preparatory step before the proof of concept with the real waste-derived substrate.

Across the conditions tested, the F/F selection strategy proved highly effective in enriching PHA-storing microorganisms, resulting in robust and adaptable cultures capable of metabolizing both simple and complex substrates.

Variations in operational conditions influenced biomass growth and polymer accumulation dynamics, demonstrating the metabolic flexibility of the microbial communities. Despite changes in organic loading

rate, carbon-to-nitrogen ratio, and sludge purge strategy, the cultures remained stable and efficient, highlighting their resilience under different operating scenarios.

The monomeric composition of the polymers during produced was largely consistent, with 3-HB predominating (~75–80%), 3HV as a minor fraction (~17–18%), and 3HHx present in very small amounts (~3–6%), despite the variations in the operating conditions.

The work in Phase II confirmed that the process remained stable under more complex substrate compositions and the hexanoic acid increment in the feedstock up to 60% COD could be correlated with an increment of the 3HHx fraction up to 16% wt. Nevertheless, this strong mismatch between the contents may result from the population preference to metabolize the C6 into C4 and C2 and store it as 3HB. Process validation with the fermented wine lees will bring more certainties on the 3HHx range that can be attained using the real feedstock.

Complementary accumulation assays for sample production provided additional insight into polymer synthesis dynamics and enabled the production of approximately 106 g of dried biomass containing around 55 g of PHA, which were supplied to project partners for downstream extraction, purification, and material characterization. In this case, the polymers produced exhibited more significant variation in composition. The ranges observed (3HB around 78–83%, 3HV approximately 7–15%, and 3HHx 3–10%) reflect the influence of the more complex substrates used as well as the operational history of the cultures. Overall, these results demonstrate that the developed MMC-based process is robust, reproducible, and suitable for sustainable PHA production from diverse CA streams rich in hexanoic acid.

III.II PHA PRODUCTION and EXTRACTION (UNIROMA)

Introduction

Polyhydroxyalkanoates (PHA) are a family of linear polyesters fully biobased and biodegradable. They are being produced intracellularly by over 300 species of microorganisms under unbalanced growth conditions (Reis et al., 2011). The interest towards PHA production has significantly grown in recent years, owing mostly to their intriguing thermomechanical properties, which are comparable to those of conventional fossil-based plastics (e.g., polyethylene, polypropylene). Therefore, in this scenario, they represent an optimal replacement of traditional plastics in specific applications and can contribute to minimize environmental plastic pollution and greenhouse gas emissions. Nevertheless, the widespread usage of this biopolymer remains hindered by their high market costs mainly associated to both the production and downstream steps. Conventional industrially established production processes involve the use of pure microbial cultures or recombinant strains characterized by high yield in terms of intracellular storage capacity (more than 90 %, with respect to dry cellular matter). However, their application negatively affects the production costs since they require sterile conditions to grow and “ad hoc” balanced substrate media to store (Meng et al., 2022). In order to reduce production costs, the use of Mixed Microbial Cultures (MMC) has been extensively investigated. The polymer production involving MMC allows to exploit renewable substrates (e.g., organic wastes or residues) reaching an intracellular PHA content up to 80 (%, wt/wt) (Oliveira et al., 2017). Another peculiar aspect on the application of MMC consists of the possibility to modulate the polymer composition tuning the organic substrates composition into desired carboxylic acids (through acidogenic fermentation), which are direct substrates for MMC-PHA production, as well as by changing process parameters (e.g., organic loading rate - OLR or hydraulic retention time - HRT). The composition of the polymer determines its thermomechanical properties, and the final polymer application in the field of bioplastic market. Polyhydroxybutyrate (PHB), the most studied polymer in this class, is characterized by high crystallinity and brittleness limiting its applications in different plastic sectors. In this view, the incorporation of a different monomer fraction (e.g., 3-Hydroxyvalerate and/or 3-Hydroxyhexanoate) inside the entire polymer chain enhances the thermal and mechanical properties (Alfano et al., 2024). However, PHA production process with MMC is still not competitive with the fossil-based plastic production one.

The downstream step represents the bottleneck of the entire process covering around 50 % of the entire production cost (Salvatori et al. 2023). Best-established PHA recovery procedures, able to achieve high recovery and purity yields, are based on extraction methods employing hazardous halogenated solvents (in particular $CHCl_3$), which are not-suitable compounds in a sustainable manufacturing chain. Moreover, cold precipitation is often used to enhance the polymer purity and recovery using non-solvents such as ethanol, acetone, or methanol (Pagliano et al., 2021). Many research efforts are focused on the use of green solvents such as ethyl acetate, alcohols, ethyl lactate (Alfano et al. 2021), or supercritical fluids, as a low or no toxicity alternative to chlorinated hydrocarbons (Haghighi et al., 2026).

In this context, in the frame of the Agriloop project, UNIROMA was in charge of producing short chain PHA (scl-PHA) at a laboratory scale adopting a novel continuous-flow process. Overall, over 150 g of PHBV were recovered through the digestion of non-polymer cell materials (NPCM) (Salvatori et al., 2023), and distributed to other project partners (in particular UM and UNIBO) to accomplish the objectives of WP4.

In addition, ethyl acetate has been investigated as a green solvent for PHA recovery. Additionally, supercritical carbon dioxide (sc-CO_2) was also investigated as a strategy to enhance the recovery of the PHA granules due to the disaggregation of non-polymeric cellular materials. For this purpose, UNIROMA used both the PHA-rich biomass obtained with the continuous-flow process and the PHA-rich biomass provided by NID.

UNIROMA also collected samples of PHA-rich biomass, which were sent to other project partners to study extraction strategies including predatory bacteria (CSIC) or larvae (ENTO).

Materials and Methods

1. **Operation of the continuous-flow process for Polyhydroxyalkanoates production with Mixed Microbial Cultures (MMC)**

The novel continuous-flow process for MMC-PHA production developed by UNIROMA consists of three main reactors. The tubular feast reactor (1L working volume) is continuously fed (Q_E) from the bottom with a synthetic carboxylic acid mixture (CA), and the effluent from this reactor is recirculated (from the top) to the famine reactor (i.e., a Continuously Stirred Tank Reactor - CSTR, 5L working volume) without the use of a pump. Once in the famine reactor, fraction of the mixed liquor is recirculated in the feast reactor via a recirculation flow rate (Q_R), and another fraction is sent to the PHA accumulation reactor (CSTR, 2L working volume), which is also continuously fed ($Q_{E'}$) with a synthetic CA mixture with the same composition as the feast reactor, but 2 or 3 times more concentrated and nutrient deficient (i.e., nitrogen-free) in order to maximize PHA production. All flow rates were accurately defined to equilibrate the whole system. The effluent from the accumulation reactor was daily harvested and sent to downstream processing, consisting of PHA stabilization, extraction, and recovery.

The overall process is represented in Figure 20, and consists of the feast and famine reactors, referred to as the continuously fed feast and famine (FF) system, for the microbial selection into PHA-storing microorganisms starting from an activated sludge (used as inoculum) collected from a municipal wastewater treatment plant, and of the continuously fed accumulation reactor.

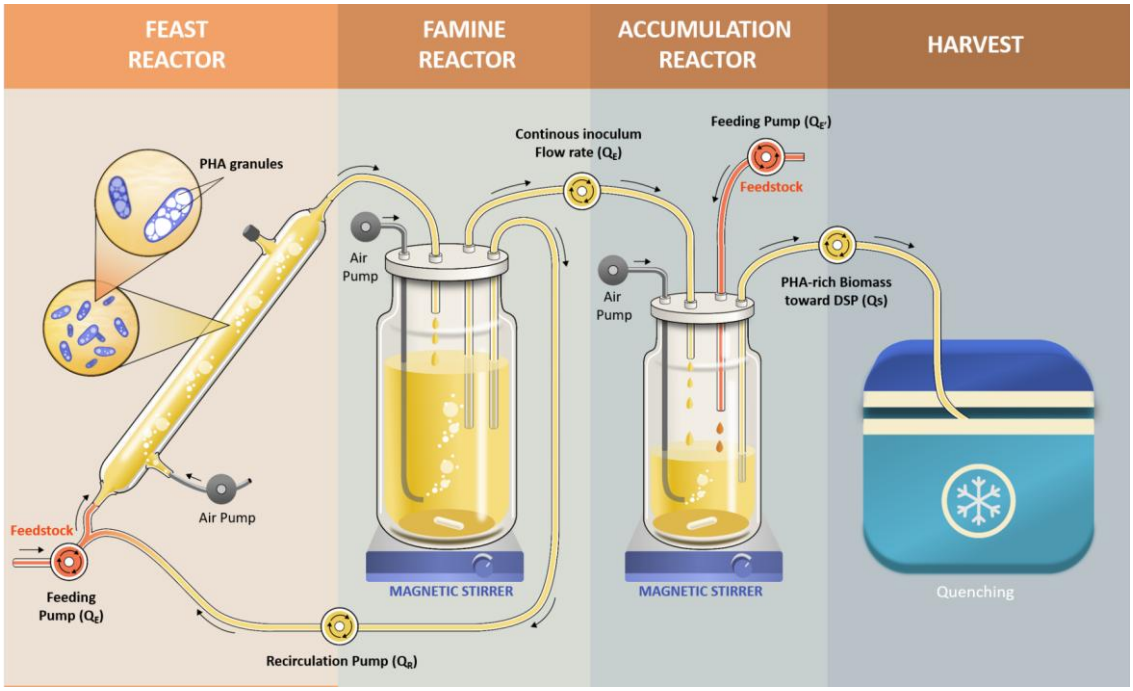


FIGURE 20: SCHEME OF THE CONTINUOUS MULTI-FLOW PROCESS FOR MMC-PHA PRODUCTION (DEPOSITED PATENT# 102024000013207, "CONTINUOUS PROCESS FOR POLYHYDROXYALKANOATES PRODUCTION WITH MIXED MICROBIAL CULTURES")

The downstream processing consisted in a succession of several steps resulting in recovering the PHA (at different purity grades in powder form) and other high added value co-products: Extracellular polymeric substances (mainly composed of Proteins and Carbohydrate).

- **Quenching**

The biomass collected from the accumulation reactor was immediately quenched in order to stop the metabolic activity of microorganisms and avoid any PHA consumption. In this regard two strategies of biomass stabilization were adopted: **Acid quenching** (bringing biomass to pH 2 using drops of concentrated H_2SO_4, 96% (w/w); and **Thermal quenching** (the biomass was collected and stored at 4°C).

- **Centrifugation**

This step is very crucial, because it allows the solid/liquid separation, and after that, the solid biomass is recovered and transferred to the next step. So, after the quenching, the quenched biomass was centrifuged (at 8000 at 20°C for 15 min) using a bench OHAUS centrifuge (model FC5916R) with capacity of around 2L per cycle.

- **Extraction**

Herein, the wet pellet of PHA-enriched biomass was treated with both a hydrolyzing and oxidizing agent: firstly NaOH (0.2 M) for 4h followed by H_2O_2 (1.5 %, w/v) for 1h. Afterward, the recovered polymer was washed and dried in an oven at 60°C (Salvatori et al., 2023).

- **NaOH residual solution precipitation for Extracellular Polymeric Substances (EPS) recovery**

In this step, following the PHA extraction with NaOH, the residual alkaline solution at pH 13 was precipitated to pH 2 using concentrated H_2SO_4 (96%, w/w) and then centrifuged at 8000 rpm and 20°C for 15 min.

The continuous-flow process for MMC-PHA production was set up and operated for approximately 2 years at a fixed recirculation factor ($R_C = Q_R/Q_E = 8$) (Tayou et al., 2023) and two organic loading rates (OLR) of 2.12 and 4.25 gCOD/Ld with a fixed C/N ratio of 35 applied for the microbial selection reactors (feast and famine, FF). The hydraulic retention time (HRT) of the FF system was equal to the sludge retention time (SRT) (HRT=SRT= 1 d), and no temperature and pH control was performed. The accumulation reactor was operated at an HRT of around 6 hours (working volume of 2L) and at an OLR of 6.12 gCOD/Ld. The feedstock for the feast and accumulation reactors was composed of a synthetic mixture of acetic (AcOH) and propionic acids (PrOH) (65% and 35% in terms of Chemical Oxygen Demand -COD, respectively). Moreover, following fungi contamination of the system, the whole system was inoculated again with a new inoculum, and operated at the lowest OLR (2.12 gCOD/Ld) for the selection maintaining unchanged the OLR of the accumulation reactor. Since the main objective here was the production of extracted PHBV copolymer with a target HV content, ranging between 25 and 35 (%, wt/wt); the feedstock composition of solely the accumulation reactor was changed during the experimentation from 65% (AcOH) and 35% (PrOH) to 75% (AcOH) and 25% (PrOH) in terms of COD. Thereby, the produced polymer was extracted, recovered (according to the protocol mentioned in the previous section) and characterized in terms of PHA purity, monomeric composition, and molecular weight. Supplementarily, the use of a real feedstock (fermented wine lees, provided by UGent) is currently being investigated. This activity has recently started with feedstock characterization and operation of the FF system using a synthetic mixture simulating the composition of the fermented feedstock, therefore related data are not included in this deliverable 3.2.

PHA extraction and EPS recovery were performed as described in Figure 21. Briefly, after the PHA extraction with NaOH (0.2 M) the alkaline suspension was centrifuged at 8000 rpm at 20°C for 10 min. The PHA-rich pellet was recovered and further purified with H_2O_2 (1.5 %, w/v) for 1h, while the alkaline supernatant was brought to pH 2 in order to precipitate the digested component (such as proteins and carbohydrates).

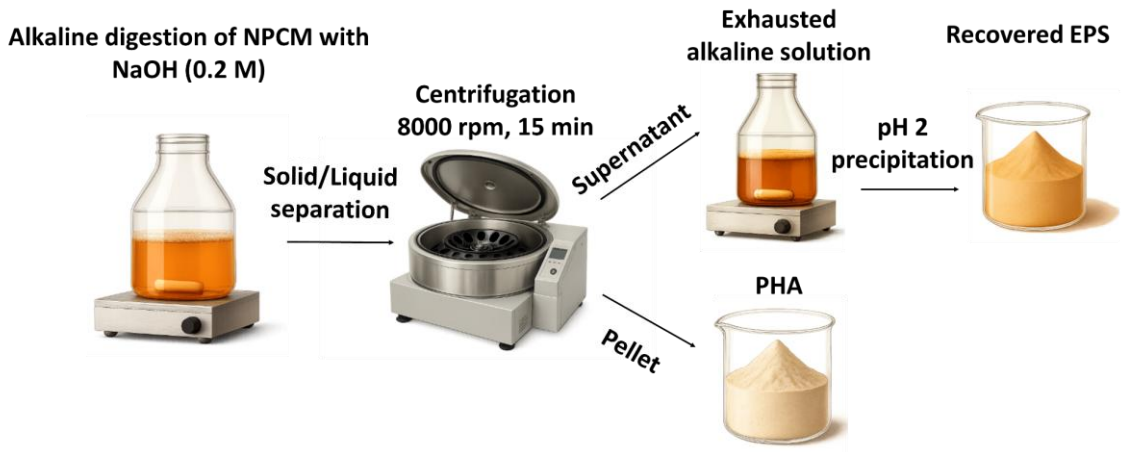


FIGURE 21: SCHEME OF THE ADOPTED PHA EXTRACTION AND EPS RECOVERY PROCEDURE

2. Analytical measurements

The concentration of carboxylic acids was determined through gas chromatographic analysis by injecting 1 µL volume of liquid phase in an Agilent GC 8860 instrument equipped with a DB- FFAP column (30 m × 530 µm × 0.25 µm), N_2 was used as carrier gas at a flow rate of 30 mL/min, the initial oven temperature was set at 60 °C, and it was increased up to 175 °C at a rate of 20 °C/min; the flame ionization detector (FID) was

set at 320 °C. PHA determination was done by sampling 5 mL of mixed liquor from biological reactors and immediately treated with 1 mL of a sodium hypochlorite solution (5 % active Cl_2) to stop the polymer degradation. Samples were then stored in the freezer (-20 °C) for following PHA analysis, which was performed according to Braunegg et al. (1978) (Braunegg et al., 1978). Then, after hydrolysis and esterification into 3-hydroxyacyl methyl esters, these were analyzed by gas chromatography GC (Agilent 8860) using a flame ionization detector (FID). The PHA concentration was quantified by a GC method injecting 1 µL volume of organic liquid phase into an Agilent 8860 GC equipped with a HP- 5 column (30 m × 320 µm × 0.25 µm), N_2 was used as carrier gas at a flow rate of 10 mL/min, the oven temperature was initially maintained at 100 °C for 1 min; then increased up to 130 °C at a rate of 12 °C/min and further increased up to 250 °C at a rate of 25 °C/min; the FID was maintained at 300 °C. The relative abundance of 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV) monomers was determined using as reference standard a commercial P(HB/HV) copolymer with 12 % (wt) HV content (Sigma–Aldrich, Milan, Italy).

Regarding the biomass, volatile suspended solids (VSS) were determined according to the standard method APHA (Rice et al. 2012). Total proteins and total carbohydrates content of the recovered EPS were evaluated by Bicinchoninic acid (BCA) method (Walker et al., 2009) (using Bovine Serum Albumin -BSA, as standard) and Phenol-sulfuric method (Dubois, et al., 1956) (using Sodium Alginate as standard), respectively. Functional groups analysis was performed with FTIR spectroscopy (FITR APEX Thermofisher). Tests of gel-forming proprieties were evaluated by dissolving the Freez-dried EPS in NaOH (0.1 M) at 10% (wt/v) and then pipetted in $CaCl_2$ (2.5 %, wt/v). The hydrogel beads were transferred in EDTA solution 2% (w/v) to evaluate their stability for 1 month.

The molecular weight (Mw) of purified samples was determined by Gel Permeation Chromatography (GPC) using a chromatograph equipped with a pump (JASCO PU-4180), a guard column and two columns in series (TSKgel®G6000-HHR TSKGel GMHHR-H), a column oven (JASCO CO-4060) and a refractive index detector (JASCO RI-4030). Chloroform was used as eluent at a flow rate of 1 ml/min. The detector was set at 35 ˚C, whereas the columns were set at 40 ˚C. Polystyrene standards (0.013–3.05 × 10^6 g/mol) were used to calibrate the system.

3. Calculations

The overall PHA concentration was obtained by summing the concentration of HB and HV monomers measured from GC analyses, whereas the intracellular PHA content was determined as the ratio between PHA concentration and the biomass concentration (i.e., VSS):

PHA (mg/L) = HB (mg/L) + HV (mg/L)

Intracellular PHA content (%, wt/wt) = [PHA (mg/L) / VSS (mg/L)]* 100

The HV content in the stored copolymer was calculated as follows:

HV (%, wt wt) = [HV (mg)/PHA (mg)]*100

The obtained PHA concentrations measured by GC were converted into a COD basis according to the stoichiometry conversion factors of 1.67 gCOD/gHB and 1.92 gCOD/gHV.

The recovery yield, defined as the ratio between the weight of extracted polymer (we × p) to the initial weight of PHA within the dry biomass before the extraction procedure (wb × fi), was calculated using following equation:

Recovery yield (%) = $[(w_e \times p) / (w_b \times f_i)] \times 100$

wherein w_e and p are the weight and purity of extracted polymer, w_b is the weight of the biomass and f_i the initial content of PHA in the biomass. Both p and f_i were evaluated by GC analysis.

Results and discussion

This section reports data obtained by UNIROMA with the continuous-flow process for MMC-PHA production. Data related to PHA and EPS recovery are also described.

PHA production and recovery

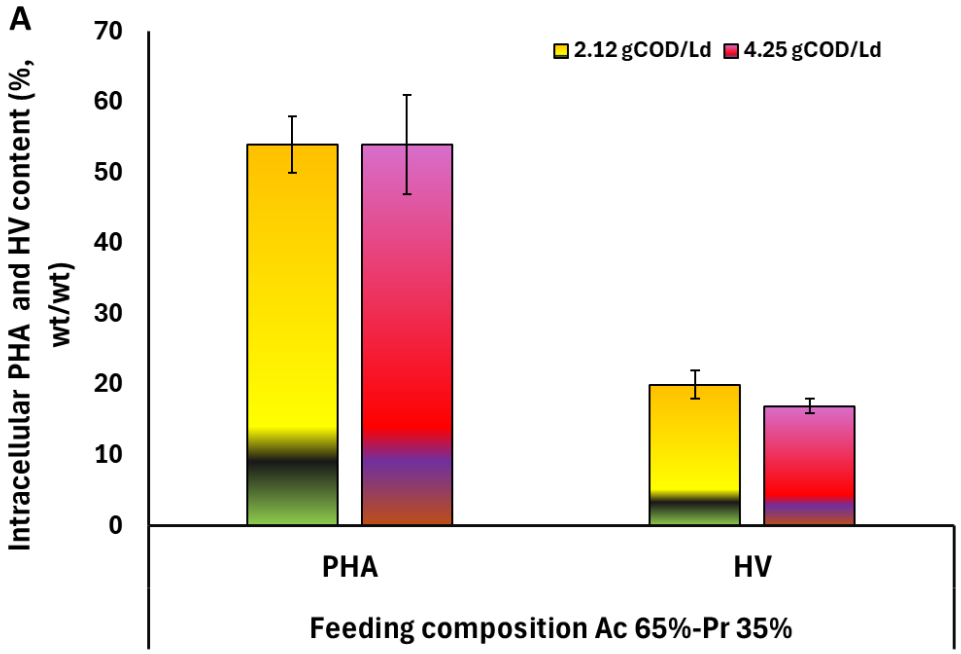
The results herein discussed represent all the data collected monitoring the whole continuous-flow process (i.e., feast, famine, and accumulation reactors) for around 2 years of operation, with main focus on the performance of the accumulation reactor. Preliminary data of these results have also been presented in M12, delivered on September 2024.

During the system operation, a fungi contamination occurred, which caused a substantial decrease in the process performance in terms of PHA storage. Therefore, the system operation was stopped and re-activated with a new inoculum

As previously stated, during the experimentation, the selection stage (feast and famine-FF reactors) was operated at two OLR (i.e., 2.12 and 4.25 gCOD/Ld) at a fixed R_C (equal to 8), which represents the ratio between the recirculation flow rate from the famine to the feast reactor and the influent flow rate to the feast reactor, whereas the accumulation reactor was operated at an applied OLR of 6.12 gCOD/Ld. Figure 22A reports the performance of the process before the fungi contamination in terms of intracellular PHA content in the accumulation reactor, with the biomass selected in the selection stage at both tested OLRs. A very similar value of the intracellular PHA content was obtained, accounting for 54 ± 4 (%, wt/wt) with the biomass selected at 2.12 gCOD/Ld and 54 ± 7 (%, wt/wt) with the biomass selected at 4.25 gCOD/Ld. During the overall experimentation, the poly(hydroxybutyrate-co-valerate) (PHBV) copolymer was obtained. Similarly, to the intracellular PHA content, the HV content also resulted in a comparable value (of 20 ± 2 and 17 ± 1 %, wt/wt) with the two investigated conditions. These results indicate that the OLR increase in the selection reactors did not significantly affect PHA productivity and consequently even the monomeric HV content.

Although the obtained HV content was in line with the targeted one (i.e., 20 - 35 %, wt/wt), the system after several months of operation underwent fungi contamination, and the FF reactors were inoculated again with a “fresh” activated sludge. After the system re-start, the same feedstock composition as for the previous operation period (i.e., 65% and 35% of acetic and propionic acid on COD basis, respectively) was adopted. Once reached the steady-state conditions for the FF reactors, the accumulation reactor was also operated again and, as reported in Figure 22B, the HV content in the stored polymer reached an average

value (47± 3 %, wt/wt) higher than that obtained prior the contamination (Figure 22A). This increase in the HV content by adopting an identical feedstock composition, could be theoretically attributed to the selection of a different community of PHA-storing microorganisms. Therefore, to decrease the HV content in the stored polymer and achieve the targeted value, it was decided to modify the feedstock composition by decreasing the propionic acid percentage from 35% to 25 % (on COD basis). This resulted in a decrease in the HV content (on average from 47 ± 3 to 26 ± 1%, wt/wt) in the polymer stored in the accumulation reactor (Figure 22B). Additionally, the intracellular PHA content increased following the feedstock composition modification (on average from 35 ± 2 to 57 ± 3 % (wt/wt) before and after the feedstock composition modification, respectively.



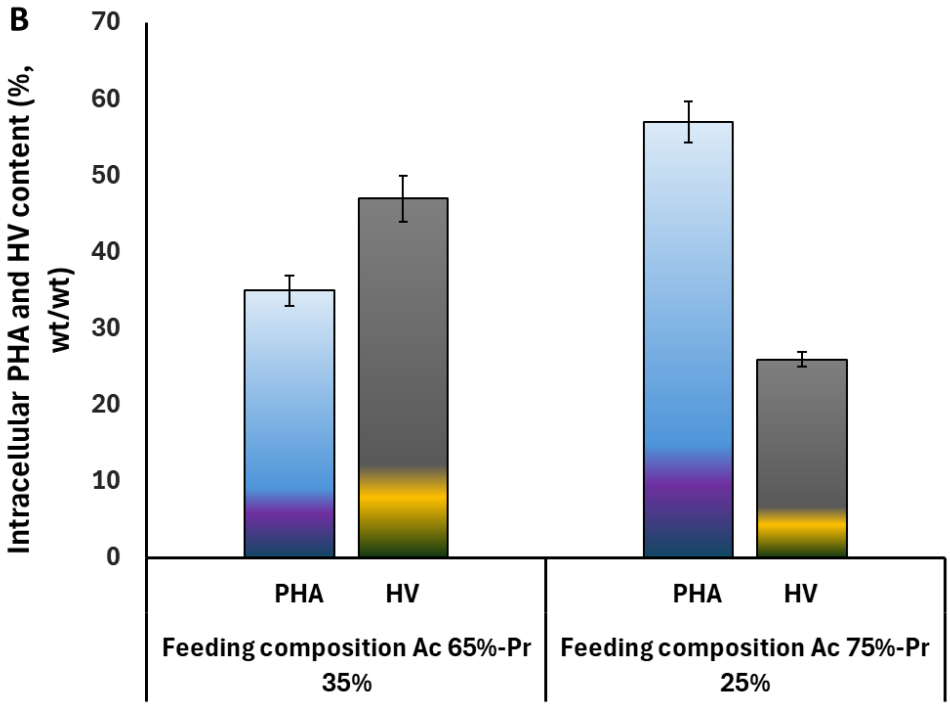


FIGURE 22: A) EFFECT OF THE OLR APPLIED TO THE FF SYSTEM ON THE INTRACELLULAR PHA CONTENT AND MONOMERIC POLYMER COMPOSITION IN THE ACCUMULATION REACTOR. B) IMPACT OF THE FEEDSTOCK COMPOSITION TO THE ACCUMULATION REACTOR ON THE INTRACELLULAR PHA CONTENT AND HV CONTENT. ERROR BARS REPRESENT ± STANDARD ERROR OF THE AVERAGE MEASUREMENTS TAKEN OVER THE COURSE OF EACH RUN.

After the process optimization to achieve the targeted PHA composition, the daily produced polymer was collected, stabilized, extracted, and purified according to the procedure described in section 1 of Material and Methods.

Indeed, once the PHA is produced, it is necessary to immediately inactivate or stabilize the biomass to avoid its consumption by the bacteria. Two stabilization procedures were tested (i.e., acid stabilization and thermal stabilization) and, after biomass stabilization, the stored polymer was recovered by using NaOH (0.2 M) and H_20_2 (1.5 %, w/v), resulting in a final PHA purity up to 82 ±7 %, wt/wt. Figure 23 reports data of polymer purity during each step of the extraction and recovery procedure applied on the biomass collected from the continuous-flow accumulation reactor to perform ad-hoc tests aimed at comparing the two investigated stabilization approaches. A higher PHA purity grade was obtained with the acid treatment, which acted as both stabilization method and pre-treatment step of the biomass due to the partial degradation of the cell membrane. In addition, these results emphasize that the H_2O_2 purification after the digestion with hydrolyzing agents was required in order to enhance the final polymer purity (over 80 %, wt/wt).

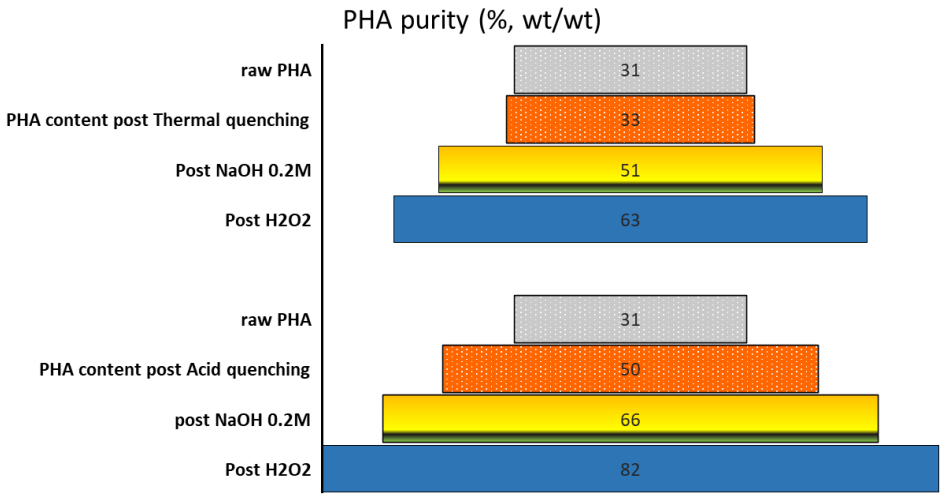


FIGURE 23: VARIATION OF PHA PURITY BASED ON THE EXTRACTION AND RECOVERY STEPS APPLIED TO THE BIOMASS HARVESTED FROM THE ACCUMULATION REACTOR (THE RAW BIOMASS REPRESENTS THE PHA-RICH BIOMASS FROM THE ACCUMULATION REACTOR).

Overall, Tables 16 and 17 summarize all samples of recovered PHA and PHA-enriched biomass which were collected during the UNIROMA activity and sent to the different project partners.

TABLE 16: EXTRACTED PHA RECOVERED THROUGHOUT THE RESEARCH ACTIVITY, CHARACTERIZED (IN TERMS OF PURITY, HV CONTENT, AND MOLECULAR WEIGHT), AND SENT TO PARTNERS INVOLVED IN WP4 (I.E., UM AND UNIBO).

Date of shipping	Type of PHA	PHA Purity (%, wt/wt)	HV content (%, wt/wt)	Quantity sent (g)	Molecular weight (kDa)	Delivered to (Partner)
26 April 2024	PHBV	44 ± 6	40± 3	25	629	UM (WP4)
4 July 2024	PHBV	74± 4	57± 4	25	-	UNIBO (WP4)
31 July 2024	PHBV	99 ± 1	27± 1	23	n. d	UM (WP4)
10 January 2025	PHBV	76 ± 1	22 ± 2	33	1700	UM (WP4)

10 January 2025	PHBV	96± 2	20± 1	5	n. d	UM (WP4)
10 January 2025	PHBV	96 ± 4	22± 4	34	2400	UNIBO (WP4)
3 June 2025	PHBV	74± 2	48± 5	15	n. d	UM (WP4)
3 June 2025	PHBV	84 ± 1	56 ± 3	6	n. d	UNIBO (WP4)

TABLE 17: PHA-RICH BIOMASS HARVESTED BY UNIROMA AND DISTRIBUTED TO OTHER PARTNERS INVOLVED IN TASK 3.4 (I.E., CSIC AND ENTO) DEDICATED TO PHA EXTRACTION.

Date of shipping	To (Partner)	Pre-treatment	Type of product	PHA content (%, wt/wt)	HV content (%, wt/wt)	Qty sent (g)
13 February 2024	CSIC (WP3)	acidified to pH2 with H_2SO_4	Dried PHA-Rich biomass	35 ± 4	20 ± 2	48
24 May 2024	ENTO (WP3)	acidified to pH2 with H_2SO_4	Fresh liquid PHA-Rich biomass	48 ± 2	52± 4	600 mL
24 May 2024	ENTO (WP3)	acidified to pH2 with HCl	Fresh liquid PHA-rich biomass	38 ± 4	42 ± 1,2	600 mL
31 July 2024	UM (WP4)	acidified to pH2 with H_2SO_4	Dried PHA-Rich biomass	42 ± 4	33 ± 2	16.7
23 January 2025	CSIC (WP3)	Quenched at 4°c	Dried PHA-Rich biomass	37,5 ± 3	21 ± 2,8	38

23 January 2025	CSIC (WP3)	Quenched at 4°c	Dried no PHA biomass	0	0	4,8
23 January 2025	CSIC (WP3)	Quenched at 4°c	Fresh no PHA biomass	0	0	28
23 January 2025	CSIC (WP3)	-	PHA production broth	-	-	500 mL
20 June 2025	CSIC (WP3)	acidified to pH2 with H_2SO_4	Fresh PHA-Rich biomass	52 ± 3	54 ±5	31
20 June 2025	ENTO (WP3)	acidified to pH2 with H_2SO_4	Fresh PHA-Rich biomass	45± 6	40± 6	50

Extracellular polymeric substances (EPS) recovery

The composition of the recovered EPS, in terms of total carbohydrates and proteins, is reported in Table 18.

TABLE 18: COMPOSITION OF THE RECOVERED EPS

EPS recovery (mg $VS_{EPS}/gVSS$)	Total carbohydrates (mg Alg/g VS_{EPS})	Total proteins (mg BSA/g VS_{EPS})	Proteins/Carbohydrates
222 ± 11	154 ± 17	752 ± 30	5.1± 0.7

The FTIR spectrum (4000–500 cm^{-1}) of the recovered EPS exhibits a broad O–H stretching band at approximately 3300 cm^{-1}, C–H stretches near 2920 cm^{-1}, and strong amide I (~1624 cm^{-1}) and amide II (~1537 cm^{-1}) bands. The bands at around 1414-1400 cm^{-1} corresponds to the symmetric stretching of -COO groups in uronic acids, while the band at 1200–900 cm^{-1} features C–O–C stretches characteristic of polysaccharides Figure 24 (Lin et al., 2013).

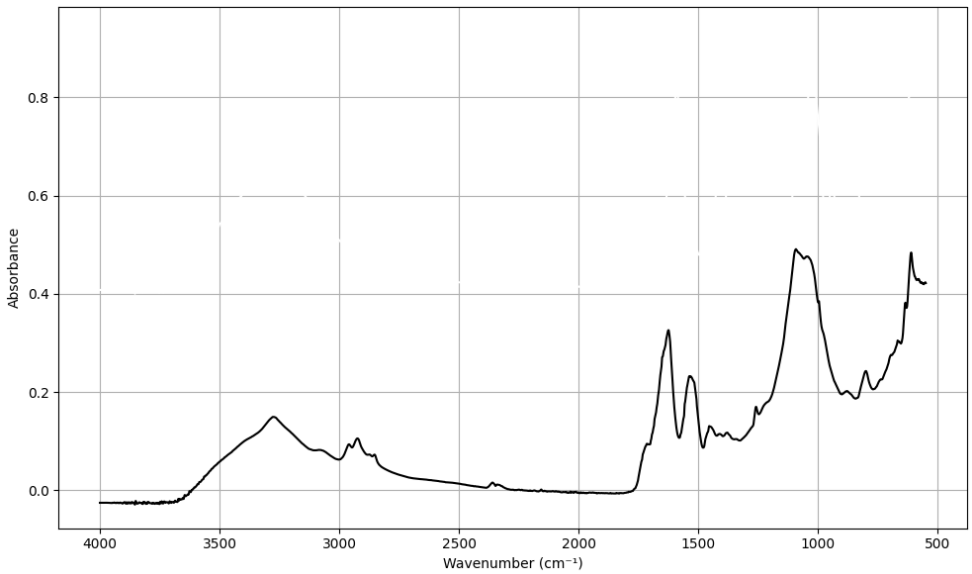


FIGURE 24: FTIR SPECTRA OF THE RECOVERED EPS

The presence of uronic acids suggests a gel-forming propriety with bivalent cation which was then investigated as described in Figure 25 (Felz et al., 2016).

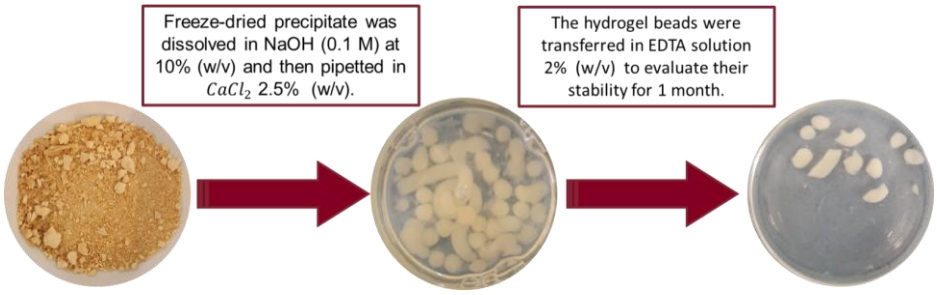


FIGURE 25: EPS- GEL-FORMING PROPERTIES TEST: HYDROGEL FORMATION AND STABILITY TEST

After 1 month in EDTA solution, the hydrogel beads maintained their shape indicating a good stability over time.

PHA extraction

This section describes two PHA extraction approaches investigated by UNIROMA in the frame of Task 3.4. Part of the herein reported results have also been included in M13, delivered on November 2024 (M24).

- PHA extraction by supercritical CO_2

A lab-scale stainless-steel Supercritical Fluid Extraction (SFE) reactor was used to carry out the cell lysis (Carlo Erba Instruments, Milan, Italy), with a cell reactor volume of 1 mL. Figure 26 shows the diagram of the laboratory system used for the sc-CO_2 treatment of microbial biomass. Liquid CO_2, stored under compression in a 30 kg cylinder, is pumped from a reservoir through a syringe pump (Carlo Erba Instruments) whose operation is controlled by a dedicated software. The liquid CO_2 is compressed inside the syringe at a higher pressure at the critical pressure (72.9 bar). The syringe pump has an outer jacket refrigerated by a thermocryostat (Julabo F12, Seelbach, Germany) which avoids the gasification of CO_2. To this point, the movement of the piston inside the syringe is controlled via software when the target pressure value is reached. Reaching the target pressure inside the syringe pump, monitored by a pressure sensor, allows the CO_2 to subsequently reach the extraction cell housed in a thermostatic chamber heated to a temperature higher than the critical temperature (TC = 31 °C). In this way, the CO_2 in the cell is under supercritical conditions. The cell lysis treatment involved the use of approximately 500 mg of dry biomass granules which were placed in the extraction cell.

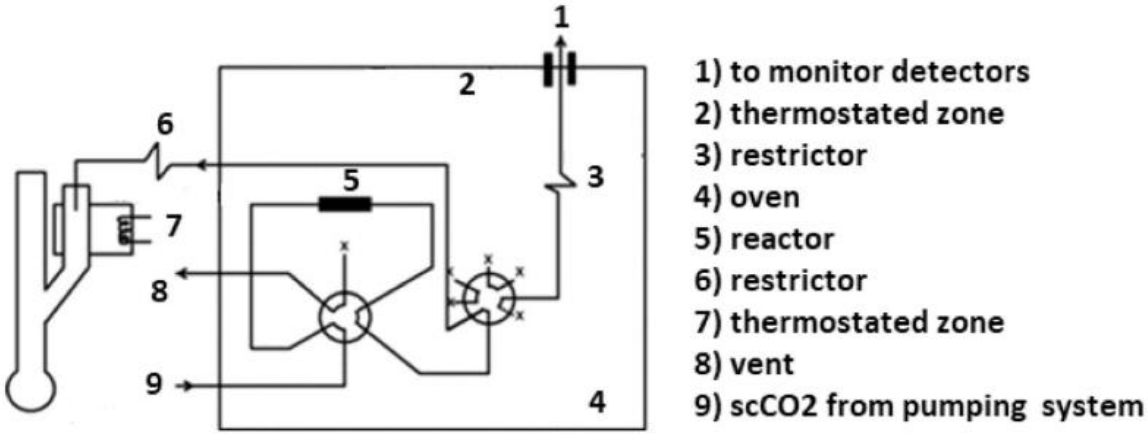


FIGURE 26: SCHEME OF THE LABORATORY SYSTEM USED FOR THE SCCO2 TREATMENT OF MICROBIAL BIOMASS (HAGHIGHI ET AL., 2026).

The extractions took place in a metal tubular reactor of 1 cm^3 where the samples were introduced. For each experiment, 300 mg of dry biomass was placed in the extraction cell. The operating parameters were varied in the pressure range of 30–40 MPa and the temperature range of 40–80 ˚C. A scheme of the extraction process is shown in Figure 27.

FIGURE 27: SFE SCHEMATIC PROCESS OF THE RAW BIOMASS ENRICHED INTO PHA

TABLE 19: SIX APPLIED PROTOCOLS WITH SC-CO_2 FOR PHA RECOVERY

n. Protocols	Procedures	samples	composition
1	20 MPa, (40 °C), 2 h + H_2O	UNIROMA	PHBV
2	H_2O_2 (2.5 % wt/v) 18h → 20 MPa,(40 °C), 2 h + H_2O	UNIROMA	PHBV
3	H_2O_2 (2.5 % wt/v) 18h → 20 MPa, (40 °C), 2 h → Lipase (50 mg/mL) + H_2O	UNIROMA	PHBV

4	30 MPa, (40 °C), 2 h + H_2O	NID	PHBVH
5	30 MPa, (80 °C), 2 h → H_2O_2 (2.5 % wt/v) + H_2O	NID	PHBVH
6	30 MPa, (80 °C), 2 h → Lipase (50 mg/mL) + H_2O	NID	PHBVH

Herein, two different PHA-rich dry biomasses (collected by UNIROMA and NID) were extracted with sc-CO_2. Both samples were collected from an accumulation step to obtain PHA-enriched biomass. In particular, the initial PHA content accounted for 43 ± 1.2 and 60 ± 1 % (wt/wt) for the UNIROMA and NID samples, respectively. In addition, UNIROMA biomass is a poly(hydroxybutyrate-co-valerate) (PHBV) copolymer with an HV content of 75 (%, wt/wt), whereas the NID biomass was a poly(hydroxybutyrate-co-valerate-co-hexanoate) (PHBVH) terpolymer with a content of HV and HH accounting for 17 and 3 (%, wt/wt). During the experimental period, six different protocols were performed on both samples. Table 19 shows the various treatments applied on the UNIROMA and NID biomass. More in detail, the treatments consist of sc-CO_2 operated at different conditions of pressure and temperature, the combination of sc-CO_2 and a chemical agent such as H_2O_2 (as a pre- and post-treatment before sc-CO_2 extraction), and the combination of sc-CO_2 and an enzyme (i.e., lipase). Each sample was characterized in terms of PHA purity and composition (%, wt/wt), measured by a GC method, as reported in analytical measurements section.

Protocols number 1, 2, and 3 (Table 19) have been applied to the PHA-rich biomass collected by UNIROMA and protocols number 4, 5, and 6 to the biomass collected by NID.

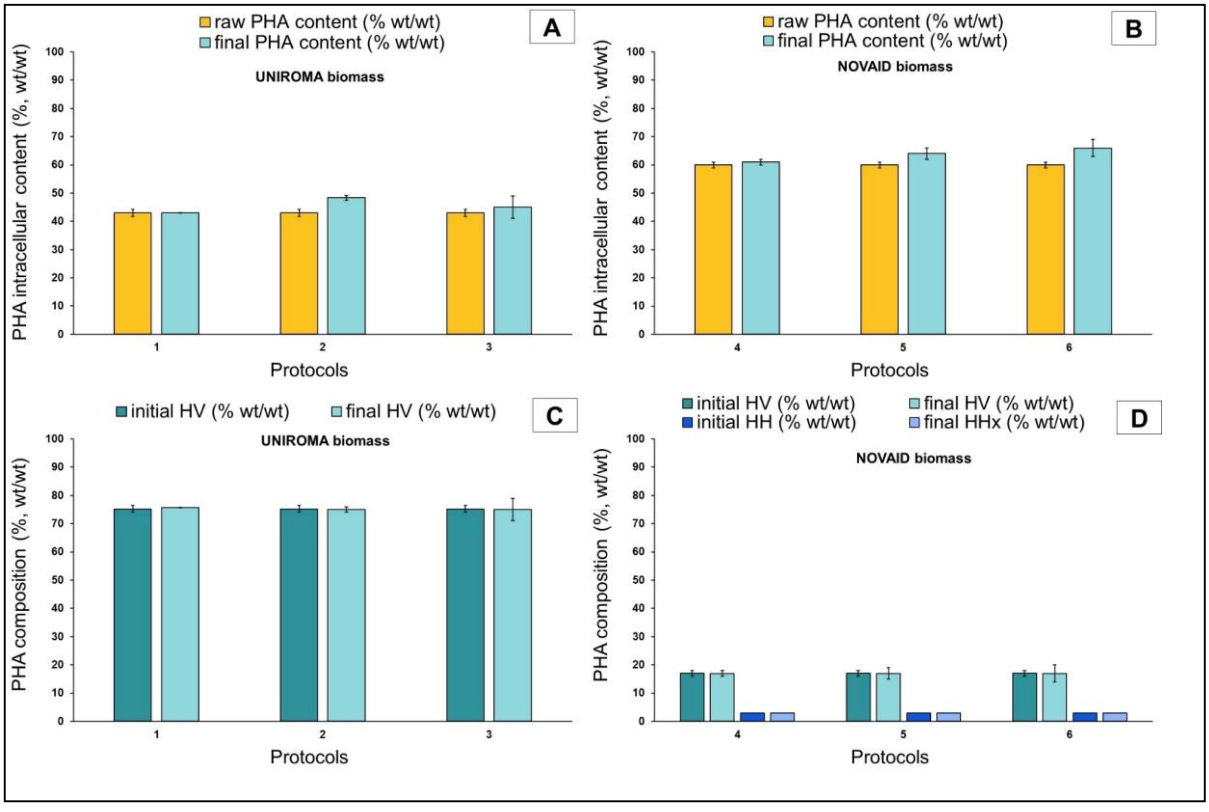


FIGURE 28: INITIAL (YELLOW BAR) AND FINAL (GREEN WATER BAR) PHA CONTENT DETERMINED IN UNIROMA (9A) AND NID (9B) SAMPLES. PHA MONOMERIC COMPOSITION OF UNIROMA (9C) AND NID (9D) SAMPLES. DATA REPRESENTS THE AVERAGE OF TWO REPLICATED TESTS.

Figures 28A and 28B highlight the results in terms of PHA content of raw biomass with any treatment used and the final PHA content determined after the treatment. Protocols 1 and 4 did not allow to increase the PHA content, suggesting that the action of sc-CO_2 likely consisted of just disintegrating the cellular materials. The other treatments with H_2O_2 (2.5 % wt/v) and lipase (50 mg/mL) (i.e., protocols 2, 3 and 5, 6) as enzymatic agents showed a slight increase in the PHA content. It is worth noting that the applied treatments did not affect the sample composition (Figures 28C and 28D).

The presented results demonstrate that sc-CO_2 treatment with the tested conditions is not efficient due to the difficult separation of polymer granules from the disrupted biomass. However, preliminary experiments have been conducted by combining sc-CO_2 method with organic solvents, as described in the following section.

- PHA extraction with Ethyl Acetate (EtOAc)

The PHA-rich biomass sample collected from UNIROMA was also tested for biopolymer recovery with green solvents. This extraction is based on the formation of a biphasic system composed of ethyl acetate (EtOAc) and water (H_2O) to obtain the separation of PHBV granules from the surrounding biomass. Hydroxy-ethyl cellulose (HEC) was also used as a thickening agent to improve the separation of PHBV granules. In both cases, this method is herein referred to as EtOAc: H_2O. More in detail, HEC allows for enhancing the separation between biomass and proteins on the granule's surface, which will result in emulsion formation.

The protocol includes four main steps, also illustrated in Figure 29:

1) Mechanical disruption of dry biomass through homogenization in EtOAc at swelling temperature (T_{sw}).

2) Swelling of PHBV granules at T_{sw} to easily separate them from the biomass

3) Addition of water or an aqueous solution of HEC (EtOAc: H_2O or HEC ratio of 1:6)

4) PHBV-rich phase withdrawal and polymer drying

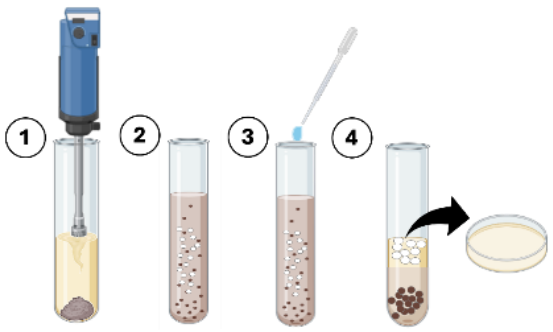


FIGURE 29: SCHEMATIC REPRESENTATION OF PHBV EXTRACTION WITH BIPHASIC ETOAC-H_2O SYSTEM.

Steps 1) and 2) were conducted at PHBV swelling temperature (T_{sw}= 65 °C), previously determined through swelling tests. The PHA-enriched biomass produced from UNIROMA was also extracted with chloroform in order to compare the results with respect to the novel developed method employing green extraction. The purified polymer films of PHBV obtained from chloroform extraction were then used for the swelling tests. The swelling tests were performed using small pieces (0.5 mm thickness) of PHBV film and put into contact with EtOAc at 25 °C. Then the temperature increased by 5°C steps and visible changes in film volumes were detected and taken as a signal of the swelling structure. The determined T_{sw} is taken as an indication of swelling phenomena, but it is worth noting that the swelling behaviour of intracellular copolymers could be slightly different due to the morphological features of PHA granules. More in detail, temperature and time of swelling for intracellular granules may be higher because of the more difficult permeation of solvents into the complex biomass structure. Anyway, preliminary extraction experiments have been conducted considering T_{sw} of PHBV films as the effective swelling temperature. The effect of different parameters, such as swelling time (t_{sw}) and HEC concentration has been studied on the UNIROMA biomass sample. Investigated t_{sw} were 5 and 30 min, while HEC concentrations were 0.25, 0.5, and 1 (%, wt/v). The alternative biphasic method has been applied also to biomass pre-treated with sc-CO_2. To verify the effectiveness of sc-CO_2 in disrupting cells, the biomass treated with only sc-CO_2 without any other chemical agent (1st protocol) has been used. Then, biomass treated with 2nd protocol (H_2O_2+ sc-CO_2+ H_2O) has been selected for successive experiments. This choice has been made based on results illustrated in Figure 28A, in which a slightly higher value of PHA final content is reported. The combination of sc-CO_2 and biphasic extraction is expected to result in an increase in PHBV recovery with respect to single methods thanks to the ability of sc-CO_2 to disrupt cell walls and the good biomass-polymers separation obtained with the biphasic system.

For these experiments, the following conditions have been used: HEC concentration of 0.5 % (wt/v), t_{sw} = 30 min, and T_{sw}= 65 °C.

Results from the chromatographic characterization of PHBV extracted with the biphasic method are reported in Figure 30.

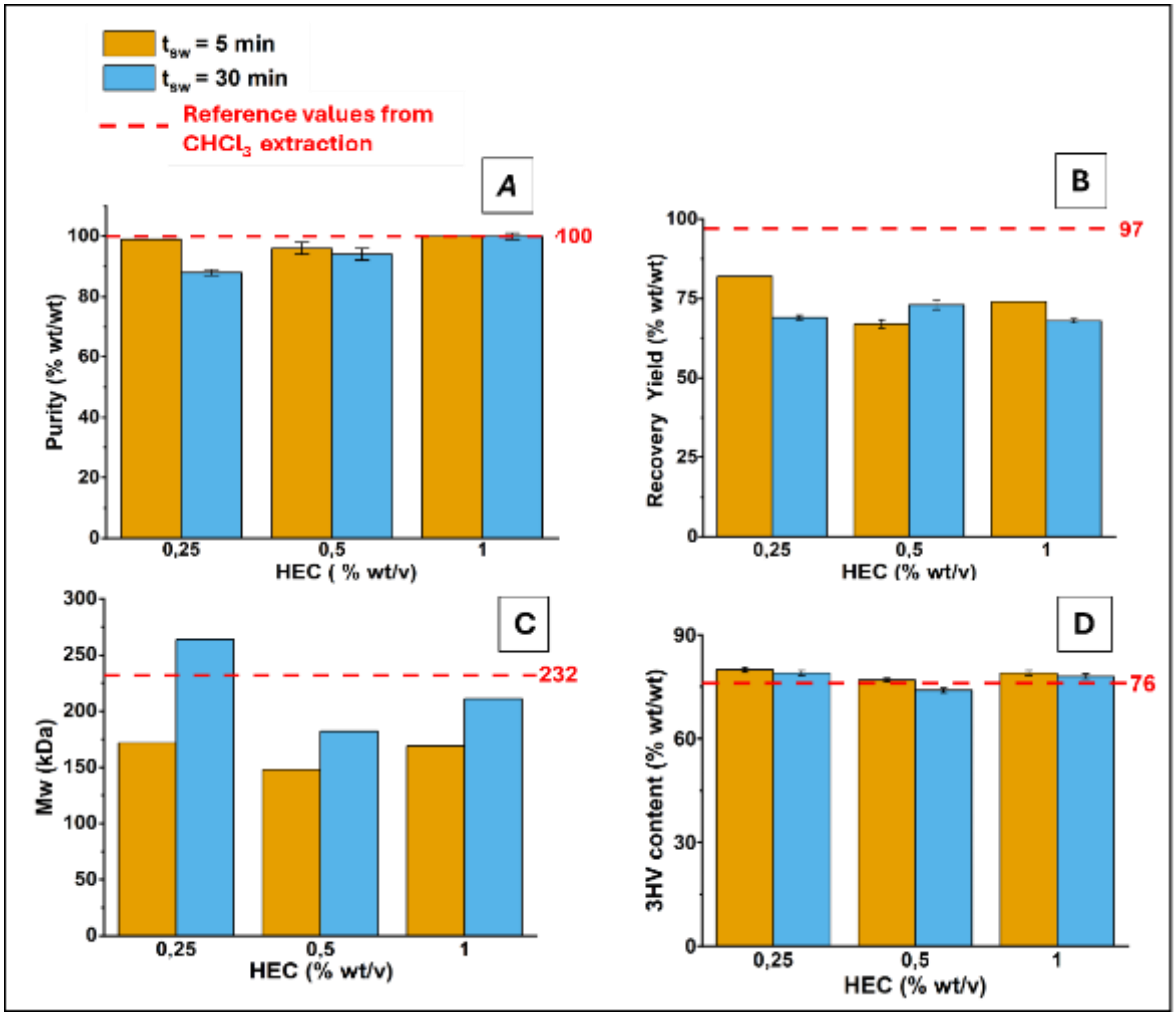


FIGURE 30: RESULTS FROM EXTRACTED PHBV SAMPLES: A) PURITY, B) RECOVERY YIELD, C) MOLECULAR WEIGHT, AND D) MONOMERIC COMPOSITION. DATA REPRESENT THE AVERAGE RESULTS OF TWO REPLICATED TESTS.

In addition, all samples were characterized in terms of average molecular weight (M_W) by GPC. Preliminary experiments were carried out using EtOAc and water. The recovered PHBV is characterized by quite high purity, slightly below the reference values from $CHCl_3$ extraction (87 ± 5 %, wt/wt with respect to 100± 1 % wt/wt). Despite the good purity, a low recovery yield was obtained (17± 4 %, wt/wt) with respect to $CHCl_3$ reference (97 %, wt/wt). This is likely due to the formation of an emulsion which hindered the effective separation of the PHBV granules from the biomass, leading to the recovery of small quantities of polymers. The optimization of this method by using HEC was then studied. The properties of recovered PHBV are reported in Figure 30. Values from $CHCl_3$ extraction are reported as a comparison. High-purity PHBV was recovered for all the HEC concentrations and t_{sw} (Figure 30A) meaning that the tested conditions did not impact the purity of the extracted polymer. The recovery yield, shown in Figure 30B, was lower than that obtained with standard chloroform extraction (about 70 % wt/wt compared to 97 % wt/wt). However, there is a significant increase compared to the recovery yield reached without HEC (17± 4 % wt/wt). Also, the extraction yield was not significantly affected by the HEC concentration and t_{sw}. All the tested conditions resulted in recovery yields higher than those obtained with the biphasic system. The green extraction method led to a slight Mw decrease (from 225 KDa to about 150 KDa), especially at 5 minutes of swelling (Figure 30C). On the other hand, passing from t_{sw} = 5 to 30 minutes Mw increased. This may be due to

slower swelling kinetics and separation of the macromolecules with higher Mw, which likely require more time. The monomeric composition (Figure 30D) was not affected by using HEC at any concentration nor by t_{sw}. For this reason, the composition was comparable to PHBV extracted with chloroform. According to these results, it is possible to conclude that using the lower quantity of HEC (0.25 %, wt/v) combined with t_{sw} = 5 min results in the recovery of a high-purity PHBV with high yields. The quality of recovered PHBV is improved by increasing the swelling time to 30 minutes which results in higher molecular weight. Additionally, no traces of HEC were found in the extracted PHBV. On the other hand, HEC was found in the biomass and preliminary tests for its recovery and reuse are being conducted.

The illustrated method has been applied to samples previously treated with sc-CO_2 (1st and 2nd protocol). The sc-CO_2 treated biomasses were used as received. No washing, filtering and/or drying steps were added to those included in the sc-CO_2 treatment illustrated in Figure 26. The properties of extracted PHBV are reported in Table 20. As it is possible to note from Table 20, the combination of sc-CO_2 treatment with EtOAc led to an improvement in purity with respect to the exclusive use of sc-CO_2. (1st protocol combined with EtOAc). However, the coupled treatment did not improve the performance of the EtOAc method. These results do not justify the choice of such a time-consuming and costly extraction procedure. For this reason, no further studies have been conducted with the coupled sc-CO_2 and green solvent extraction.

TABLE 20: PURITY, RECOVERY YIELDS, 3HV CONTENT, AND MOLECULAR WEIGHT OF THE SAMPLES TREATED WITH SC-CO_2 AND A COMBINATION OF THE LATTER METHOD WITH ETOAC.

SAMPLE	Purity (% wt/wt)	Recovery Yield (%wt/wt)	3HV content (wt/wt)	Mw (kDa)
Reference ($CHCl_3$)	100 ± 1	97	76	232
1st protocol	43 ± 0.1	-	75	-
1st protocol +EtOAc	100	55	78	160
2nd protocol	48 ± 0.9	-	75	-
2nd protocol + EtOAc	99 ± 2	66	74	175

By taking into account the above reported results, the optimal protocol applied on UNIROMA samples involves 5 minutes of biomass homogenization, swelling at T_{sw} at approximately 65°C for 30 minutes and HEC concentrations fixed at 0.5 (%, wt/v). This protocol has been tested on the PHA-enriched biomass produced from the continuous-flow process (described in section 2). In particular, a set of PHBV samples characterized by HV monomeric content ranging from 26 to 74 (%, wt/wt) was recovered with the biphasic strategy aiming to identify the possible effect of the copolymer composition on the extraction efficiency. All the recovered samples were characterized in terms of purity, monomeric composition, recovery yield and molecular weight. In addition, their properties and recovery yields were compared with those obtained from the standard benchmark extraction procedure adopting chloroform. In Table 21. Results from the characterization of the samples purified with green solvent (i.e., EtOAc) are reported.

Table 21: Characterization of the samples extracted with the benchmark method (first row) and with biphasic method (second row, in bold).

sample name	PHA raw biomass (%, wt/wt)	HV (%, wt/wt)	Purity (%, wt/wt)	Recovery (%, wt/wt)	Mw (KDa)	PDI
PHBV_26	48	26.0	100.0	63.4	542.0	2.0
		26.0	**65.0**	**57.0**	**525.0**	**2.0**
PHBV_32	40	32.0	100.9	67.0	558.0	3.4
		32.0	**59.0**	**54.8**	**624.0**	**2.3**
PHBV_56	78	56.0	100.0	n.d.	486.0	2.6
		56.0	**81.0**	**20.4**	**578.0**	**2.9**
PHBV_74	43	74.0	100.0	96.6	232.0	1.6
		74.0	**82.0**	**73.0**	**181.9**	**2.0**

As shown in Table 21, the HV content of PHBV recovered by biphasic method is comparable to that obtained from chloroform extraction, indicating that the proposed method does not affect monomeric composition and, consequently, the final properties. However, both purity and recovery yields were lower than the values obtained with standard chloroform extraction. In particular, the polymer purity reached values of 82 and 81 % (wt/wt) without any pre-treatment. Further investigations are required to understand if the low recovery yield values could be associated to the complex biomass morphology which hinder the complete release of PHBV granules. On the other hand, it should be considered that few milligrams of biomass were used for each experiment and a significative loss of extracted PHBV (possibly taking place during PHBV-rich organic phase withdrawal) likely occurred. To verify this hypothesis, the extraction protocol should be tested on larger biomass amounts.

Alfano and colleagues reported higher values of recovery yield when EtOAc extraction was applied to the PHA-enriched biomass (Alfano 2021). Notably, in the previous work, the extraction was conducted at higher temperature and pressure (several temperatures ranging from 100 to 150 °C were tested, reaching a maximum pressure of 7 atm for T= 150 °C). Under these conditions, the recovery yield reached values of 66-71 (%, wt/wt) (depending on the temperature) suggesting a better dissolution of the PHA-enriched biomass into the solvent. Figure 31 highlights the performance of green solvent extraction on the biomass characterized by different HV monomeric content. The monomeric content did not substantially affect the performance of green extraction. In particular, these findings suggest the possibility to apply this procedure also on the biomass enriched into medium chain length polymer (e.g., PHBVH).

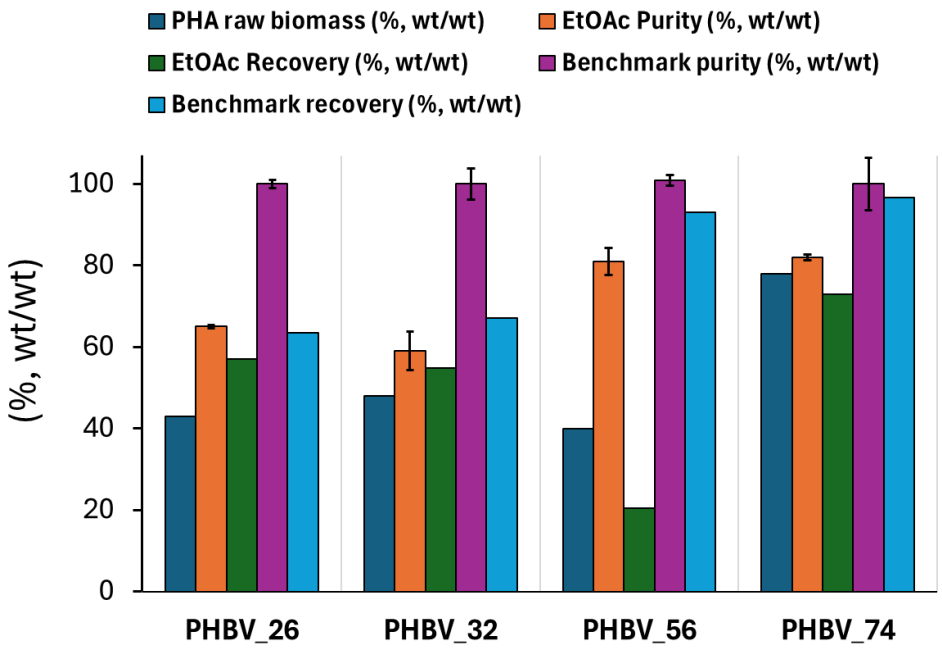


FIGURE 31: RESULTS OF THE PURITY AND RECOVERY OF THE PHBV-ENRICHED BIOMASS OBTAINED WITH ETOAC AND BENCHMARK EXTRACTION PROCEDURES.

Furthermore, the molecular weight (Figure 32) showed no significant differences between samples extracted with the two different methods. These findings suggested that the swelling temperature -fixed at around 65°C- did not induce polymer degradation.

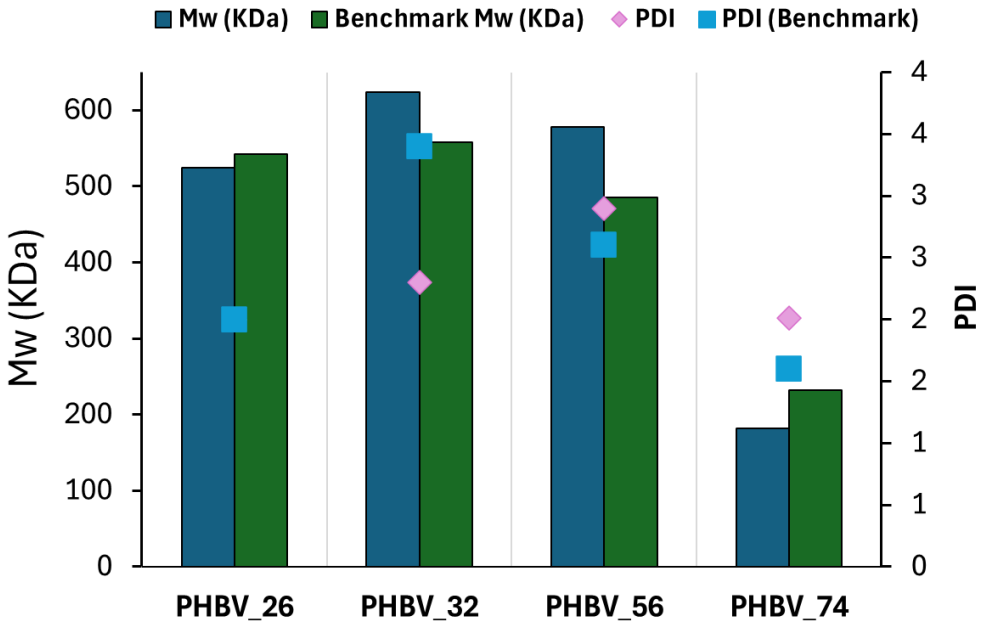


FIGURE 32: RESULTS OF MOLECULAR WEIGHTS (MW) ANALYSIS OF THE PHBV-ENRICHED BIOMASS OBTAINED WITH ETOAC AND BENCHMARK EXTRACTION.

Overall, the observed differences in purity, recovery yields and molecular weight of extracted samples does not show a coherent trend related to the 3HV content. These diversities may therefore be ascribed to the biomass morphology or technical limitations due to biomass quantity and lab-scale equipment. Based on these findings, it could be assumed that the monomeric composition of the PHBV contained in the raw biomass does not affect the entire extraction process.

In this view, the composition of the copolymer, characterized by low or high HV monomeric content (i.e., 26 to 56 %, wt/wt), did not affect the extraction process.

In addition, the PHA-enriched biomass produced from NID was also recovered applying the biphasic protocol.

The extracted sample was characterized in terms of PHA content, composition (terpolymer, PHBVH), and Mw. Then the results were compared with chloroform extraction (Table 22). The purity value of EtOAc extraction was 72 %, wt/wt not so far from the value obtained in the previous experiments on PHA-enriched biomass produced by UNIROMA. However, both chloroform and EtOAc extraction resulted in low recovery yields (27 and 28 %wt respectively). These findings may be attributed to the powdered form of biomass, a factor that may limit the complete polymer separation.

Furthermore, the composition of terpolymer did not affect the green extraction performance.

TABLE 22: CHARACTERIZATION OF THE PHA-ENRICHED BIOMASS PRODUCED FROM NID PARTNER AND RECOVERED WITH CHLOROFORM AND ETOAC EXTRACTION METHODS.

Sample	PHA content (%, wt/wt)	HV content (%, wt/wt)	HHx content (%, wt/wt)	Mw (KDa)	PDI
Raw biomass	49	20	4	—	—
$CHCl_3$ extraction	104	19	4.4	1135	2.6
EtOAc extraction	72	20	4	1560	2.1

Conclusions

The results discussed in the present section of the Deliverable emphasize the feasibility to produce PHA-enriched biomass with an intracellular biopolymer content over 55 % (wt/wt) exploiting a continuous-flow multi reactor process. In addition, the produced PHBV copolymer was characterized by the target HV monomeric content ranging from 20 to 35 % (wt/wt). Since the feedstock used during the experimentation consisted of a mixture of synthetic carboxylic acids, current research is being focused on the use of real feedstock (i.e., fermented of wine lees provided by UGent). Furthermore, applying the combined treatment of NaOH and H_2O_2 on the PHA-rich biomass, other high added value compounds along with PHA recovery can be obtained, such as Extracellular Polymeric Substances (EPS) or alginate like extracellular polymers having similar properties with commercial alginate. This highlights the sustainability of the continuous-flow process to be used for a wide range of applications to recover valuable side products.

The role of sc-CO_2, the application of green solvent (i.e., EtOAc) as well as a combination of both treatments was evaluated for PHA extraction from microbial cells. The obtained results point out that, regarding the combined treatment, the application of sc-CO_2 did not allow to enhance the performance of the green solvent procedure. Indeed, high results in terms of PHA purity, comparable with the standard benchmark procedure (i.e., chloroform), were collected applying solely the green solvent on the biomass without the sc-CO_2 pre-treatment. In addition, the novelty of green solvent approach with EtOAc consists of reducing the contact time between solvent and biomass. In this context, the polymer recovery is facilitated by its swelling into the organic phase. This could positively affect the extraction process costs as well as the sustainability of the process that is usually performed with chlorine solvents.

Furthermore, the green solvent extraction was also applied to the biomass produced from NID. In this scenario, the HHx monomer content in the produced PHBVH terpolymer did not affect the performance of the adopted extraction procedure in terms of PHA purity and Mw.

IV. Polyhydroxyalkanoates recovery from biomass

IV.I. By predatory bacteria (CSIC)

3.1 Preliminary assays to determine predatory capacity (pure and mixed cultures)

The biomass enriched into PHA was treated using the predatory bacterium *Bdellovibrio bacteriovorus* HD100. The PHA-accumulating biomass was quenched by acid treatment at the facilities of the partners responsible for biomass collection (i.e., UNIROMA and NID) to prevent polymer degradation. In addition, UNIROMA biomass was oven-dried to ensure its stability and to facilitate sample delivery. Bacterial predation is known to be a multifactorial process, relying on a wide range of enzymes that act on multiple targets, including active metabolites and prey-membrane morphology (Caulton and Lovering, 2023). Therefore, considering that this acid pre-treatment and the use of rehydrated prey might affect the ability of *B. bacteriovorus* to prey upon the PHA producers, preliminary tests were applied on *Pseudomonas putida* KT2440, as a model prey, in order to evaluate the impact on predation capacity. Following the protocol previously established by UNIROMA, after PHA production, the pH of the culture broth is reduced to 2 by adding sulphuric acid. The biomass is then collected by centrifugation and subjected to a drying or freeze-drying process.

Due to *Bdellovibrio*'s predatory lifestyle, several methods have been developed to quantify these bacteria, including the double-layer technique. However, this method requires extensive preparation and, more importantly, does not allow real-time monitoring of predation. Flow cytometry offers a more efficient way to track predation in real time. Therefore, as a first approach, the recombinant strain BdG (*B. bacteriovorus* HD100 with a genomic insertion containing the gene encoding the msGFP protein) has been specifically designed for the Agriloop project using hierarchical assembly cloning (GG technique). Predatory capacity after acid pretreatment was analysed using both fresh and oven-dried *P. putida* KT2440 To obtain a PHA accumulating biomass, *P. putida* was previously cultured in 0.1N M63 minimal medium supplemented with 15 mM octanoate as the carbon source for 24 h (Martinez et al., 2011). Each prey sample was then prepared as a 10X concentrated solution in Hepes+ buffer (H+, 25 mM Hepes, pH 7.8, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$) to homogenise cell numbers and subsequently, each prey was subjected to the appropriate treatment. Finally, cells were rehydrated in a concentrated H+ solution. For predation assays, each prey was incubated with the BdG predator in shake flasks for 48 hours at 30°C. BdG growth was analysed by flow cytometry by counting green fluorescence events, while predation was analysed as the decrease in viable prey cells on the plate (Figure 33).

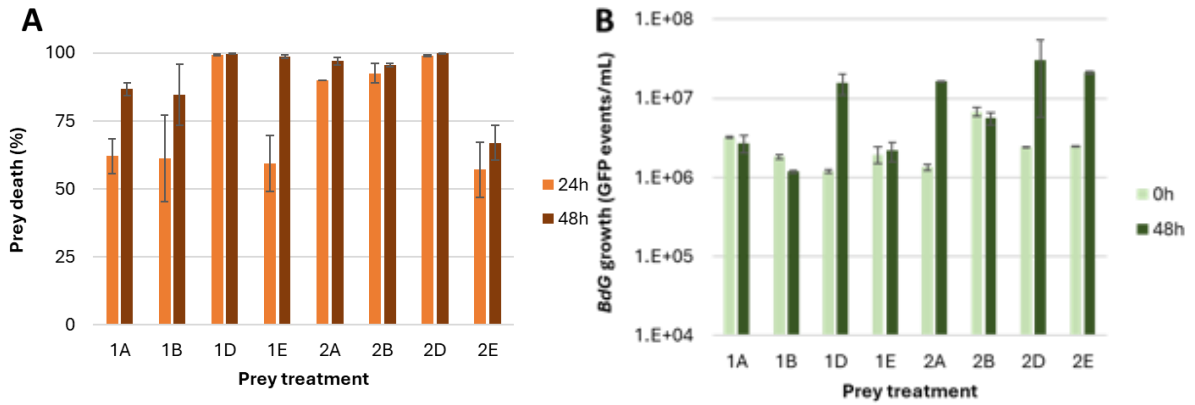


FIGURE 33: PREDATORY ASSAYS WITH A MODEL PHA-RICH QUENCHED (Q) BIOMASS. A) Percentage decrease in viable prey cells during incubation with BdG. B) Evolution of BdG progeny with the pretreated prey at different

levels. All experiments were performed with *B. bacteriovorus* (BdG) as predator and *P. putida* KT2440 as prey without PHA (denoted A) and with PHA (denoted B). Prey treatment abbreviatures: A) Fresh biomass-Q; B) Dry biomass rehydrated-Q; D) Fresh biomass (as control); E) Dry biomass rehydrated. Data represent the average of three biological replicates.

Preliminary results suggest that the predatory capacity is not significantly affected by the acid pre-treatment of the biomass. Compared to the control condition, where the prey colony-forming units (CFU) decreased by 99% after 24 hours of predation, the CFU decrease was 60% when acid-pre-treated prey was used (Figure 33A). Moreover, flow cytometry data indicate that BdG can also prey on rehydrated dry cells (Figure 33B). Further experiments are required to quantify the efficiency of this process.

Then, both predation capacity and PHA recovery were evaluated on metabolically inactive cells of *P. putida* KT2440. Progeny was analyzed using flow cytometry, and prey cell viability was measured to determine the death rate. In addition, co-cultures were observed by phase-contrast and fluorescence microscopy. Fresh biomass was used as a reference. Predation yield was calculated as the number of predator cells produced per prey cell consumed, according to Equation 1 (Figure 34). Polymer recovery was calculated as the increase in yield relative to the values obtained from fresh biomass (Figure 34 and 35). As a control, prey cells were incubated alone under the same conditions as the co-cultures.

(1) Predation yield= $(BdG_{48h} - BdG_{0h})/(Prey_{48h} - Prey_{0h})$

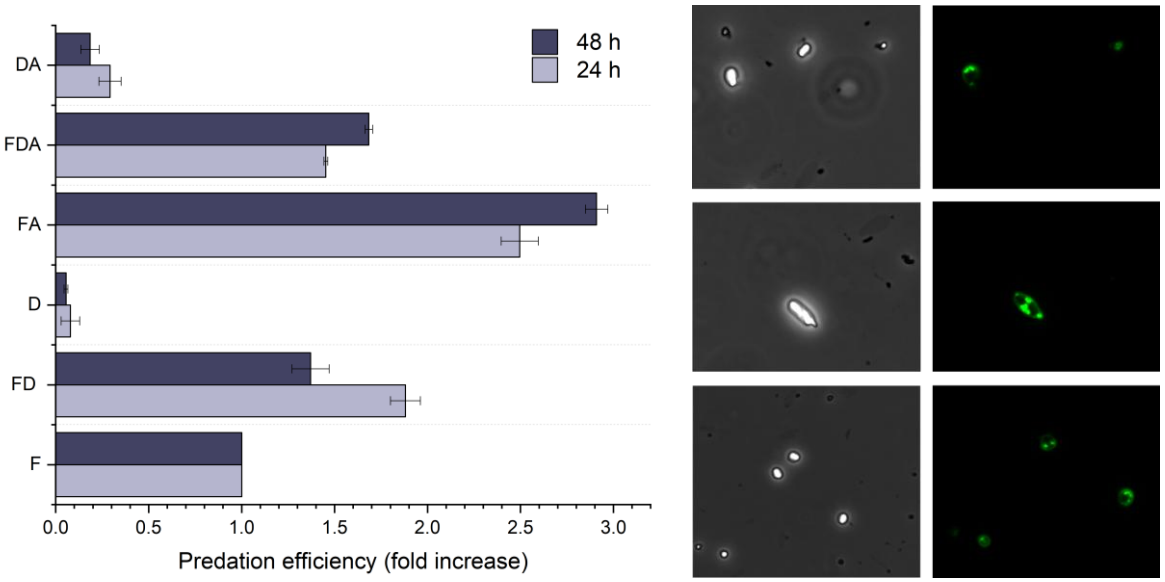


FIGURE 34. ANALYSIS OF THE PREDATORY CAPACITY OF *B. BACTERIOVORUS* BDG ON PRETREATED *P. PUTIDA* KT2440 PHA-ACCUMULATING CELLS. Left panel shows the predation yield obtained for each treatment applied to the biomass. Right panel presents phase-contrast and fluorescence microscopy images of BdG invading lyophilized biomass cells after 48 hours of incubation. Abbreviations: DA (dried acidified); FDA (freeze-dried acidified); FA (fresh acidified); D (dried); FD (freeze-dried) and F (fresh).

The highest yields were obtained after 24 hours with just lyophilised biomass and after 48 hours with acidified fresh biomass. These results indicate that BdG is capable of preying on treated and metabolically

inactive cells. Moreover, after predation, the polymer can be easily recovered, and biomass pretreatment further facilitates its recovery.

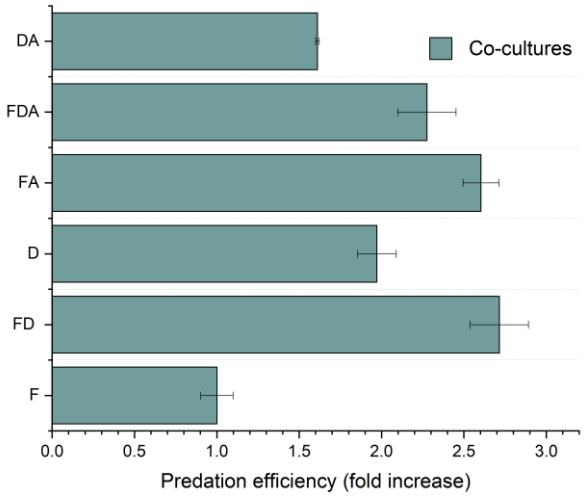


FIGURE 35: POLYMER RECOVERY AFTER INCUBATION OF TREATED PREY WITH BDG. Data shown after 48 hours of incubation. Abbreviations: DA (dried acidified); FDA (freeze-dried acidified); FA (fresh acidified); D (dried); FD (freeze-dried) and F (fresh).

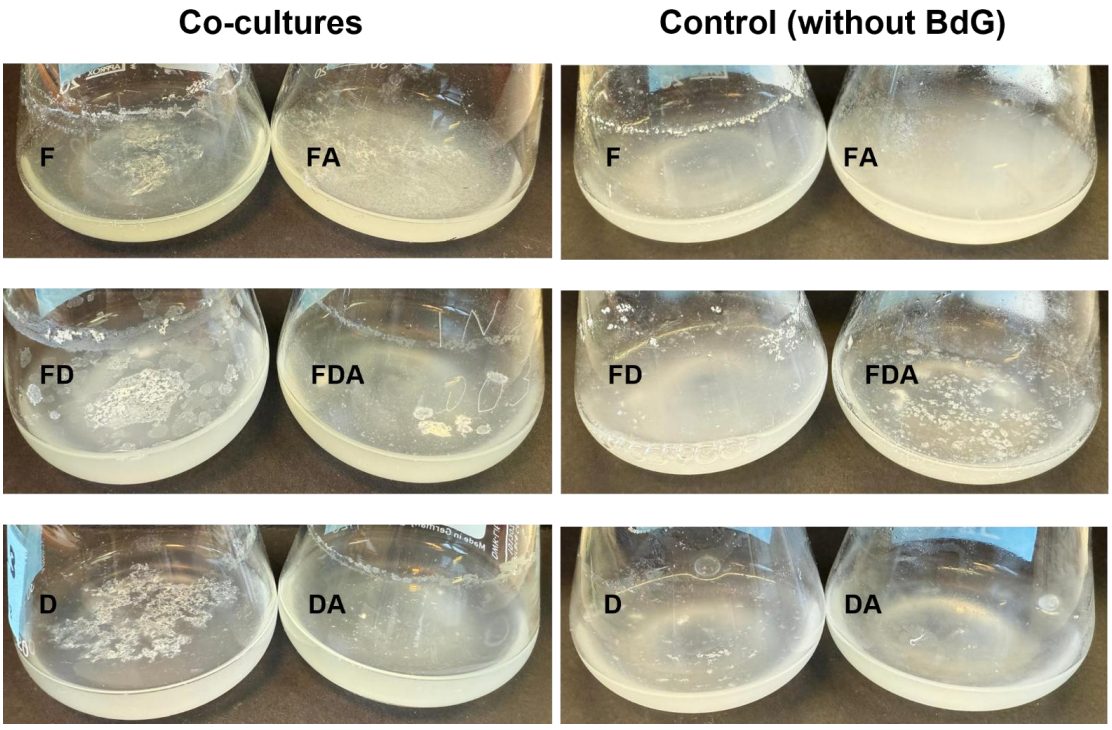


FIGURE 36: APPEARANCE OF THE BIOMASS AFTER 48 HOURS OF INCUBATION. The left panel shows the co-cultures, while the right panel shows the control flasks, in which only the prey was incubated following the respective treatments. The polymer can be observed as a whitish layer on the surface of the predation buffer. Abbreviations: DA (dried acidified); FDA (freeze-dried acidified); FA (fresh acidified); D (dried); FD (freeze-dried) and F (fresh).

In addition, two different *Pseudomonas* species from NOVA-ID (*Pseudomonas oleovorans* and *Pseudomonas resinovorans*) were tested as potential prey and *P. putida* KT2440 was used as a control. Predator progeny was analysed by flow cytometry as the increase in the number of fluorescence cells after applying different prey pre-treatments (fresh, freeze-dried, oven-dried and freeze) (Figure 37). Co-cultures were incubated for 48 hours in an H+ buffer.

BdG was able to prey on both *P. oleovorans* and *P. resinovorans* regardless of the prey treatment. No significant difference was observed in the number of predatory progeny when *P. oleovorans* was used compared to the control strain *P. putida* KT2440 (Figures 37A and 37B). However, a significant increase in the number of BdG cells was observed after 48 hours of incubation with *P. resinovorans* (Figure 37C). Regarding prey treatments, a slight decrease in BdG progeny was observed when freeze-dried prey was used compared to fresh and frozen prey. Further experiments are required to determine the optimum prey/predator ratio under PHA production conditions.

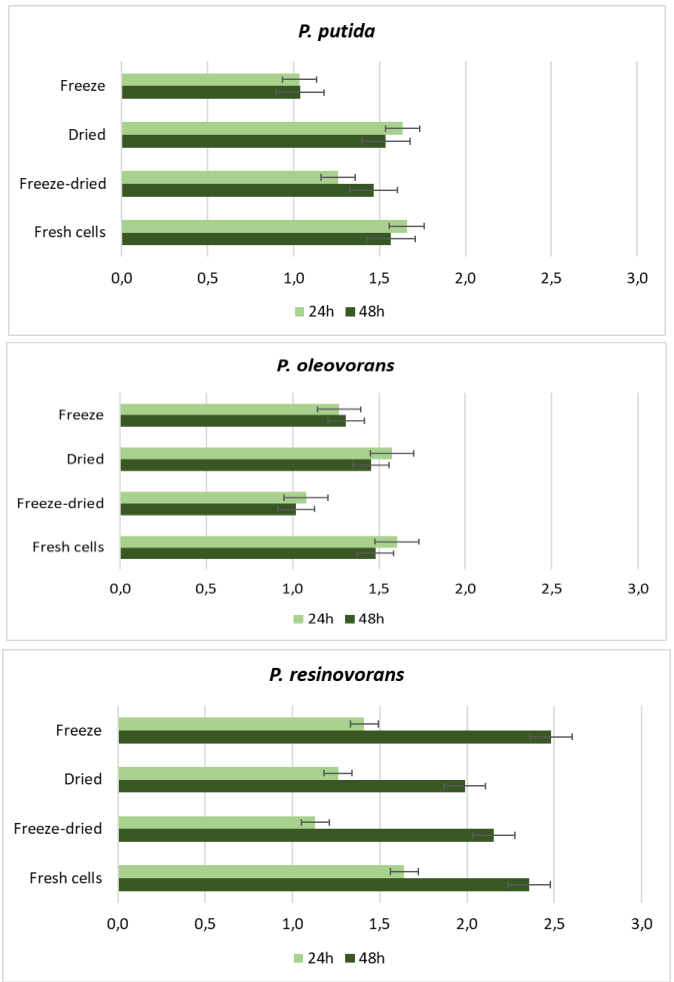


FIGURE 37: PREDATORY ABILITY OF B. BACTERIOVORUS OVER DIFFERENT TREATED *PSEUDOMONAS* STRAINS. A) BdG progeny using *P. putida* KT2440 suspensions. B) BdG progeny using *P. oleovorans* suspensions. C) BdG progeny using *P. resinovorans* suspensions. Predator progeny was analysed by the MASQuant 16 flow cytometer (Miltenyi) as the number of fluorescence cells. Data represent the average of three biological replicates.

3.2 Predatory ability over treated mixed microbial cultures (MMC)

The efficiency of predation was also tested in complex populations using biomass from UNIROMA following PHA accumulation. In addition, predation was analysed using the culture broth obtained after PHA production in UNIROMA, replacing the commercial HEPES buffer to integrate the predatory system into the same bioreactor immediately after PHA production.

BdG demonstrated the ability to prey upon dried complex biomass, showing an active predatory response even under conditions that mimic post-fermentation substrates since predatory activity was not inhibited by the UNIROMA supernatant (Figure 38A). The presence of PHA within the prey appeared to enhance progeny formation during the initial 24 hours of predation, even with a high MMC initial concentration, suggesting that the polymer may provide an additional energy source or structural advantage for predator replication (Figure 38B), although a fraction of prey cells remained resistant after 48 hours, overall (Figure 38C). These results indicate that the PHA extraction process could potentially be integrated directly after fermentation, allowing the use of the same culture broth for both production and recovery stages.

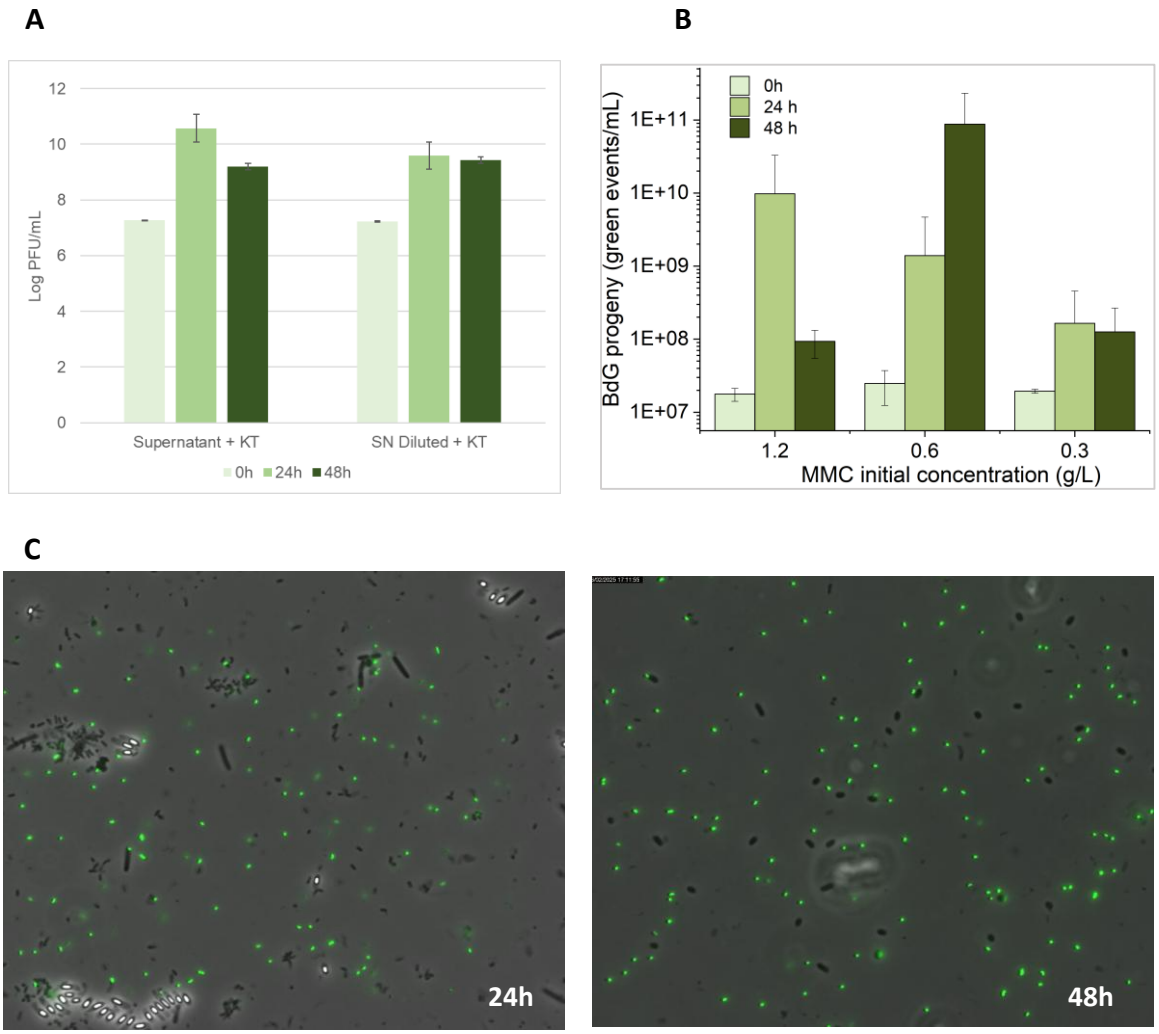


FIGURE 38: PREDATORY ACTIVITY OF BDELLOVIBRIO BACTERIOVORUS BDG ON DRIED COMPLEX BIOMASS AND POST-FERMENTATION SUBSTRATES. The figure summarizes progeny formation and prey resistance under different conditions, highlighting the effect of PHA presence and the feasibility of performing polymer extraction directly from the fermentation broth.

3.3 Identification of potential hydrolytic enzymes by proteomic analysis

Proteomic and functional analysis of the *B. bacteriovorus* HD100 genome allowed the identification of two novel ORFs with putative PHA depolymerase (PhaZ) functionality. For this purpose, an in-silico analysis was carried out considering the characteristic catalytic domains of scl-PhaZs: a signal peptide (SP) to allow the secretion, a large catalytic domain type I that contains the catalytic triad and a linker domain between the catalytic domain and the C-terminal substrate binding domain (SBD) (Figure 39). Using in silico signal peptide prediction to identify a Sec or TAT signal peptide, we identified 169 proteins that were considered to be the secretome of *B. bacteriovorus*. The hydrolase activity was then assigned by Gene Ontology, and the depolymerase activity was predicted using profile hidden Markov models based on their α/β fold structure. This method provides a robust statistical approximation for identifying functions in proteins from their sequences.

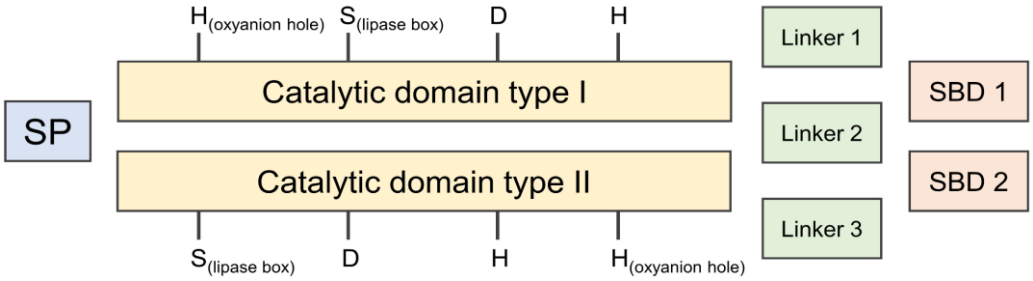


FIGURE 39: SCHEME OF THE MULTIDOMAIN STRUCTURE OF SCL-PHAZS. e-scl-PhaZs consist of a signal peptide (SP), a catalytic domain (type I or type II), a linker (1: Threonine-rich linkers, with two to six threonine residues; 2) Fibronectin type III (Fn3) linker; 3) An acid-long cadherin-like sequence) and a substrate binding domain (SBD).

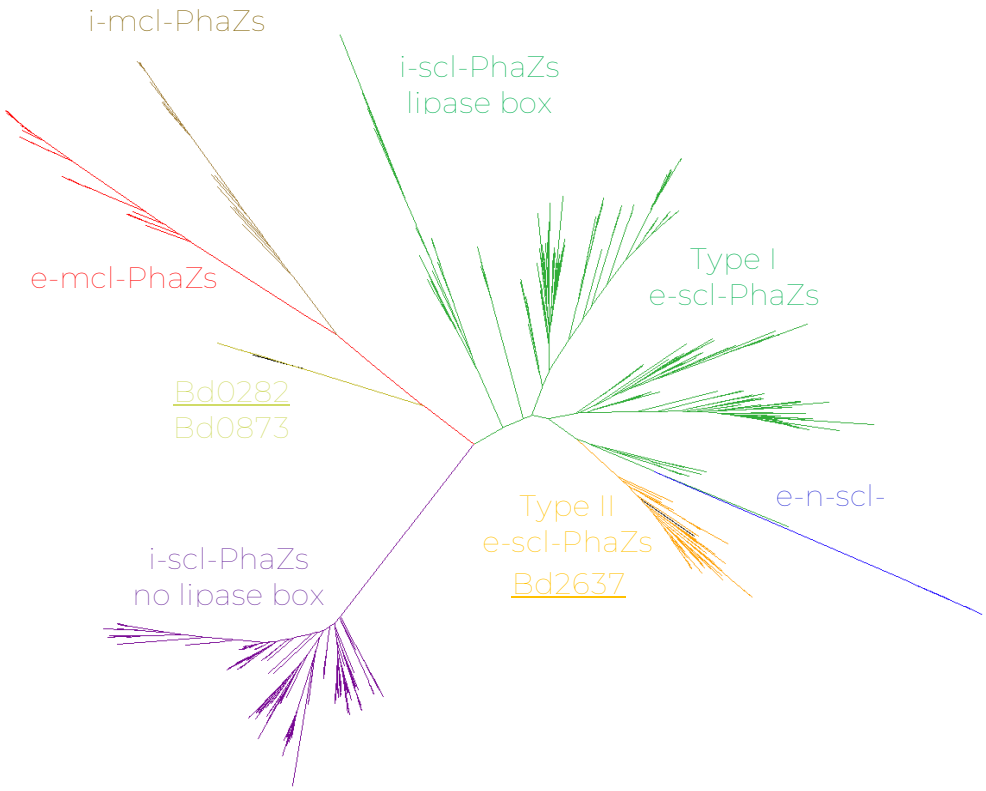


FIGURE 40: PHYLOGENETIC TREE OF THE PHAZS RETRIEVED FROM THE PHA ENGINEERING DATABASE, ALONG WITH CANDIDATE PHAZS FROM *B. BACTERIOVORUS* HD100. The colours of the branches indicate the type of PhaZ and the putative novel PhaZ is highlighted and underlined.

The α/β fold was found in four proteins: Bd0282, Bd0873, Bd3610 and Bd2637. Bd3610 was excluded because this protein uniquely contains an Fn3 domain (see Figure 39, Linker Type 2). Bd2637 was previously reported as an extracellular short-chain depolymerase. Bd0282 and Bd0873 were aligned with all PhaZs in the PHA Engineering database using the Clustal Omega algorithm to construct a phylogenetic tree (Figure 40). As Bd0282 and Bd0873 clustered on the same branch as a reported medium-chain extracellular PhaZ (Bd3709), both proteins were considered for further experiments.

3.4 Purification and characterisation of novel PHB polymerase enzymes

The PHA extraction protocols discussed herein regarding the employment of mixed microbial cultures and considering that the proteomic analysis of the *Bdellovibrio* HD100 genome allowed the identification of new and potential ORFs (open reading frames) encoding PhaZ depolymerases, we proceeded to the heterologous expression of the phaZ ORFs identified in silico. To recombinantly express PhaZ depolymerases from *B. bacteriovorus*, the coding sequence codons were optimized and synthesized by Twist Biosciences. Gene bank numbers BD_RS04025, BD_RS01305 and BD_RS12045 coded for Bd0873, Bd0282 and Bd2637 proteins, respectively. The restriction targets NcoI at 5' and XhoI at 3' were used for its introduction into the vector pCri9a (Addgene). The obtained vectors were transformed into chemocompetent *E. coli* Bl21(DE3). Positive clones were selected by plating the transformants into LB agar plates containing 50 µg/mL of kanamycin (Km), 1 mM isopropyl β-D-thiogalactoside (IPTG) and an overlay of amorphous PHB. The PHB suspension was prepared by solving 30 mg PHB in 5 mL dichloromethane, sonicated in the presence of 15 ml 0.2 % N-sarkosyl, and evaporating the solvent. This solution was centrifuged and concentrated 10-fold and mixed with 1% agar to make the PHB overlay. Positive clones were selected for the production of a clear halo in the PHB.

For PhaZ depolymerase expression, positive BL21 (DE3) clones were grown in LB supplemented with Km and induced with 1 mM IPTG for 4 h at 37ºC when 0.5 OD600 was reached. Protein expression was monitored by activity with amorphous PHB plates after concentrating the culture supernatant (Figure 41). The three enzymes were successfully expressed as extracellular enzymes fused to the PelB signal peptide. All of them degraded amorphous PHB prepared by dissolving the polymer in dichloromethane with N-sarkosyl as a surfactant. Still, they could not degrade crystalline PHB suspensions prepared by direct sonication of PHB powder into aqueous suspensions.

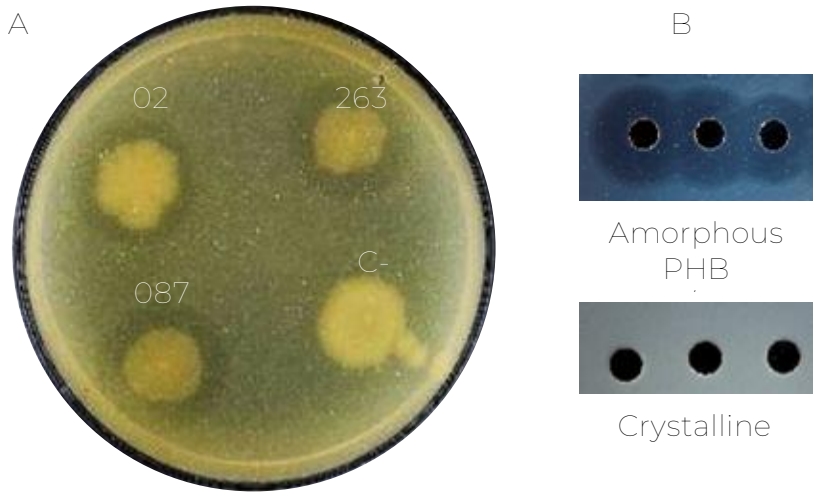


Figure 41: Recombinant PhaZ depolymerases analysis. A) Whole-cell activity assay using E. coli grown in LB plates plus amorphous PHB (dichloromethane/sarkosyl). B) Activity assay using Bd0282 concentrated supernatants incubated in amorphous PHB (DCM/sarkosyl) and crystalline PHB.

3.5 Genetic engineering approaches to decrease natural polymer hydrolysis

In silico analysis of the *Bd* HD100 genome allowed the identification of three ORFs (open reading frames) *bd0282*, *bd0873* and *bd2637* with putative PHB depolymerase functionality. To control PHA degradation activity by the predator in PHA-producing cultures, we have developed a methodology to generate Bd*phaZ* mutant strains (ΔBd*phaZ* strains) each carrying a single gene mutation in one of the identified *phaZ* genes. We have followed two approaches to obtain the mutant strains:

3.5.1 Generation of Bd*Δ2637 strain*

We have obtained a Bd*phaZ* mutant strain in *bd2637* (BdΔ*2637)* by double homologous recombination assays using the plasmid pK18mobsacB-GG, adapted in our laboratory to the Golden Gate cloning technology (GG) (Salgado et al., 2024). The GG method allows the cloning of homologous DNA fragments obtained by PCR amplification from the genome of BdG, in a backbone GG-plasmid, in a step reaction. The recombinant plasmid is transferred by conjugation to the BdG genome, where the knockout of the tagged genes will be produced by homologous recombination. After that, two recombination steps should be performed: the first allows the selection of Km (50 µg/mL) resistance trans-conjugants, and the second enables the selection of bacteria that have lost the integrative plasmid, by resistance to sucrose (5%).

After several days of incubation, the recombinant Bd halos were visible. Several of these halos were then picked and grown in liquid media before to verified by PCR analysis the *pha*Z knockout (Figure 42A).

This strategy was also used to generate defective Bd*phaZ* strains in *bd0282* and *bd0873*. However, after the second recombination event and selection on sucrose plates, we were unable to recover any mutant strains, either Δ*bd0282* or Δ*bd0873*. Therefore, to generate these mutant strains, we employed an alternative strategy based on the disruption of the target genes through simple homologous recombination.

3.5.2 Generation of Bd*Δ0282 and* Bd*Δ0873 strains*

A 600 bp DNA fragment containing homologous sequences to *bd0282* or *bd0873*, respectively, was cloned into the plasmid pK18mob and transformed into the donor strain *E. coli* DH5λpir. The recombinant plasmid is transferred by conjugation in a tri-parental mating assay to the BdG recipient strain, where the knockout of the target genes was produced by integration of the whole plasmid by a simple homologous recombination event. The transconjugants were recovered by Km resistance on double-layer agar plates (50 µg/mL), selective medium. Bd recombinant halos were grown, and the insertion of the recombinant plasmid into the BdG genome was verified by PCR analysis and genome sequence. (Figure 42B).

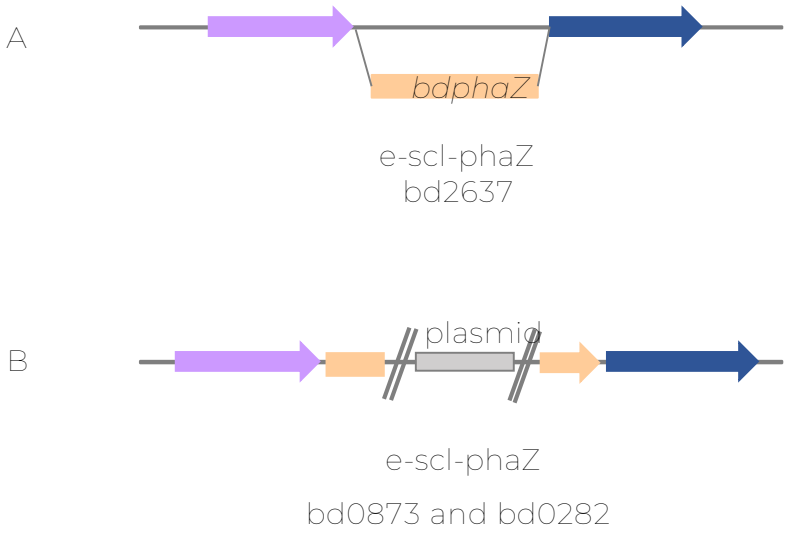


FIGURE 42: SCHEME OF THE GENOME OF BD*PHAZ* MUTANT STRAINS. A) Generation of Bd*Δ2637* by deletion of the target gene. B) Generation of Bd∇0873 and Bd∇0282 strains by disruption of the target genes.

Over the coming months, the predatory capacity of the recombinant strains BdGΔ2637, BdΔ0282 and BdΔ0873 on PHA-accumulating prey will be tested and evaluated, with the wild-type strain B. bacteriovorus HD100 used as a control. The predation efficiency and polymer recovery capacity of the recombinant strains will be compared to that of the wild-type strain after knocking out the genes encoding potential depolymerases that can hydrolyse the polymer and reduce the efficiency of the process.

Conclusions

This study demonstrates that *B. bacteriovorus* HD100 and its recombinant fluorescent derivative BdG maintain an active predatory capacity towards *Pseudomonas* spp., even after the prey biomass has undergone acid or drying pre-treatments. The predator effectively attacked metabolically inactive or rehydrated cells, enabling PHA recovery without the need for living prey. Furthermore, BdG exhibited robust predatory behaviour towards complex post-fermentation biomasses, demonstrating compatibility with fermentation supernatants. This suggests that PHA extraction could be integrated directly into the production stage, thereby improving process efficiency and polymer recovery yields.

In parallel, proteomic and functional analyses of the *B. bacteriovorus* HD100 genome identified three open reading frames (bd0282, bd0873 and bd2637) with putative PHA depolymerase (PhaZ) activity. Successful heterologous expression of these enzymes in *E. coli* confirmed their extracellular activity against amorphous PHB, highlighting their potential role in polymer degradation during predation. To control this

depolymerase activity and improve PHA recovery, knockout mutant strains (BdΔ2637, Bd∇0282 and Bd∇0873) were generated using homologous recombination strategies. Ongoing experiments aim to evaluate the predatory performance and polymer recovery efficiency of these mutants compared to the wild-type strain.

This integration could streamline downstream processing, enhancing both process efficiency and polymer recovery yields. Future studies should focus on optimising predator-prey ratios, assessing long-term stability under industrial conditions and quantifying the energy and material balances of this biotechnological approach.

IV.II. Biological digestion with *Hermetia illucens* (Hi) larvae of the PHA enriched biomass (ENTO)

The ability of the larvae of *Hermetia illucens* (Hi), a cosmopolitan species originally from the new world, to digest the biomass enriched into biopolymer (i.e., PHA) with consequent PHA release has been investigated.

The larvae of the insect are known to be at certain point as bacteriophages and thus can digest the bacterial biomass while leaving behind a residue as PHA untouched. The experiment was carried out to check on the biological digestion from 4 different broths provided from NID and UNIROMA, each acidified with H_2SO_4 and HCl to reach a low pH to stop microbial growth. The references of the broth were NIDSul, NIDClo, URSul and URClo. All the broths were neutralized with NaOH solution 0,1N up to achieve pH 6.

Material and Methods

The first step was to get broth samples to measure the dry weight (DW). To realize the process, small larvae with an average weight of 3.5 mg were set in a recipient of 150 cm^2 containing a sustention material of synthetic yarn (Figure 43). The weight of the yarn was recorded. 100 ml of the solution is added to the container together with 100 small larvae, thus at digestion capacity of 1 ml of broth per larva. After 48 hours, the larvae were extracted manually to measure weight. The residual liquid was collected and then weighed.

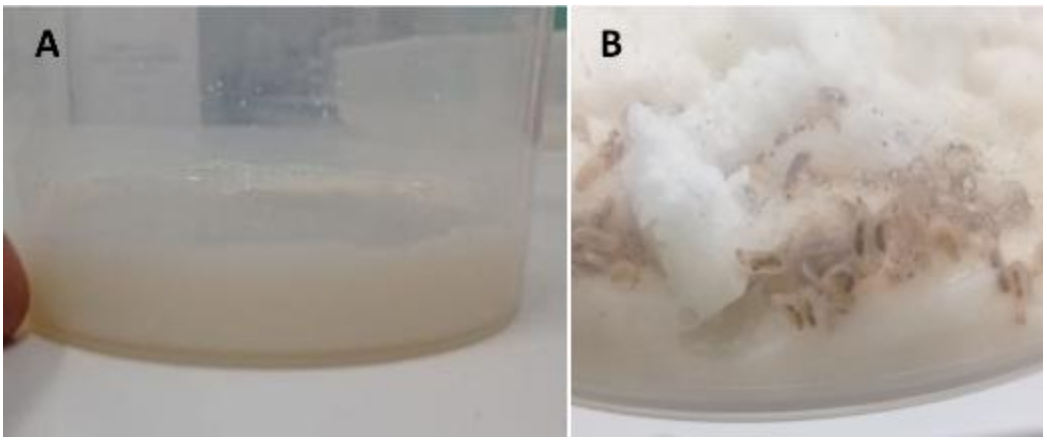


FIGURE 43: LARVAE DIGESTION SETTING. BACTERIAL BROTH IN THE RECIPIENT BEFORE ADDING THE YARN AND THE LARVAE (A); LARVAE, SYNTHETIC YARN AND BACTERIAL BROTH (B).

The liquid collected was then centrifuged at 3500 r.p.m. for one hour and then the liquor was rejected, and the solid portion was dried.

Digested biomass was estimated by:

$$Digested\ solids\ (\%) = \frac{Initial\ dried\ solids\ (g) - Final\ dried\ solids\ (g)}{Initial\ dried\ solids\ (g)} \times 100$$

Solvent extraction of the PHA

The process to extract PHA from the dry digested bacterial broth was as follows:

1. Dry digested biomass was added to a vial together with Anisole solution at 60g/l and put in ultrasound bath at 20 – 25 °C at the start.
2. Ultrasound was applied and temperature increased up to 120ºC.
3. When the temperature reached 120ºC the temperature was kept in the bath for 3 hours.
4. Temperature decreased in cold baths until it was lowered to 40ºC.
5. The solution was then filtered in a filter paper of 13 µm pore size, and then rinsed with more Anisole solution.
6. Solution was warmed up again to 80 ºC until volume was reduced by evaporation at half the starting volume (16 hours after)
7. The solution was cooled again to 45ºC.

Alcohol precipitation of PHA

This step is required for the recovery of PHA into the Anisole-PHA solution, and the procedure was as follows.

1. Alcohol solution (Isopropanol at 12.5 vol.) was added to the reactor and was let it to warm up to 40ºC while agitation in the ultrasonic bath for about 30 minutes until some solid parts were observed.
2. The suspension of PHA-iso-propanol-anisole was cooled in ice-bath until it reached 5ºC for an hour agitating time to time.
3. The suspension was then put in the centrifuge
4. To centrifuge at 3 500 rpm for 30 minutes to recover solids.
5. The pellet obtained was extracted and put in a watch glass to dry at 80ºC until it is constant.

Preliminary results related to the action of the *Hermetia illucens (Hi)* on the PHA recovery are shown in **Table 23**. It could be observed how the original weight of the digestated broth was reduced during the process at approximately 65% of the original volume. It is due to water evaporation assisted by the larvae digestion action.

The digested biomass was reduced already more than 48% with the heist digestion for the NIDClo broth (55.81). Alcohol precipitated applied on the Anisol solution enriched into PHA was dependent on the original PHA concentration, and a maximum extraction expressed in terms of grams of polymer recovery accounted for 0.37 g was reached for NIDSul sample.

The larvae weight obtained was about 8.5 mg per larvae for NID treatments while for UR treatments was 1.85 mg. This difference could be related to the amount of nutrients in the starting broth. In addition, larvae body weight increase means that the bacterial broth was nutritive, and the feeding behavior was not affected by the acid used to preserve the media and PHA consumption.

TABLE 22. RESULTS EXPRESSED IN TERMS OF DRIED DIGESTATED, PERCENTAGE OF DIGESTATED, AND PHA.

SAMPLE	Initial DW (g)	Digestated broth (g)	DW digestated (g)	% digested	Largain (mg)	PHA (g)
NIDSul	1.85	64.43	0.88	54.64	9.1	0.370
NIDClo	1.72	63.89	0.76	55.83	8.3	0.344
URSul	0.38	68.3	0.19	50.12	1.9	0.076
URClo	0.33	65.28	0.17	49.32	1.8	0.066

Larvae digestion scaling up

Once demonstrated the capability of the larvae to digest the microbial biomass and release the PHA, it was prepared an experiment to pave the way for the scaling up of the process. For that process, a concentrated bacterial biomass was dispersed inside a plastic recipient together with clay pebbles used as structural carrier material. Larvae with an average individual weight of 6 mg were added directly onto the substrate to start feeding, and the system was kept at 28 °C for 48 hours. The feeding activity was visually noticeable: after 24 hours, the substrate became progressively darker due to bacterial digestion, and after 48 hours, digestion was considered complete.

FIGURE 44 : LARVAE SUBSTRATE EXPERIMENT. FROM LEFT TO RIGHT: A) PURE CONCENTRATED BACTERIAL BIOMASS IS DISPERSED IN PLASTIC RECIPIENT WITH PEBLES CLAY AS CARRIER; B) LARVAE OF AN AVERAGE WEIGHT 6 MG ARE ADDED TO THE RECIPIENT TO START FEEDING ON THE SUBSTRATE AND KEEP AT 28ºC FOR 48 HOURS; C) AFTER THE 24 HOURS SUBSTRATE BECOME DARKER SHOWING LARVAE DIGESTION AND AT 48 HOURS THE DIGESTION IS FINISH

Once the 48-hour digestion phase was finished, demineralized water was added to the plastic recipient. The mixture was gently agitated and then passed through a 350-micron mesh, which allowed the recovery of the liquid fraction while separating the larvae and the clay pebbles.

The collected liquid containing the digested biomass was dispensed into 15-mm Petri dishes and placed in an oven at 99 °C for 24 hours to fully dry the material. (Figure 45).

FIGURE 45: DIGESTED SUBSTRATE RECOVERY.

The dried digested biomass (Figure 45) was then collected manually. This solid fraction, already biologically pre-treated by the larvae, represents the material from which PHA can be extracted in the subsequent chemical steps.

Solvent extraction of PHA

Extraction of the PHA from the dried digestate was performed using acetone as the main solvent. The dry mass was transferred into 50-mL Falcon tubes and filled with acetone, maintaining contact for 24 hours at room temperature, with periodic agitation to enhance solubilisation.

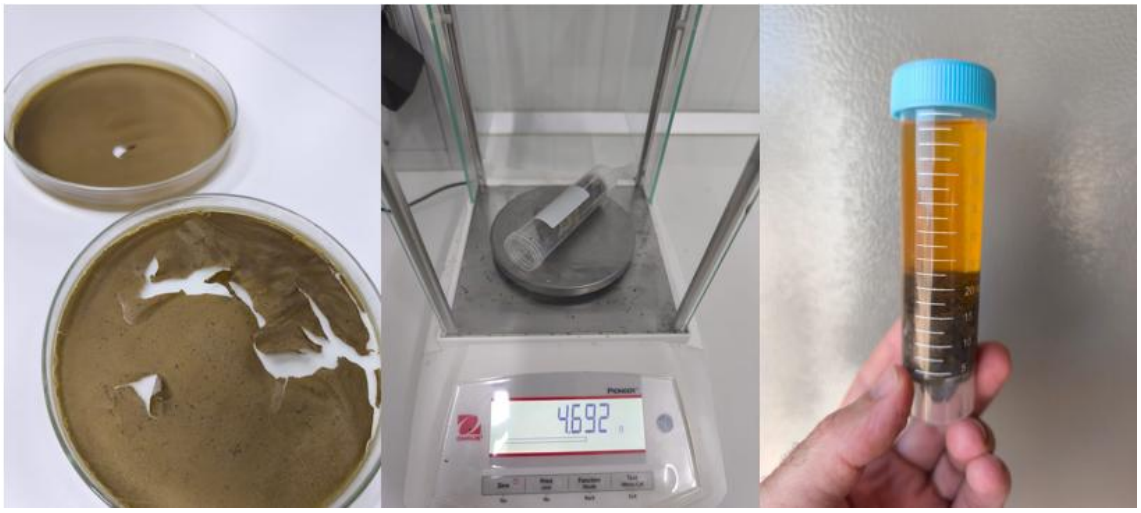


FIGURE 46: FROM LEFT TO RIGHT: A) THE DRY MASS IS COLLECTED; B) DRY MASS OBTAINED; C) ACETONE IS ADDED TO FILL UP THE 50 ML FALCON TUBE, AND INCUBATED 24 HOURS AT ROOM TEMPERATURE AND AGITATED PERIODICALLY.

After extraction, the solvent was filtered through standard filter paper to remove the remaining solids and obtain a clean acetone-PHA solution (Figure 47).

FIGURE 27: PHA SOLVENT FILTRATION

PHA recovery by water-induced precipitation

To promote PHA condensation, demineralised water was added to the acetone solution under agitation. A gummy solid rapidly appeared as the polymer precipitated. This solid was then separated from the liquid phase using a 75-micron nylon mesh.

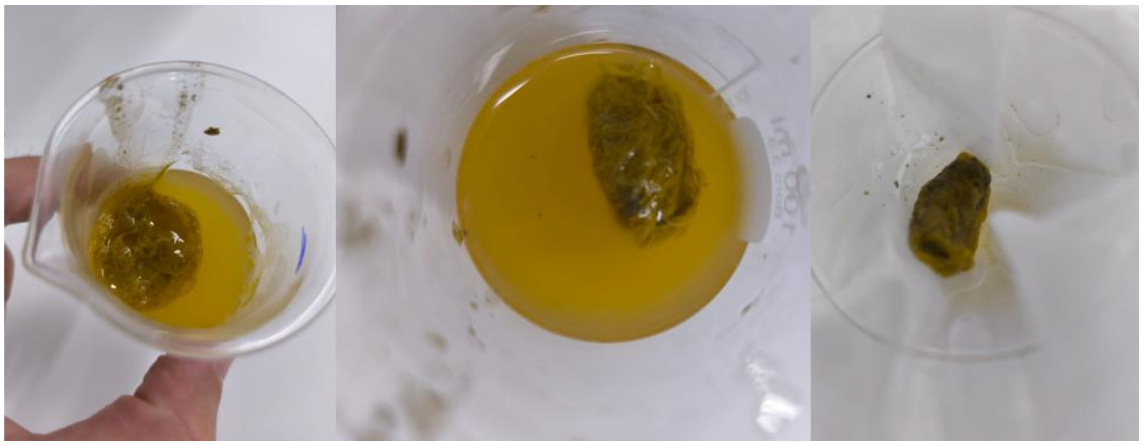


FIGURE 48 : PHA CONDENSATION. A) AND B) DEMI WÁTER IS ADDED WHILE AGITATED AND A GUMMY SOLID APEAR; C) THE SOLID IS SEPARATED FROM THE LIQUID USING A NYLON MESH OF 75 MICRONS

The recovered PHA pellet was finally dried in an oven at 50 °C for 5 hours until it reached a constant weight (Figure 48). In the test shown in the, 0.962 g of PHA were obtained from 70 g of concentrated bacterial biomass, demonstrating the effectiveness of the combined biological–chemical extraction system.

FIGURE 49 : FINAL PHA PELLET YELLOW PHA PELLET INDICATED CERTAIN IMPURITIES AND TST GAVE A TOTAL PURITY OF 95%.

Conclusions

The larvae of *Hermetia illucens* effectively digested the bacterial biomass, reducing the organic matrix and facilitating the release and accessibility of the PHA. The subsequent solvent extraction using acetone and water-induced precipitation allowed a clean recovery of the polymer.

This integrated bioprocess demonstrates the potential of combining biological digestion with conventional solvent extraction to obtain PHA from microbial biomass in a simplified and efficient way.

General Conclusions

The results presented in this deliverable collectively demonstrate significant progress toward the sustainable valorization of agro-industrial residues through the integrated production of high-value bioproducts—medium-chain carboxylic acids (MCCAs), microbial protein (MP), and polyhydroxyalkanoates (PHAs), within a circular bioeconomy framework. Each technological pathway developed by the project partners showcases both scientific innovation and practical feasibility for industrial upscaling.

UGent successfully developed a highly selective and productive MCCA production process using an expanded granular sludge bed (EGSB) reactor fed with ethanol-rich wine lees. The system achieved productivities up to 46.5 gCOD L^{-1} d^{-1} and caproate selectivity of 76–78%, operating stably even at low hydraulic retention times (12 h). Ethanol served as an effective electron donor, enabling efficient chain elongation and substrate conversion (caproate yield = 0.69 gCOD $gCOD^{-1}$). The MCCA-rich effluent produced under these optimized conditions was further used for downstream PHA production, confirming the valorization potential of this agro-waste stream.

The enzymatic hydrolysis of grey starch (GS), a potato-processing byproduct, was optimized to produce fermentable hydrolysates, by AVECOM, for microbial protein synthesis by UGENT on task 3.2. Although initial hydrolysis trials presented technical challenges, process adjustments, especially pH control and improved mixing, resulted in 40.6 L of concentrated hydrolysate (COD = 122.5 g L^{-1}), later scaled for protein production. Modeling suggests that further optimization could yield COD conversions above 80% and feed concentrations exceeding 250 g COD L^{-1}. Fed-batch cultivation of *Cupriavidus necator* H1 G+3 on hydrolyzed GS resulted in biomass concentrations up to 9.6 ± 2.5 g L^{-1}, with protein contents between 50–73% and conversion yields of 0.38 ± 0.02 gCDW $gCOD^{-1}$, confirming GS is a good substrate for microbial protein production. The resulting biomass exhibited an amino acid composition comparable to literature-reported profiles, reinforcing its nutritional quality and potential for animal feed applications.

NID demonstrated the feasibility of converting lipidic fractions of tomato pomace (TP) and brewer's spent grain (BSG) into PHAs using both single strains and mixed microbial cultures (MMCs). Among the strains tested, *Burkholderia thailandensis* E264 and *Cupriavidus necator* DSM 428 showed the highest PHB yields, achieving up to 87.6% polymer content with BSG oil under bioreactor conditions. Parallel MMC-based studies confirmed the robustness of the enrichment and selection strategy for mcl-PHA production from carboxylic acid-rich substrates (e.g., hexanoic acid mixtures and fermented wine lees provided by UGENT). The developed process achieved intracellular PHA contents exceeding 50% (wt/wt) with PHBV copolymers containing 20–35% 3HV, demonstrating scalability and flexibility toward real waste streams.

UNIROMA developed a MMC-based process for PHA production from carboxylic acid (CA) streams rich in hexanoic acid. Complementary accumulation assays yielded 106 g of dried biomass containing about 55 g of PHA, which was supplied for downstream extraction and characterization. The polymers exhibited variable compositions (3HB: 78–83%, 3HV: 7–15%, 3HHx: 3–10%), reflecting the influence of complex substrates and operational conditions. The continuous multi-reactor process achieved PHA-enriched biomass with over 50% intracellular polymer and PHBV copolymers containing 20–35% 3HV. Applying a combined NaOH–H_2O_2 treatment enabled the simultaneous recovery of other high-value compounds, such as EPS and alginate-like polymers. Green solvent extraction using ethyl acetate (EtOAc) provided high PHA purity comparable to chloroform methods, with reduced contact time and improved sustainability, while supercritical CO_2 pretreatment showed no added benefit. The EtOAc extraction also proved effective for NID-produced PHBVH, with HHx content not affecting polymer purity or molecular weight. Overall, these findings confirm the efficiency, scalability, and environmental compatibility of the continuous PHA production and recovery process for circular biorefinery applications.

CSIC advanced an innovative biological extraction approach using the predatory bacterium *Bdellovibrio bacteriovorus* HD100. The predator effectively preyed on acidified, dried, or rehydrated PHA-producing

cells, releasing polymer granules without chemical solvents. Proteomic analyses identified three novel depolymerase candidates (Bd0282, Bd0873, Bd2637), which were successfully expressed in *E. coli* and shown to degrade amorphous PHB. Mutant strains lacking these enzymes (Δbd0282, Δbd0873, Δbd2637) were engineered to improve polymer recovery efficiency and prevent unwanted degradation during predation. This innovative bioprocess represents a major step toward eco-friendly PHA extraction integrated directly into production systems.

Ento demonstrate that *Hermetia illucens* larvae can efficiently digest bacterial biomass to reduce the organic matrix and enhance PHA accessibility, enabling a simplified downstream process in which conventional solvent extraction yields a clean and effective polymer recovery.

Data Management Plan follow-up

N°	Dataset name	Open Data	Closed Data	Means of dissemination	Maximum delay before access	Data set access
22	WP3 -Task 3.3 NID (Partner #10.	Data have been elaborated in excel files, available on request in the AgriLoop Dataverse	Data reported in the deliverable are available on request	Scientific publications, International and national conferences and dissemination events	Once published and at the latest 2 years after the end of the project	https://entrepot.recherche.data.gouv.fr/dataverse/agriloop_wp3
3	WP3-T3.3 and 3.4 Biopolymer (PHA) production from bacteria and Extraction (Partner #11)	Data have been elaborated in excel files, available on request in the AgriLoop Dataverse	pseudonymized data set containing sensitive personal data (GDPR)	Scientific publications, International and national conferences and dissemination events	Published 8 days before the submission to reviewers	https://doi.org/10.57745/QYHLPM
21	WP3 -Task 3.1 UGENT (Partner #15)	Data have been elaborated in excel files, available on request in the AgriLoop Dataverse	Data reported in the deliverable are available on request	Scientific publications, International and national conferences and dissemination events	Once published and at the latest 2 years after the end of the project	https://entrepot.recherche.data.gouv.fr/dataverse/agriloop_wp3

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This table above sums up the main information regarding the data produced for this deliverable, where is it stored and are the specific rules to respect concerning access, publication and FAIR principles.

N°	Dataset name	Owner	Name of the current contact	IPR issues	Use of third-party	Restrictions on data sharing (Y/N)
22	WP3 -Task 3.1 NID (Partner #10)	NID	Cristiana Torres Maria Reis	To be defined according to the Institution regulation	no	Yes, compliance with GDPR
8	WP3-Task 3.3 and Task 3.4 UNIROMA (Partner #12)	UNIROMA	Lionel Nguemna Tayou Gaia Salvatori Luca Di Leva Marianna Villano	To be defined according to the Institution regulation	no	Yes, compliance with GDPR
21	WP3 -Task 3.1 and Task 3.2 UGENT (Partner #15)	UGENT	Ramon Ganigué Korneel Rabaey	To be defined according to the Institution regulation	no	Yes, compliance with GDPR
	WP3 -Task 3.1 Avecom (Partner #18)	AVECOM	Kim Windey Lars Stegemüller	To be defined according to the Institution regulation	no	Yes, compliance with GDPR
	WP3 -Task 3.4 CSIC (Partner #13)	CSIC	Auxiliadora Prieto Natalia Hernández Ana Hernández Arriaga	To be defined according to the Institution regulation	no	Yes, compliance with GDPR
	WP3 -Task 3.4 Entomotech(Partner #12)	ENTO	Juan Cortès Ortiz	To be defined according to the Institution regulation	no	Yes, compliance with GDPR

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This table above sums up the main information regarding potential Intellectual property protection or GDPR issues.

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