EVALUATION OF POLYHYDROXYALKANOATES (PHA) PRODUCTION CAPACITY OF DIFFERENT ORGANISMS

Indrita Mahalanabish*[1], Api Rani Arpita[2], Kazi Rohan[3] and Md.Salatul Islam Mojumder[4]

[1] *Student, Shahjalal University of Science & Technology, Bangladesh, e-mail: indritamn@gmail.com*
[2] *Student, Shahjalal University of Science & Technology, Bangladesh, e-mail: arpitaapi.cep.sust@gmail.com*
[3] *Student, Shahjalal University of Science & Technology, Bangladesh, e-mail: rkazi9417@gmail.com*
[4] *Professor, Shahjalal University of Science & Technology, Bangladesh, e-mail: salatul-cep@sust.edu*

***Corresponding Author**

ABSTRACT:

Polyhydroxyalkanoates (PHAs) are biodegradable polymers with potential to replace petrochemical plastics, however their large-scale adoption is limited by high production costs, polymer degradation, and brittleness associated with high crystallinity. This study evaluates the use of mixed carbon substrates, comprising hydrolyzed sucrose and propanoic acid to enhance PHA production using *Cupriavidus necator (C. necator)*. The focus is on achieving higher PHA yield with reduced crystallinity, thereby improving polymer flexibility and thermal properties. To further reduce production costs, tap water was used as an alternative to laboratory-grade reverse osmosis (RO) water, and showed no significant effect on PHA yield or composition. The findings demonstrate that mixed substrates result in higher PHA yields with reduced crystallinity, improving the polymer's overall performance. Under nitrogen-limited conditions, excess carbon was preferentially diverted toward PHA accumulation rather than biomass formation. The highest dry cell weight (DCW) of 4.152 g/L was achieved using a sole-substrate system comprising 20 g/L hydrolyzed sucrose and 2 g/L ammonium sulfate, indicating an optimal carbon-to-nitrogen ratio enhances microbial biomass growth and PHA accumulation. A comparative analysis was conducted among *Pseudomonas putida (P. putida)*, *C. necator* strains from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), specifically DSM 545 and DSM 428. Among these strains, DSM 428 exhibited the highest performance, with a DCW of 2.80 g/L along with a PHA content of 42.124%, indicating its superior capacity for biomass production and polymer accumulation under the tested conditions. Two extraction methods—solvent-based (using chloroform) and bio-based (employing cell-lysing bacteria)—were applied to the harvested PHA biomass. The extracted polymers were then analyzed for thermal properties and purity using thermogravimetric analysis (TGA), and chloroform-based extraction resulted in higher polymer recovery efficiency compared to the bio-based method. Overall, this research demonstrates a cost-effective and sustainable strategy for enhanced PHA production with improved polymer properties, supporting PHAs as viable green alternative to petroleum-based plastics.

Keywords: *Polyhydroxyalkanoates; Substrate; Extraction; Thermal Properties; Biodegradable*

1. INTRODUCTION

Plastics play an indispensable role in our day-to-day lives due to their quality and diverse applications. In recent years, the growing population has worsened polymer waste issues, drawing attention to the impacts of accumulating non-biodegradable plastics. Over the next five years, the capacity of the world's bioplastics production is expected to more than triple, from around 2.4 million tones in 2021 to 7.5 million tones in 2026 (Döhler et al., 2022). The rise in bioplastics reflects a response to environmental issues from traditional plastics and drives research toward sustainable, cost-effective production of commonly produced biopolymer PHAs. PHAs are biodegradable, renewable polyesters produced by microorganisms as storage biopolymers. They decompose naturally under aerobic or anaerobic conditions, making them eco-friendly alternatives to petroleum based plastics (Kourmentza et al., 2017). They are used in various applications, including packaging, agricultural films, and biomedical devices, due to their biocompatibility and biodegradability. Since PHAs can be produced biologically from renewable feedstocks, they offer a potential solution to reduce plastic pollution and the environmental impact of conventional plastics has attracted considerable industry attention as biodegradable and biocompatible thermoplastics (Anderson & Dawes, 1990). PHAs have properties similar to that of conventional plastics produced from petroleum resources (Rasheed et al., 2013). Compared to Polyhydroxybutyrate (PHB)—a subset of PHAs offers enhanced mechanical flexibility, thermal stability, and tailored properties, making them more versatile for diverse applications, including medical devices and flexible materials. While PHB is favored for its cost efficiency and established production methods, its high crystallinity (60–70%) makes it brittle, limiting its adaptability (Nikolaivits et al., 2021). In contrast, the ability to modify the monomer composition of PHAs provides greater adaptability for industrial demands. PHAs are a family of biodegradable polymers with a broad range of applications (Philip et al., 2007). PHAs can be produced in open, mixed microbial cultures as part of biological treatment of municipal and industrial wastes and residuals (Serafim et al., 2008). By integrating waste management with PHA production, researchers aim to develop scalable and eco-friendly solutions for tackling plastic pollution (Fernandez-Bunster & Pavez, 2022). The choice of substrate, including pure, mixed, or waste-based options, plays a pivotal role in optimizing production. Glucose have proven to be efficient, economical options for carbon sourcing (Zhao et al., 2021). Produced from renewable resources, PHAs also offer more sustainable and environmentally friendly production processes. To achieve sustainable and efficient commercial PHA production, several key factors must be considered. These include the steady availability and composition of feedstock, their cost, ease of collection, transportation, and storage, as well as the importance of avoiding competition with food and feed production. The global shift toward PHAs represents a convergence of scientific innovation and environmental stewardship. By leveraging sustainable feedstocks and cost-effective production methods, PHAs offer a viable solution to the plastic pollution crisis while paving the way for a greener future. This study explores the potential of various available substrates, feeding strategy and sustainable PHA production, emphasizing advancements, challenges, and industrial applications. Polyhydroxyalkanoates (PHAs) are biodegradable biopolymers with potential as sustainable plastic alternatives. However, their adoption is challenged by high production costs, hydrolysis of polymer, lower yield, polymer stability and undesirable material properties like high crystallinity, which leads to brittleness.

My study provides insights to develop a cost-effective and scalable strategy for producing high-performance PHAs by employing mixed carbon substrates and optimized feeding regimes. Specifically, the study investigates the use of hydrolyzed sucrose and propanoic acid to reduce crystallinity and tailor monomer composition, aiming to enhance the polymer flexibility and commercial applicability of PHAs. This research has led to a focus on optimizing feeding strategies to minimize polymer degradation during hydrolysis and improve production efficiency. By controlling substrate feeding more effectively, it is expected that PHB stability will improve, resulting in higher and more consistent yields. The thermal properties of the final product are systematically evaluated to assess their suitability for industrial processing and end-use application.

2. METHODOLOGY

2.1 Organisms & Strains

PHA production was studied with *C. necator* DSM 545, DSM 428, and *P. putida*, which were obtained from the laboratory culture collection of the department of Chemical Engineering and Polymer Science. These strains are capable of utilizing mixed carbon sources like hydrolyzed sucrose (Madison & Huisman, 1999). DCW and PHB content were compared for DSM 545 grown on hydrolyzed sucrose alone and in combination with propanoic acid.

2.2 Carbon Sources and Culture Media

A saturated solution (700g/L) of commercially available "FRESH" company sugar was hydrolyzed through autoclaving at 121°C for 15 minutes in the autoclave machine (BIOBASE BKQ-Z30I) to convert the sugar into glucose and fructose (Cui et al., 2016). This resulting hydrolyzed syrup was subsequently diluted, and a final concentration of 20 g/L hydrolyzed sucrose was used as the carbon source in all fermentation experiments. Propanoic acid was supplemented at concentrations ranging from 0.0496 to 0.0992 g/L to promote poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) in strains such as *C. necator* (Munir & Jamil, 2018).

The mineral media composition was based on the formulation by (Blaszczyk, 1993), with $MgSO_4$ concentration was modified to 0.8 g/L. The growth media (Table 2.2) were prepared in 500 mL Erlenmeyer flasks (as bioreactor) containing 100 mL working volume. Hydrolyzed sucrose, either alone or with propanoic acid, served as carbon sources, while ammonium sulfate $(NH_4)_2SO_4$ was used as the sole nitrogen source. The initial pH was maintained between 7.2 and 7.4 using 3 M NaOH or 6 M HCl as necessary. Media were sterilized by autoclaving at 121 °C for 15 min in the autoclave machine (BIOBASE BKQ-Z30I), and substrates were aseptically added to the sterile culture media using sterile syringes inside a laminar flow cabinet (BIOBASE V1300).

Table 2.2 - Growth Media Composition

Constituents	**Components**
Carbon Source	Hydrolyzed Sucrose (20g/L) Propanoic Acid (0.0496g/L or 0.0992 g/L)
Nitrogen Source	$(NH_4)_2SO_4$ = variable
Minerals	K_2HPO_4 = 7.0g/L KH_2PO_4 = 3.0 g/L $Na_3C_6H_5O_7$. $2H_2O$ = 0.5 g/L $MgSO_4.7H_2O$ = 0.8 g/l $FeSO_4.7H_2O$ = 0.05 g/L

2.3 Inoculum Preparation

Stock cultures of *C. necator* DSM 545, DSM 428, and *P. putida* were maintained on LB agar plates (LB 10 g/L, agar 20 g/L). Seed cultures were initiated by aseptically transferring a loopful of bacterial colony from the agar plates into 500 mL Erlenmeyer flasks containing 100 mL of mineral growth medium supplemented with the respective substrates. Seed cultures were incubated at 30 °C and 120 rpm in a shaking incubator (Biobase BJPX-1102C) for 24 h. For pure carbon source batches, the medium contained 20 g/L hydrolyzed sucrose, while mixed batches included 20 g/L hydrolyzed sucrose supplemented with 0.049g/L or 0.09 g /L propanoic acid. Subsequently, 5 mL of the actively grown seed culture was aseptically transferred inside laminar flow cabinet (BIOBASE V1300) into the 100 mL of fresh mineral production medium in 500 mL Erlenmeyer flasks (bioreactors) containing pre-prepared substrates. The inoculum dose was 5% v/v.

2.4 Reactor Configuration

Fed-batch experiments used 500 mL flasks with a working volume of 100 mL mineral media (K_2HPO_4 7 g/L, KH_2PO_4 3 g/L, sodium citrate 0.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.8 g/L, $FeSO_4 \cdot 7H_2O$ 0.05 g/L, and variable concentrations of $(NH_4)_2SO_4$). The pH was adjusted (7.2-7.4), flasks sealed with cotton corks, wrapped

in foil, and autoclaved at 121 °C for 15 min. Hydrolyzed sucrose was sterilized separately and, under aseptic conditions, added with bacterial seed before incubation at 30 °C and 120 rpm.

2.5 Feeding Strategy

Carbon sources were added after 24 h to maintain carbon-limited conditions. Each batch consisted of five flasks where one for seed culture preparation and the remaining four for 96 h fed-batch incubation. One flask was sampled every 24 h for analysis. Substrate was replenished based on residual carbon concentration to sustain limitation, while pH kept at 7.4. The carbon source ratio was adjusted according to consumption patterns to achieve maximum PHA accumulation.

2.6 Extraction of PHB

The quantification of PHB was carried out using an approach inspired by the work of (Yilmaz et al., 2005) with a few thoughtful adjustments to enhance the methodology. The harvested sludge was centrifuged to separate the liquid, allowing the biomass containing the polymer to settle at the bottom of the falcon tubes. The solid biomass was either frozen overnight in a deep freezer for preservation and then dried at 60°C in an oven to obtain dried biomass. Extraction was carried out using two methods: chloroform and biological extraction.

2.6.1 Chloroform Extraction Process:

About 3–5 g of dried biomass was ground and stirred in 100 mL chloroform for 2 h, then filtered to separate the polymer-rich filtrate. After evaporating chloroform to a thick consistency, 20 mL cold ethanol was added to precipitate the polymer, which was finally filtered to obtain the pure product.

2.6.2 Biological Extraction Process:

Approximately 3–5 g of dried biomass was ground and treated with *Bacillus pumillus* FH9 (Fcells) to lyse cells and release PHB. A 100 mL Fcells broth was prepared by inoculating a 500 mL flask with 6 g/L yeast extract using a loopful of a preserved colony. Dried cell powder was sterilized by autoclaving at 121 °C for 15 min in basal medium (0.4 g/L KH_2PO_4, 0.4 g/L K_2HPO_4, 0.5 g/L $MgSO_4$, 1 g/L yeast extract) with pH adjusted to 7.4 using 1 M NaOH. After inoculation, the Fcells culture was shaken at 37 °C, 120 rpm for 24 h, then used for cell lysis in basal medium. The flask was incubated 72 h, centrifuged at 4500 rpm for 10 min, and the pellet dried at 50 °C for 12 h.

3. RESULT & DISCUSSION

3.1 Effect of Nitrogen Conc. On the Growth of DSM 545 & Subsequent Production of Polymer Using Hydrolyzed Sucrose as Substrate

3.1.1 With 1g/L $(NH_4)_2SO_4$

The growth of DSM 545 was studied in a fed-batch system with 20 g/L hydrolyzed sucrose and 1 g/L $(NH_4)_2SO_4$ in 100 mL of media (500 mL flask). Over 96 h, glucose in the supernatant was measured every 24 h, replenished to 20 g/L, and pH maintained at 7.2.

OD and DCW steadily increased, reaching 3.11 and 1.7854 g/L at 96 h, showing active growth. Glucose dropped from 20 to 10.93 g/L in 48 h, then rose to 19.92 g/L after adding 10 g/L sucrose, before falling to 16.92 g/L by 96 h, confirming effective feeding. Even under nitrogen limitation, DSM 545 remained metabolically active and redirected metabolism to PHB accumulation (Merugu et al., 2012).

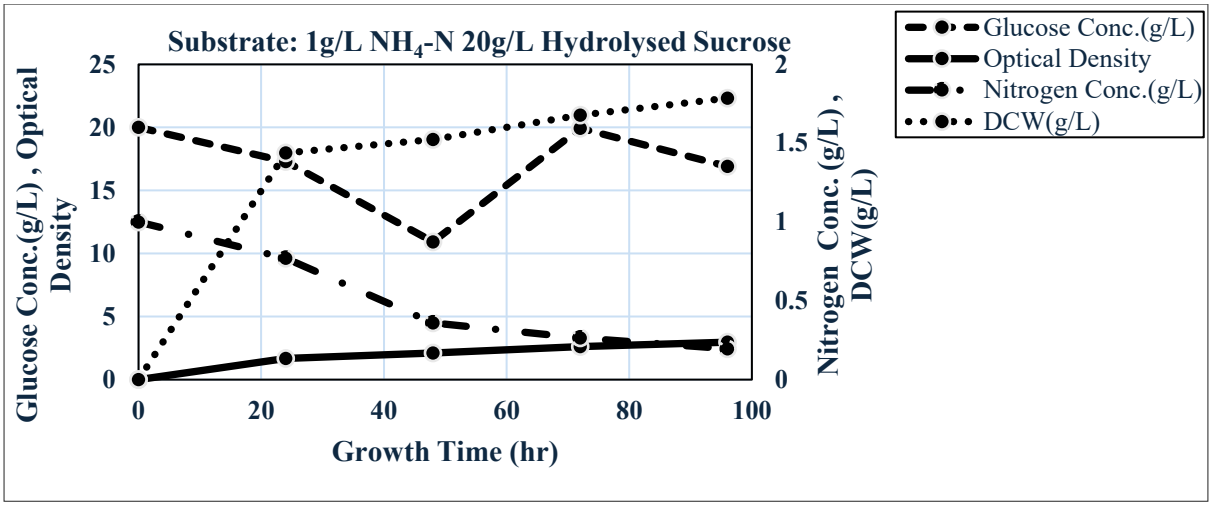

Figure 3.1.1 Optical Density, Dry Cell Weight (DCW) with the concentration profile of ammonium-nitrogen (NH4-N) & glucose (Using RO-water)

3.1.2 With 2g/L $(NH4)_2SO_4$

The growth of DSM 545 was studied in a fed-batch system with 20 g/L hydrolyzed sucrose and 2 g/L $(NH_4)_2SO_4$ in 100 mL of media (500 mL flask). Over 96 h, glucose in the supernatant was measured every 24 h, replenished to 20g/L, and pH maintained at 7.2.

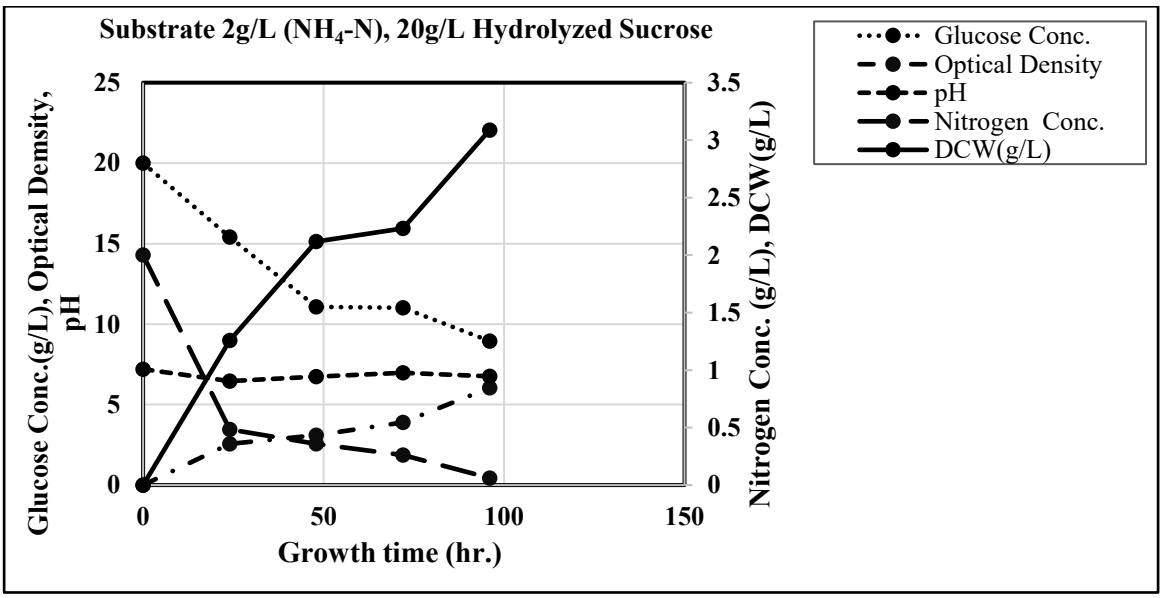

Figure 3.1.2 Optical Density, Dry Cell Weight (DCW), pH with the concentration profile of ammonium-nitrogen (NH4-N) & glucose (Using RO-water)

DSM 545 showed steady growth over the 96-hour fed-batch cultivation using hydrolyzed sucrose (20 g/L) and ammonium sulfate (2 g/L). Biomass increased from 0 g/L to 3.09 g/L, with OD rising from 0.00 to 6.04, indicating active cell growth. Glucose levels decreased progressively due to microbial consumption, from 20.00 g/L to 8.95 g/L and were replenished periodically. Ammonia dropped sharply to 0.062 g/L, confirming nitrogen-limited conditions that promote intracellular PHA accumulation. Ammonium sulfate was varied to induce nitrogen limitation, a known trigger for PHA synthesis. An effective range of 1–2 g/L $(NH_4)_2SO_4$ was identified, with 2 g/L yielding the highest dry cell weight (4.15 g/L) and thus considered optimal under the studied conditions. The pH remained stable, supporting sustained biomass growth.

Other parameters such as temperature, pH, agitation speed, and carbon concentration were kept constant to isolate the effect of nitrogen limitation. These parameters are known to influence PHA synthesis and are suggested for future optimization studies.

3.2 Comparative PHB Yields from Different Bacterial Species Cultivated on Hydrolyzed Sucrose

A comparative study was performed using *C. necator* strains DSM 545, DSM 428, and *P. putida* to evaluate their PHB accumulation capabilities. The process employed mineral media with RO water was supplemented with 20 g/L hydrolyzed sucrose and 2 g/L $(NH_4)_2SO_4$. PHB content at 1 g/L $(NH_4)_2SO_4$ was not quantified as this condition was used solely for biomass growth assessment. PHB analysis was performed only at the optimal $(NH_4)_2SO_4$ concentration (2 g/L) .

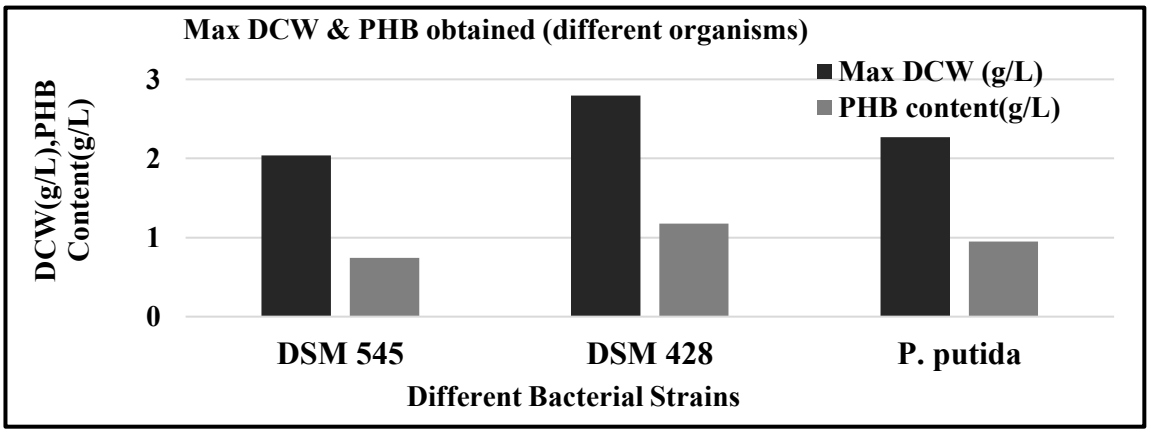

Figure 3.2 Comparative analysis of maximum DCW, PHB content obtained from different bacterial strains using hydrolyzed sucrose substrate

The Batch cultivation was conducted over 96 hours under standardized conditions. In this fed-batch fermentation process, DSM 428 exhibited the highest PHB accumulation (42.124%) and biomass production (2.80 g/L), demonstrating strong efficiency in utilizing hydrolyzed sucrose and promising for industrial-scale biopolymer production. *P. putida* followed closely with a PHB content of 41.842% and a DCW of 2.27 g/L, indicating good potential, though slightly less efficient in biomass generation. In contrast, DSM 545 showed the lowest performance, with a PHB yield of 36.498% and a DCW of 2.04 g/L, making it the least effective under the given conditions. Overall, DSM 428 proved to be the most productive strain, while DSM 545 showed some potential but lags in productivity.

3.3 Effect of Using Different Water Sources for the Preparation of the Growth Media

To evaluate cost-effective alternatives, the influence of different water sources on bacterial growth and biomass yield, the maximum dry cell weight (DCW) was measured for *C. necator* DSM 545 cultured with two substrate conditions: hydrolyzed sucrose alone and hydrolyzed sucrose supplemented with propanoic acid. The experiment was carried out using two water sources RO water and regular tap water from “SUST”.

In the batch where hydrolyzed sucrose was the only substrate, RO-water yielded the highest DCW (4.152g/L), while tap water produced a lower value (2.88 g/L). However, addition of propanoic acid reduced DCW for both with RO-water (2.13 g/L) and tap water (2.01g/L). Although RO water gave better results overall, its high cost makes it less idea for large scale use. Besides, tap water, despite lower performance could be a cost-effective alternative if optimized. Therefore, RO-water with hydrolyzed sucrose is the better condition, but improving tap water usability remains a promising edition.

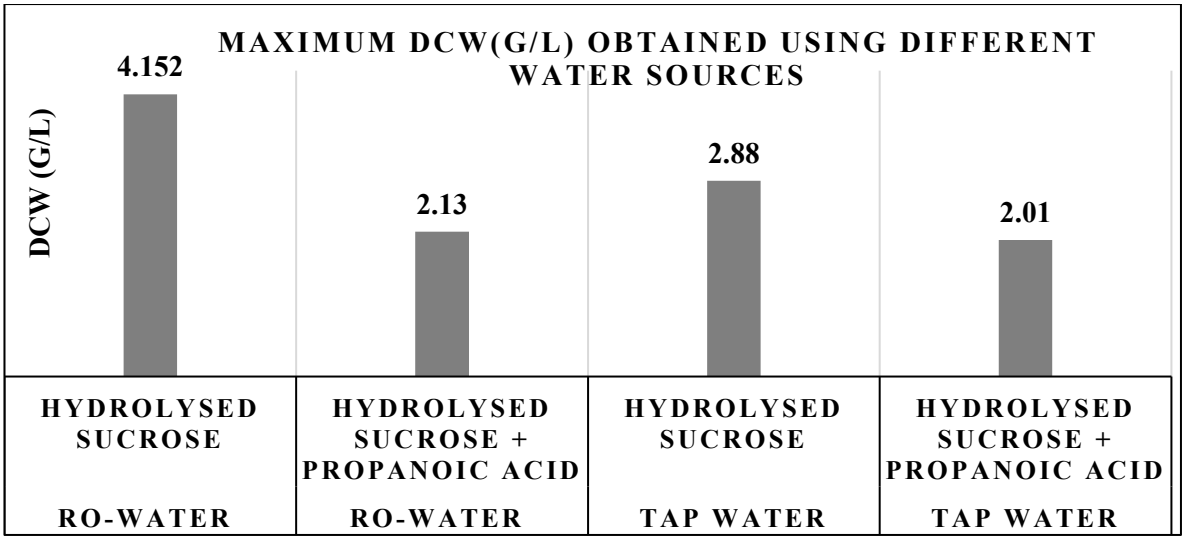

Figure 3.3 Comparative analysis of effect of using RO-water and Tap water on microbial growth and PHB production.

3.3.1 Experimental Results for Different Water Sources

3.3.1.1 Batch 1 & 2: PHA producing using RO-water

RO-water was used to prepare the mineral media. One batch used hydrolyzed sucrose as the sole substrate, while another used a mix of hydrolyzed sucrose and propanoic acid. The pure substrate batch ran for 72 hours, yielding a maximum DCW of 4.152 g/L, while the mixed substrate batch ran for 67 hours. Trends of optical density and DCW over time are shown in the figure below.

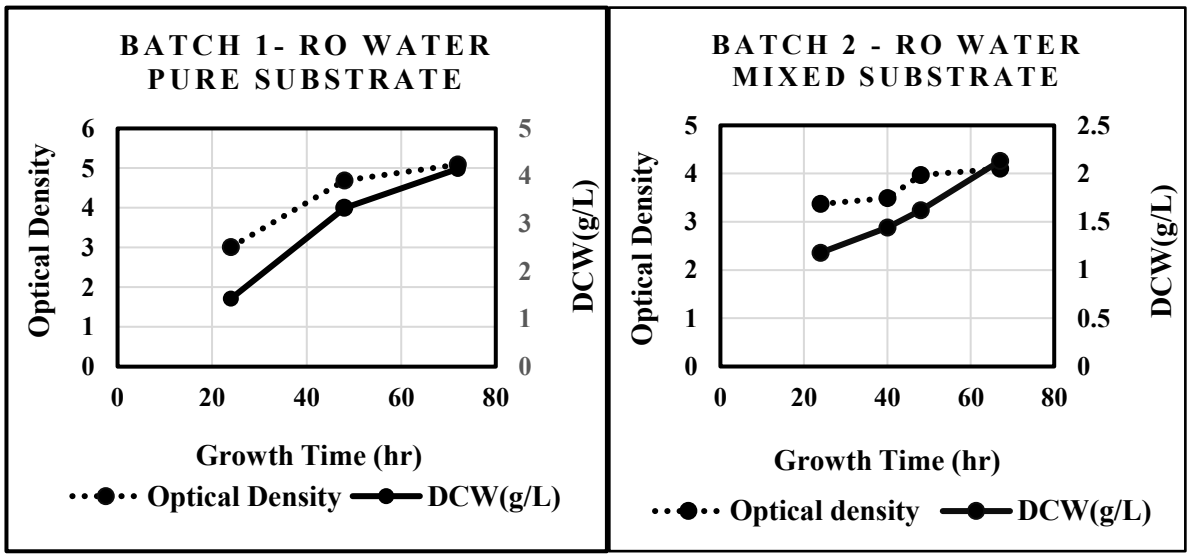

Fig 3.3.1.1 Growth profile of DSM 545 using RO water (Batch#1, Batch#2)

3.3.1.2 Batch 3 & 4: PHA producing using tap water

Mineral media were prepared using tap water from SUST. One batch utilized only hydrolyzed sucrose, while another combined hydrolyzed sucrose with propanoic acid. The pure substrate batch was operated for 96 hours and reached a maximum DCW of 2.01 g/L, whereas the mixed substrate batch ran for 60 hours. The growth patterns for optical density and DCW over time are illustrated in the figure below.

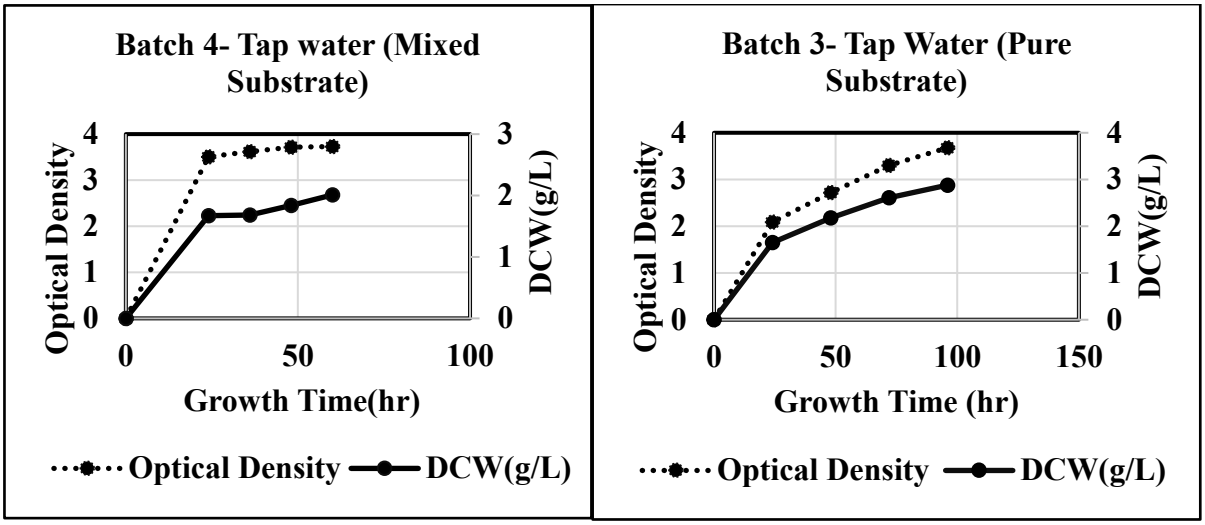

Figure 3.3.1.2 Growth profile of DSM 545 using Tap water (Batch#3, Batch#4)

The lower DCW observed in tap water may be due to dissolved salts, residual chlorine, and variable mineral composition, which are known to affect microbial growth.

3.4 Effect of Mixed Substrate on PHA Accumulation from DSM 545

Propanoic acid enables DSM 545 to produce poly 3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) instead of just PHB by incorporating 3-hydroxyvalerate (3HV) monomers, improving polymer flexibility and toughness. When combined with hydrolyzed sucrose, it provides both energy and tailored building blocks, boosting PHA yield and enhancing material properties.

3.4.1 20g/L Hydrolyzed Sucrose with 0.0496g/L Propanoic Acid

A lower concentration of 0.0496 g/L propanoic acid was employed in a controlled 60-hour batch cultivation, using 20 g/L hydrolyzed sucrose as the primary carbon source and 2 g/L $(NH_4)_2SO_4$ as the sole nitrogen source. Hydrolyzed sucrose was fed every 48h, while pH was maintained at 7.4.

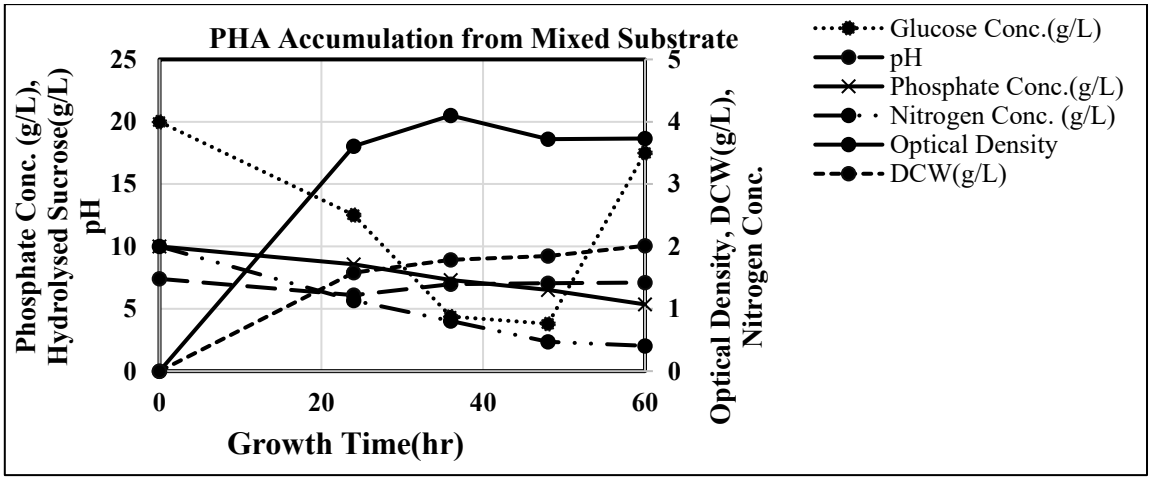

Figure 3.4.1 Optical Density, Dry Cell Weight (DCW), pH with the concentration profile of ammonium-nitrogen (NH4-N), phosphate & glucose (using mixed substrate)

In this batch, DSM 545 showed moderate growth, with DCW reaching 2.01 g/L and OD peaking at 3.73. Glucose was largely consumed for 36 hours. Ammonia declined steadily, indicating nitrogen uptake. Phosphate concentration showed a consistent decline over time (10.00 g/L to 5.35 g/L) by 60 hours. This gradual reduction indicates active microbial uptake during growth and PHB accumulation, reflecting its role as a vital nutrient in cellular metabolism. The pH dropped sharply at 24 hours (6.09)

but maintained at 7.4 periodically, emphasizing the need for pH regulation. Despite reduced propanoic acid levels, no significant PHA accumulation was observed, suggesting limited metabolic incorporation under these conditions.

3.5 Influence of Feeding Regimes on Microbial Growth & PHB Production by DSM 545

In this investigation DSM 545 was grown in two parallel fed-batch systems, each with four 500 mL flasks containing 100 mL working volume. The medium contained mineral salts, 20 g/L hydrolyzed sucrose, and 2 g/L ammonium sulfate. Seed inoculum was prepared from DSM 545 agar plates in the same medium and incubated at 30 °C, 120 rpm for 48 h. Each flask was inoculated with 5 mL seed culture and cultivated for 96 h at 30 °C, 120 rpm under aerobic conditions

The two batches differed only in their carbon feeding strategies:

1. Batch 1: Fed with 10 g/L hydrolyzed sucrose every 24 hours.
2. Batch 2: Fed with 20 g/L hydrolyzed sucrose every 48 hours.

Both setups were maintained at pH 7.4 under identical conditions. Batch 1 showed medium hydrolysis after 48h due to substrate buildup, osmotic stress, and metabolic imbalance. Batch 2 remain stable over 96h, indicating balanced carbon supply, optimal PHA production, aiding process optimization and reducing stress. This comparison highlights frequent feeding causes instability and lower PHA yield, while 48h feeding strategy ensures stable growth and efficient activity.

Figure 3.5 Hydrolyzed product of batch 1 and non-hydrolyzed product of batch 2

3.6 Thermal Properties Analysis: Thermogravimetric Analysis

The TGA curves provide insight into the polymer's thermal degradation temperature (T_{deg}) and the percentage of weight loss during complete degradation, which reflects the PHA content.

3.6.1 TGA Data: Product of DSM 545 Cultivated on Hydrolyzed Sucrose Substrate

The TGA analysis of DSM 545 cultivated on hydrolyzed sucrose substrate revealed a primary thermal degradation starting at 198.35°C and concluding at 385.22°C. This indicates the onset and completion of major polymeric decomposition, likely associated with intracellular PHA degradation. A total weight loss of 2.849 mg**,** which corresponds to 36.498% was observed. This significant loss reflects the percentage of polymeric content within the biomass, suggesting moderate PHA accumulation.

3.6.2 TGA Data: Product of DSM 545 Cultivated on Hydrolyzed Sucrose & Propanoic Acid Substrate

The TGA analysis of the pre-extracted DSM 545 sample grown on hydrolyzed sugar and propanoic acid revealed a primary degradation starting at 242.48ºC and ending at 429.21ºC. The weight loss during this thermal degradation was 6.833mg accounting for 38.039% of the initial mass. The temperature shows the thermal breakdown of intracellular biopolymers mostly PHAs. The substantial weight loss suggests of polymeric content.

3.6.3 TGA Data: Product of DSM 428 Cultivated on Hydrolyzed Sucrose Substrate

The TGA curve of the pre-extracted DSM 428 biomass grown on hydrolyzed sucrose it can be clearly seen that the thermal degradation starting at 236.56ºC and concluding at 457.69ºC. The total loss was 8.212 mg which corresponds to 42.124% of the sample's initial mass. This significant weight reduction reveals that 42.124% of the sample consists of PHA content.

3.6.4 TGA Data: Product of *P. putida* Cultivated on Hydrolyzed Sucrose Substrate

The TGA of the pre-extraction *P. putida* biomass grown on hydrolyzed sucrose shows that thermal degradation starting at 211.17°C and ending at 412.47°C. During this period, the sample exhibited a total weight loss of 5.873 mg, which corresponds to 41.842% of the initial mass. Hence, it indicates that this sample comprises 41.842% PHA; although the monomer type was not experimentally verified in this study, its identity could be determined through GC-MS analysis. According to literature review, the products synthesized by *C. necator* and *P. putida* strains are presented below.

Table 3.6.4 Literature-reported monomer compositions of various bacterial strains

Bacterial Strain	Substrate	Monomer Composition (based on literature review)	References
Cupriavidus necator (DSM 428)	Hydrolyzed Sucrose	3-hydroxybutyrate(HB)	(Haas et al., 2015)
Cupriavidus necator (DSM 545)	Hydrolyzed Sucrose	3-hydroxybutyrate(HB)	(Haas et al., 2015)
Cupriavidus necator (DSM 545)	Hydrolyzed Sucrose & Propanoic Acid	3-hydroxybutyrate-co-3-hydroxyvalerate (HBV)	(Bird et al., 2020)
Pseudomonas putida	Hydrolyzed Sucrose	mcl-hydroxyalkanoates, 3-hydroxyhexanoate (HHX), 3-hydroxyoctanoate (HO)	(Li et al., 2025; Wang et al., 2011)

3.6.5 TGA Data: Extracted Sample (bio-extraction) of DSM 545 (Hydrolyzed Sucrose Substrate)

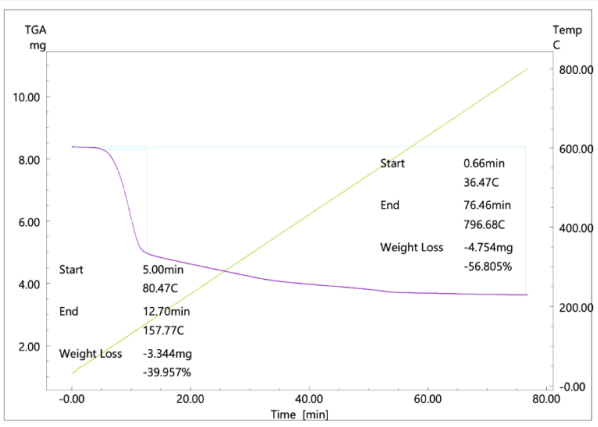

Fig 3.6.5 TGA of extracted sample (bio-extraction) of DSM 545 (hydrolyzed sucrose substrate)

The TGA of the bio-extracted DSM 545 sample, exhibited two distinct stages of thermal degradation. The first degradation phase began at 80.47°C and ended at 157.77°C, resulting in a weight loss of 3.344 mg, which corresponds to 39.957% of the initial mass. This phase is primarily associated with the removal of residual moisture and the degradation of light cellular components remaining after extraction. The second, broader degradation range extended from 36.47°C to 796.68°C, accounting for

an additional 4.754 mg loss or 56.805% of the original weight. This indicates the decomposition of remaining organic constituents, such as proteins, cell wall fragments, and non-PHA materials.

3.6.6 TGA Data: Extracted Sample (via Chloroform) of DSM 545 (Hydrolyzed Sucrose Substrate)

The TGA of the chloroform-extracted DSM 545 sample shows degradation between 270.01°C and 319.84°C, with a weight loss of 2.494 mg (84.257%), indicating high PHA purity. The overall weight loss from 31.38°C to 692.50°C was 2.801 mg (94.628%), suggesting minimal non-polymeric residues. Compared to the pre-extraction sample (41.842% PHA), the sharp dominant PHA degradation peak and low residual mass confirm that chloroform effectively isolated high-purity PHA from the biomass.

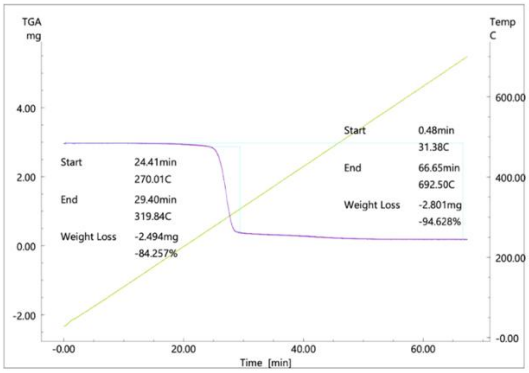

Figure 3.6.6 TGA of extracted sample (via chloroform) of DSM 545 (hydrolyzed sucrose substrate)

4. CONCLUSION

Producing Polyhydroxyalkanoates (PHAs) can be made more affordable by using tap water instead of RO water, with little loss in yield. The best results came from feeding hydrolyzed sucrose and propanoic acid together, leading to a dry cell weight (DCW) of 2.13 g/L with 38% PHA. Using only hydrolyzed sucrose, PHA accumulation ranged from 36% to 42% across different strains. Limiting nitrogen boosted PHA production, with 2 g/L nitrogen yielding the highest DCW of 4.15 g/L. High substrate amounts caused pH to drop, degrading PHA, but lower substrate levels kept pH stable and prevented this. Periodic feeding worked better than bulk addition, keeping conditions steady and reducing polymer loss. DSM 428 performed best overall for scaling up. Chloroform extraction gave higher polymer recovery than bio-extraction.

5. ACKNOWLEDGEMENT

Authors gratefully acknowledge the SUST Research Centre, SUST for financial support (Project ID: AS/2025/1/05).

6. DECLARATION OF USE OF AI

The authors declare that artificial intelligence (AI) tools were used solely for language editing, grammar correction, and improvement of clarity in the manuscript. No AI tools were used in the research design, data collection, data analysis, interpretation of results, or generation of scientific conclusions. All scientific content, results, and interpretations are the original work of the authors.

7. REFERENCES

Anderson, A. J., & Dawes, E. A. (1990). Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiological Reviews*, *54*(4), 450–472. https://doi.org/10.1128/mr.54.4.450-472.1990

Bird, F., Clarke, A., Davies, P., & Surkovic, E. (2020). Ammonia: zero-carbon fertiliser, fuel and energy store. In *The Royal Society*. https://royalsociety.org/-/media/policy/projects/green-ammonia/green-ammonia-policy-briefing.pdf

Blaszczyk, M. (1993). Effect of medium composition on the denitrification of nitrate by Paracoccus denitrificans. *Applied and Environmental Microbiology*, *59*(11), 3951–3953. https://doi.org/10.1128/aem.59.11.3951-3953.1993

Cui, Y. W., Zhang, H. Y., Lu, P. F., & Peng, Y. Z. (2016). Effects of carbon sources on the enrichment of halophilic polyhydroxyalkanoate-storing mixed microbial culture in an aerobic dynamic feeding process. *Scientific Reports*, *6*(August). https://doi.org/10.1038/srep30766

Döhler, N., Wellenreuther, C., & Wolf, A. (2022). Market dynamics of biodegradable bio-based plastics: Projections and linkages to European policies. *EFB Bioeconomy Journal*, *2*, 100028. https://doi.org/10.1016/j.bioeco.2022.100028

Fernandez-Bunster, G., & Pavez, P. (2022). Novel Production Methods of Polyhydroxyalkanoates and Their Innovative Uses in Biomedicine and Industry. *Molecules*, *27*(23), 1–30. https://doi.org/10.3390/molecules27238351

Haas, C., Steinwandter, V., De Apodaca, E. D., Madurga, B. M., Smerilli, M., Dietrich, T., & Neureiter, M. (2015). Production of PHB from chicory roots - Comparison of three Cupriavidus necator strains. *Chemical and Biochemical Engineering Quarterly*, *29*(2), 99–112. https://doi.org/10.15255/CABEQ.2014.2250

Kourmentza, C., Plácido, J., Venetsaneas, N., Burniol-Figols, A., Varrone, C., Gavala, H. N., & Reis, M. A. M. (2017). Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production. *Bioengineering*, *4*(2), 1–43. https://doi.org/10.3390/bioengineering4020055

Li, M., Doudin, K., Robins, D. B., Tetradis-Mairis, G., Wong, T. S., & Tee, K. L. (2025). Microbial synthesis of polyhydroxyalkanoate blends with engineered Pseudomonas putida. *New Biotechnology*, *88*(June), 161–170. https://doi.org/10.1016/j.nbt.2025.05.004

Madison, L. L., & Huisman, G. W. (1999). Metabolic Engineering of Poly(3-Hydroxyalkanoates): From DNA to Plastic. *Microbiology and Molecular Biology Reviews*, *63*(1), 21–53. https://doi.org/10.1128/mmbr.63.1.21-53.1999

Merugu, R., Pratap Rudra, M. P., Girisham, S., & Reddy, S. M. (2012). PHB (Polyhydroxy butyrate) production under nitrogen limitation by Rhodobacter capsulatus KU002 isolated from tannery effluent. *International Journal of ChemTech Research*, *4*(3), 1099–1102.

Munir, S., & Jamil, N. (2018). Polyhydroxyalkanoates (PHA) production in bacterial co-culture using glucose and volatile fatty acids as carbon source. *Journal of Basic Microbiology*, *58*(3), 247–254. https://doi.org/10.1002/jobm.201700276

Nikolaivits, E., Pantelic, B., Azeem, M., Taxeidis, G., Babu, R., Topakas, E., Brennan Fournet, M., & Nikodinovic-Runic, J. (2021). Progressing Plastics Circularity: A Review of Mechano-Biocatalytic Approaches for Waste Plastic (Re)valorization. *Frontiers in Bioengineering and Biotechnology*, *9*(June), 1–31. https://doi.org/10.3389/fbioe.2021.696040

Philip, S., Keshavarz, T., & Roy, I. (2007). Polyhydroxyalkanoates: Biodegradable polymers with a range of applications. *Journal of Chemical Technology and Biotechnology*, *82*(3), 233–247. https://doi.org/10.1002/jctb.1667

Rasheed, R., D., L., Ramachandran, D., & R., G. G. (2013). Characterization of biopolymer producing Streptomyces parvulus, optimization of process parameters and mass production using less expensive substrates. *International Jornal of Bioassays*, *2*(04), 649–654.

Serafim, L. S., Lemos, P. C., Albuquerque, M. G. E., & Reis, M. A. M. (2008). Strategies for PHA production by mixed cultures and renewable waste materials. *Applied Microbiology and Biotechnology*, *81*(4), 615–628. https://doi.org/10.1007/s00253-008-1757-y

Wang, H. H., Zhou, X. R., Liu, Q., & Chen, G. Q. (2011). Biosynthesis of polyhydroxyalkanoate homopolymers by Pseudomonas putida. *Applied Microbiology and Biotechnology*, *89*(5), 1497–1507. https://doi.org/10.1007/s00253-010-2964-x

Yilmaz, M., Soran, H., & Beyatli, Y. (2005). Determination of poly-β-hydroxybutyrate (PHB) production by some Bacillus spp. *World Journal of Microbiology and Biotechnology*, *21*(4), 565–566. https://doi.org/10.1007/s11274-004-3274-1

Zhao, J., Cui, Y. W., Zhang, H. Y., & Gao, Z. L. (2021). Carbon Source Applied in Enrichment Stage of Mixed Microbial Cultures Limits the Substrate Adaptability for PHA Fermentation Using the Renewable Carbon. *Applied Biochemistry and Biotechnology*, *193*(10), 3253–3270. https://doi.org/10.1007/s12010-021-03587-9