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Polyhydroxybutyrate production from non-recyclable fiber rejects and acid whey as mixed substrate by recombinant *Escherichia coli*

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Abstract

Producing polyhydroxybutyrate (PHB) from agro-food processing waste has the potential to mitigate the global synthetic plastic pollution crisis and reduce greenhouse gas emissions. Additionally, it offers a promising solution to the challenges associated with high feedstock and production costs. This study aims to explore the use of two such wastes, non-recyclable fiber rejects (FR) (solid waste) and acid whey (AW) (liquid waste), as cost-effective and sustainable carbon sources for PHB production. Fiber rejects contains up to 50% carbohydrates that can be hydrolyzed to fermentable sugars. The AW is composed of lactose, lactic acid, fats, proteins, and mineral salts which could be used as carbon sources for PHB. The focus of this work was a comprehensive evaluation of substrate utilization, cell growth, and PHB inclusion in recombinant *E. coli* during the fermentation of various blends of acid whey and hydrolysate obtained from fiber rejects. Two approaches were investigated: i) produce FR hydrolysate and mix it with AW in various ratios (1:2, 1:1, and 2:1), and ii) use acid whey to replace water during the hydrolysis of FR. Combining acid whey with the hydrolysate achieved the highest PHB yield in a shorter duration compared to using only the hydrolysate. Replacing acid whey with water during the enzymatic hydrolysis of pretreated fiber rejects and utilizing it further for fermentation resulted in the highest PHB yield of 5.2 g/L, with a 45.4% PHB inclusion rate. Additionally, the inherent lactic acid content in acid whey eliminates the need for adding acetic acid to adjust pH levels during hydrolysis, thereby saving freshwater and acid.

Keywords Polyhydroxybutyrate, Fiber rejects, Pretreatment, Acid whey, Recombinant *Escherichia coli*

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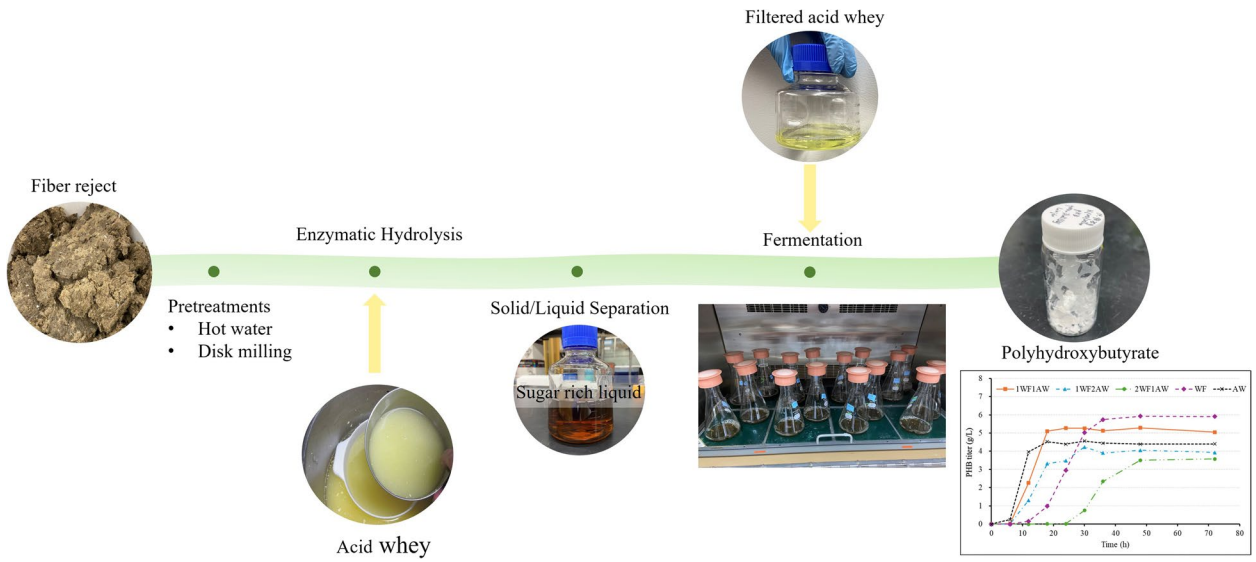
Graphical Abstract



Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible biopolymers synthesized by bacteria through fermentation and accumulated in the form of intracellular granules., Due to their production from renewable feedstocks and universal biodegradability, they represent a high-potential alternative degradation-resistant petroleum-based plastics and a sustainable solution to address fossil energy use during conventional plastic production and the environmental impact associated with their disposal [1, 2]. Among more than 160 types of PHAs, a short-chain polymer, polyhydroxybutyrate (PHB), is the most common polymer to replace traditional plastic due to its specific properties and production advantages. It exhibits good thermoplasticity, excellent hydrophobicity, and resistance to hydrolysis, providing performance characteristics similar to those of polypropylene [3]. It biodegrades entirely into CO_2 and H_2O, and the degradation rate is faster compared to other bioplastics, such as polylactic acid (PLA) [4]. PHB has been widely used in packaging, agriculture, and biomedical applications such as tissue engineering and drug delivery [5, 6]. PHB production and application have been limited on a commercial scale despite considerable interest and effort, mostly because of challenges of scalable supply of carbon sources and high production costs ($5–6 vs. $1.3–1.9 for synthetic polymers) [7]. Currently, the majority of PHB is synthesized using carbon sources obtained from food-based feedstocks such as corn starch, sugarcane, and vegetable oil, which are high-cost feedstocks, present capacity limitation issues, and compete with food production.

Abundant and underutilized waste streams from agro-food processing industries can serve as alternative carbon sources for PHB production, offering both economic and environmental benefits. These waste streams, typically an economic burden, provide an opportunity for upcycling, reducing disposal costs while potentially lowering PHB production expenses. However, challenges persist, including low sugar recovery from cellulosic wastes, inhibitory compounds, feedstock composition variability, and insufficient local supply of a single feedstock. Developing chemical-free pretreatment methods, employing robust microorganisms capable of metabolizing diverse carbon sources, and utilizing mixed feedstocks can address many of these issues. This study explores a novel bioprocess scheme for blending two abundant wastes from New York State—non-recyclable fiber rejects from recycled paper mills and acid whey from dairy processing—as sustainable and cost-effective carbon sources for PHB biosynthesis.

FR, a lignocellulosic by-product from recycled paper mills, offers significant potential for PHB production due to its high carbohydrate content [8]. After repeated recycling, the fibers lose bonding capacity and must be rejected, creating an environmental burden for the industry [9–12]. These fiber rejects are rich in carbohydrates, making them ideal for fermentation for the production of ethanol, bioplastics, and other valuable products, but require efficient pretreatment [13, 14]. A chemical-free

hydrothermal pretreatment followed by enzymatic hydrolysis has proven effective in recovering up to 50 g/L of reducing sugars [9]. Similarly, AW, a by-product of cottage cheese and Greek yogurt processing, consists of lactose (38–49 g/L), lactic acid (5.1–8 g/L), proteins (4.2–10 g/L), fats, and mineral salts [15]. Its high lactose and lactate content make it a valuable feedstock for bioplastic production. Utilizing AW not only offers a renewable carbon source but also helps address the environmental challenges associated with its disposal.

Using co-substrates for PHB production has gained attention as an effective strategy to enhance PHB yield and efficiency. This approach can balance nutrient availability, promote cell growth, and increase overall PHB productivity. Most importantly, utilizing a blend of biomass as feedstock can maximize the use of locally available waste and increase carbon supply to enhance production capacity. Tourang et al. (2023) reported that using acetate or high concentrations of glucose as sole carbon sources resulted in low growth rates. A co-substrate strategy combining glucose and sodium acetate mitigated the inhibitory effects of high substrate concentrations and significantly improved growth rates, with optimal conditions (50 g/L glucose and 20 g/L sodium acetate) leading to 83.4 g/L biomass and 31.7 g/L PHB in bioreactor cultivation [16]. Sugarcane vinasse and molasses were used as co-substrates by *Cupriavidus necator* increasing the maximum specific growth rate of 0.36 h^{-1} compared to 0.19 h^{-1} with molasses alone due to the presence of organic acids. Using a blend of vinasse and molasses resulted in enhanced biomass concentration and PHB accumulation in the bioreactor, achieving a PHB concentration of 11.7 g/L and a PHB accumulation of 56% [17]. In the current study, a mix of acid whey and FR hydrolysate will produce a mix of various carbon sources; sugars (glucose, xylose, galactose, lactose) and acids (acetic acid and lactic acid). The efficient fermentation of this mix to PHB requires use of a robust strain that can consume all these carbon sources.

In our previous study, we have demonstrated that *Escherichia coli* LSBJ with plasmid pBBRSTQKAB is able to metabolize all these carbon sources [8, 18]. It also possesses β-galactosidase which is involved in hydrolyzing lactose into a mixture of glucose and galactose, positioning it as an ideal candidate for PHB production using acid whey as substrate [18]. Moreover, *E. coli* LSBJ is a derivative of *E. coli* LS5218, which is known for its enhanced resistance to the common fermentation inhibitor acetate at higher concentrations, making it highly suitable for utilizing lignocellulosic biomass-derived sugars [19, 20]. The focus of this work is a comprehensive evaluation of the substrate utilization, cell growth, and PHB inclusion in recombinant *E. coli* during the fermentation of various blends of acid whey and hydrolysate obtained from fiber rejects. Two approaches were investigated: i) produce FR hydrolysate and mix it with AW in various ratios (1:2, 1:1, and 2:1), and ii) add acid whey to replace water during the hydrolysis of FR. In addition to the previously discussed advantages of mixed substrates, the novel mixing strategy of using AW during hydrolysis (second approach) provides additional benefits of reducing water use and chemical use (reduce acetic acid use for pH adjustment before hydrolysis) in the process, improving process sustainability.

Results and discussion

Pretreatment, hydrolysis and fermentation of FR hydrolysate

The results presented in this section are previously published and, therefore are briefly discussed here as they are critical for the comparison with other fermentation conditions [8]. Fiber rejects contained 35.5%, 9.0%, 16.6% glucan, xylan, and lignin, respectively. The ash and extractives were 25.0% and 9.7%, respectively. Pretreatment is a crucial step to improve the saccharification efficiency of lignocellulosic biomass. As reported in our previous paper, the hydrothermal pretreatment at 150 °C, combined with three cycles of disk refining, resulted in a high glucose yield (39.6 g/L) during enzymatic hydrolysis, with a cellulose conversion of 83.2%. Total reducing sugars in hydrolysate were found 47.8 g/L. With FR hydrolysate as the sole carbon source, the initial carbon sources in the medium were 16.1 g/L, 3.9 g/L, and 10.4 g/L of glucose, xylose, and acetate, respectively (Table 1). The cell growth, PHB accumulation, and substrate consumption were monitored throughout the fermentation process, and are illustrated in Fig. 1. Detailed discussion on the results from the fermentation of fiber rejects hydrolysate can be found in a previously published paper [8].

Production of PHB using acid whey

Before being used as a carbon source during fermentation, the raw acid whey was adjusted to pH 7, resulting in observable precipitation, presumably due to protein precipitation. Therefore, the pH-changed acid whey was filter sterilized before use. The composition of acid whey was found to contain 37.4 g/L lactose, 6.4 g/L galactose, and 8.3 g/L lactic acid. For the fermentation experiments, acid whey was diluted to obtain 20 g/L total sugars (17.1 g/L lactose, 2.9 g/L galactose). At that dilution level, the concentration of lactic acid was found 3.8 g/L. Cell biomass growth, PHB accumulation, and sugar consumption are shown in Fig. 2a and b. The results indicate that lactose consumption began

Table 1 Initial carbon sources concentration in the fermentation medium

Initial carbon sources (g/L)	WF	AW	AWWF[a]	1WF1AW[b]	1WF2AW[b]	2WF1AW[b]
Glucose	16.12	-	12.87	8.42	5.68	11.05
Xylose	3.88	-	1.90	2.03	1.37	2.66
Acetic acid	10.36	-	2.25	5.46	3.69	7.17
Lactose	-	17.08	4.25	8.08	10.90	5.29
Galactose	-	2.92	0.98	1.38	1.87	0.91
Lactic acid	-	3.80	0.85	1.80	2.42	1.17

[a] AWWF: scenario when acid whey is added to replace water during hydrolysis

[b] Represents the ratio (volume basis) of fiber reject hydrolysate and acid whey

immediately, with galactose and lactose being preferential sugars for *E. coli*, rapidly metabolizing within 12 and 18 h, respectively (Fig. 2b). In contrast, lactic acid uptake commenced only after these two sugars were nearly depleted. During the period of lactic acid consumption, there was a greater accumulation of PHB compared to cell growth (Fig. 2a). This observation aligns with findings from our previous study, where when lactate was used as the sole substrate, the PHB content and titer were higher compared to the experiment using lactose alone as the substrate. This difference in PHB titer can be attributed to the longer metabolic pathway required to convert lactose to PHB, as opposed to the more direct conversion pathway of lactate [18]. The cell dry weight (CDW) and PHB content continued to increase until all carbon sources were consumed, reaching 8.7 g/L and 53% at 30 h, respectively, and maintaining these levels until 72 h. The stability of PHB production after reaching its peak can be attributed to the absence of genes responsible for PHA depolymerase biosynthesis in *E. coli* LSBJ, which prevents the degradation of the accumulated polymer [21]. This genetic characteristic ensures that once PHB production reaches its maximum, it remains stable without further breakdown, thereby optimizing yield consistency over time. This is a significant advantage of using recombinant *E. coli*, which has been extensively utilized in research employing whey as a carbon source [22]. Additionally, the recombinant *E. coli* possesses intrinsic β-galactosidase activity, eliminating the need for chemical or enzymatic conversion of whey lactose into glucose and galactose before fermentation, thereby reducing production costs [23]. In this study, the PHB titer obtained from acid whey using

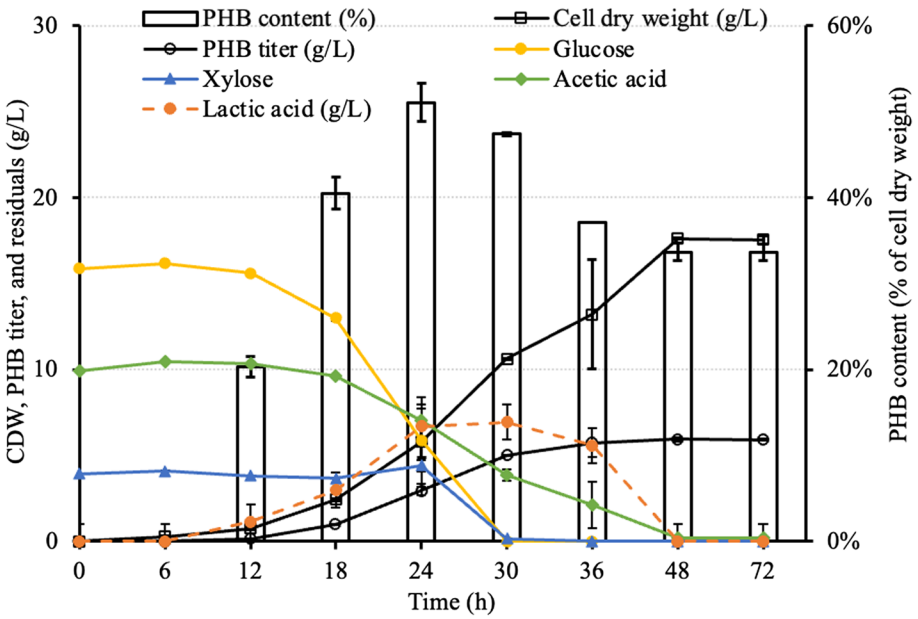


Fig. 1 Residual glucose, xylose, acetic acid, and lactic acid, Cell dry weight (CDW), PHB content, PHB titer produced from pretreated fiber reject hydrolysate using recombinant *E. coli* LSBJ. The results were presented in mean ± SD, $n = 2$ [8]

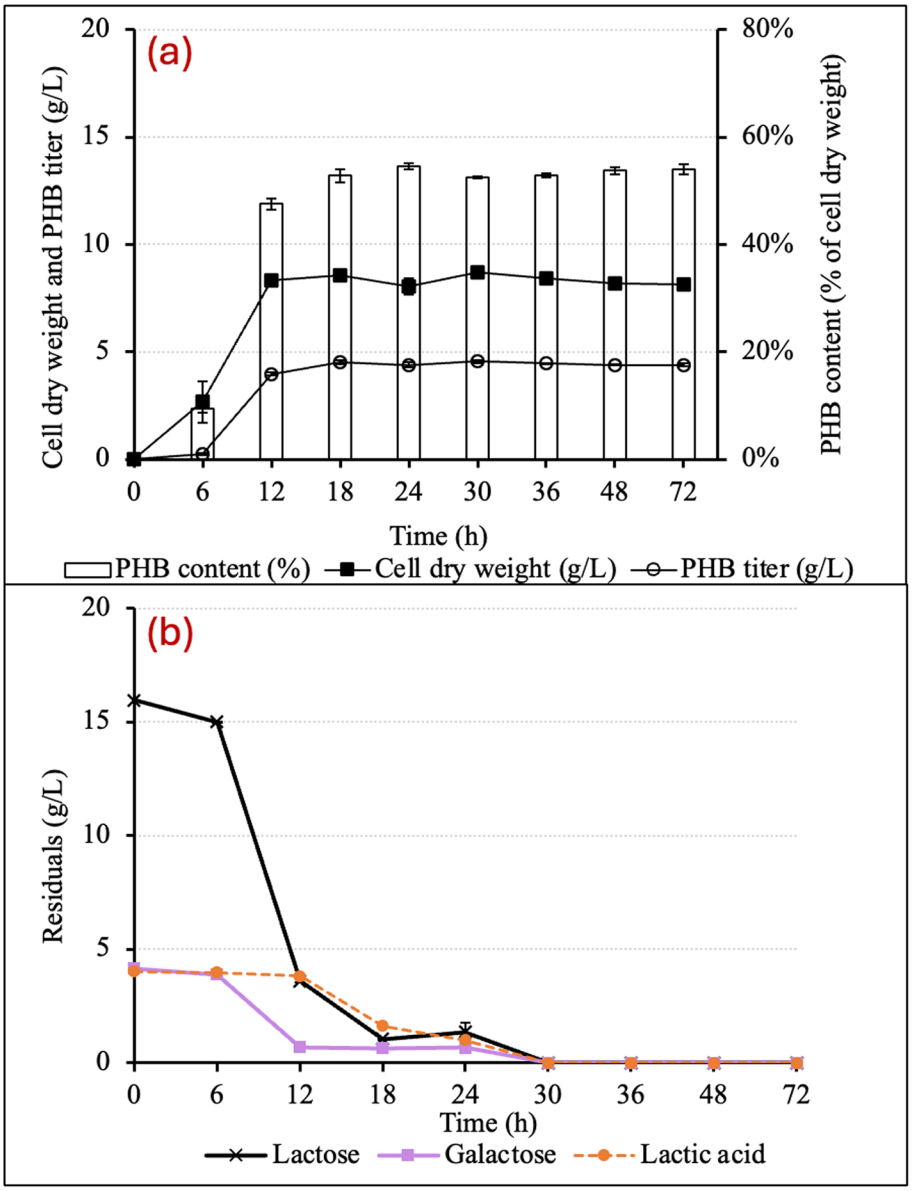


Fig. 2 Time evolution of (**a**) cell dry weight, PHB inclusion, and PHB titer (**b**) Substrate consumption and intermediate concentration during PHB production on 20 g/L of pure AW

E. coli LSBJ was higher than the previously reported PHB titer of 2.2 g/L using native *Bacillus megaterium* strain Ti3 under the optimized condition [24].

Compared with hydrolysate, the PHB content was higher when whey was used as the substrate, but the final PHB titer was lower due to the lower CDW. This discrepancy is attributed to the significant amount of acetate present in FR hydrolysate, which also results in a higher CDW but inhibits bacterial growth, leading to a prolonged lag phase [25]. Consequently, cells exhibited faster growth and a shorter lag time on acid whey compared to FR hydrolysate. Additionally, lactate was formed as a byproduct when using FR hydrolysate as a substrate, and the transition of glucose to lactate delayed the growth of *E. coli* [26]. Another reason for the lower PHB titer using AW as substrate could be the significant drop in pH to 5.2 at 12 h due to metabolic activities of the *E. coli* LSBJ, which inhibited PHB synthesis. This finding aligns with previous studies showing that a pH drop to 5.3 sharply decreases PHB content in cells to 7.0%, and a further reduction to 5.1 results in a PHB content as low as 0.52% [18]. Compared with

the work using acid whey (AW) as a fermentation substrate [18], the use of Luria–Bertani (LB) medium instead of M9 minimal medium in this study resulted in higher PHB production at lower substrate concentrations. However, the PHB content was relatively low. This can be attributed to the rich nutrient composition of LB medium, which provided readily available nutrients, leading to reduced PHB accumulation as cells prioritized growth over PHB synthesis. In contrast, the limited nutrients in M9 medium would stress the bacteria triggering higher accumulation of PHB as a storage compound.

Production of PHB using mixture (FR:AW 1:1)

To study the effect of various blends of acid whey and hydrolysate obtained from fiber rejects on cell growth and PHB inclusion in *E. coli* LSBJ during fermentation, the two substrates, hydrolysates and acid whey were mixed in a 1:1, 2:1 and 1:2 (v/v) ratios and used as the substrate for PHB production (composition shown in Table 1). For the 1:1 ratio, the results showed that CDW started to increase rapidly from 6 h until it reached a maximum of 14.5 g/L at 24 h, and then remained stable (Fig. 3). The trend of PHB content was similar to that of using FR hydrolysate alone, increasing to a maximum of 44% at 18 h and then decreasing to 36% at 24 h subsequently stabilizing (Fig. 3a). Under this condition, the maximum PHB titer was 5.3 g/L. Figure 3b demonstrates the consumption of multiple carbon sources. The consumption priority of different sugars, such as D-glucose, lactose, and D-xylose, follows a hierarchical order. This carbon source preference is regulated by intracellular cAMP-CRP and transcription factors [27–29]. The presence of glucose inhibits the expression of the lactose operon, leading to delayed utilization of lactose [18]. A similar observation was made in this study that lactose consumption primarily began after glucose was nearly exhausted when the mixture was used as a substrate. It is noteworthy that no significant increase in lactate concentration was detected during the fermentation process. Therefore, significant pH reduction was not observed compared to the mixtures with other ratios. Once the lactate in the medium was consumed, the pH of the culture increased. This observation suggests that the metabolic flux of this mixture of FR hydrolysate and acid whey through the PHB biosynthesis pathway differs from other substrates. However, further investigation is warranted to fully understand this phenomenon.

Production of PHB using mixture (FR:AW 1:2)

The two substrate FR hydrolysate and whey were mixed in a ratio of 1:2 (v/v) and used as substrates for PHB production. Since the ratio of acid whey was higher than hydrolysate in this case, the amount of glucose (5.7 g/L) was much lower than lactose (10.9 g/L) from acid whey. The concentrations of other sugars are given in Table 1. As shown in Fig. 4a, CDW in this case continued to increase from 6 h until it reached a maximum of 12.0 g/L at 48 h, after which it stabilized. It is noteworthy that a lag phase was observed between 18 and 24 h, likely due to the peak concentration of lactic acid (8.3 g/L) occurring at 18 h. The accumulation of lactic acid led to a pH drop to 5.3, consequently inhibiting cell growth and reducing the activity of enzymes involved in PHB synthesis [18, 30]. As lactate was converted to pyruvate by lactate dehydrogenase, protons (H^+) were consumed, thereby reducing the concentration of free hydrogen ions in the medium, causing the pH to increase from 18 h. The PHB content peaked at 42% at the 24 h mark, subsequently decreased to 34%, and then remained stable (Fig. 4a). Due to the consumption of acetic acid after 24 h, cell growth was observed but PHB production did not increase further, overall reducing the PHB content of the cells. Under this condition, the maximum titer of PHB was 4.2 g/L, which was about 20.7% lower than the 1:1 ratio of hydrolysate and acid whey. This can be attributed to low initial glucose concentrations in the fermentation media.

Production of PHB using mixture (FR:AW 2:1)

FR hydrolysate and AW were combined in a 2:1 (v/v) ratio to serve as the substrates for the synthesis of PHB (composition in Table 1). As shown in Fig. 5, the cell dry weight exhibited a prolonged lag phase up to 24 h, followed by an exponential from 24 to 72 h, reaching a maximum of approximately 10.2 g/L at 72 h mark. Similarly, PHB content as a percentage of cell dry weight displayed a delayed but sharp increase, peaking at around 40.0% at 36 h. Since a longer lag phase was observed with pure hydrolysate compared to mixtures with acid whey, similar trends were anticipated when the ratio of hydrolysate was higher in the mixture. However, the lag phase in this condition was longer than expected, warranting further investigation. Under this condition (FR:AW 2:1), the maximum PHB titer was 3.6 g/L which was significantly ($p < 0.05$) lower than that of 5.3 g/L (FR:AW 1:1) and 4.2 g/L (FR:AW 1:2).

Production of PHB using acid whey during hydrolysis

Due to the high ash content (~ 20%) in FR predominantly composed of calcium carbonate, the pH of the prepared slurry for hydrolysis increased to above 10. This pH level is significantly higher than the optimal condition (pH 5) required for cellulase and hemicellulase activity. Consequently, acetate needs to be added to adjust the pH to 5 prior to hydrolysis. Acid whey, characterized by its low pH due to lactic acid

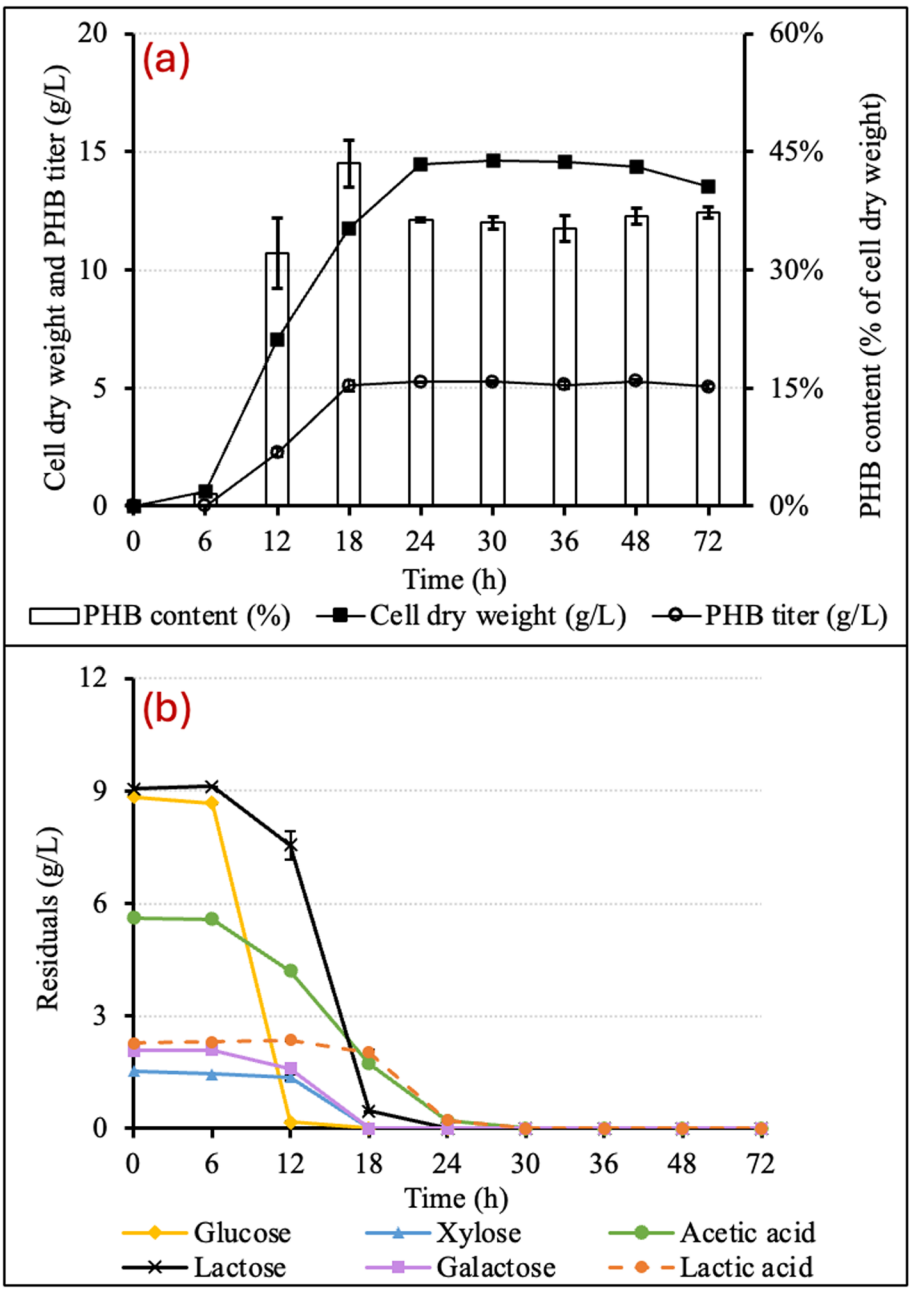


Fig. 3 Time evolution of (**a**) cell dry weight, PHB inclusion, and PHB titer (**b**) Substrate consumption and intermediate concentration during PHB production on 20 g/L of mixture (FR:AW 1:1)

presence, was used as a substitute for water in the enzymatic hydrolysis process. This substitution significantly reduces the required dosage of commercial acetic acid to one-quarter of the original amount, enriches the sugar concentration in the mixed hydrolysate (AWWF), and improves the C/N ratio. Following enzymatic hydrolysis, a total sugar yield of 59.7 g/L was achieved, comprising 38.4 g/L glucose, 5.7 g/L xylose, 12.7 g/L lactose, and 2.9 g/L galactose as well as 6.7 g/L acetic acid and 2.5 g/L lactic acid. Figure 6 illustrates the dynamics of cell growth, PHB production, and substrate consumption during fermentation using a mixture of AWWF as the substrate. From 6 h onwards, rapid cell growth and PHB accumulation were observed. PHB content peaked at 47.1% at 18 h, coinciding with complete sugar consumption.

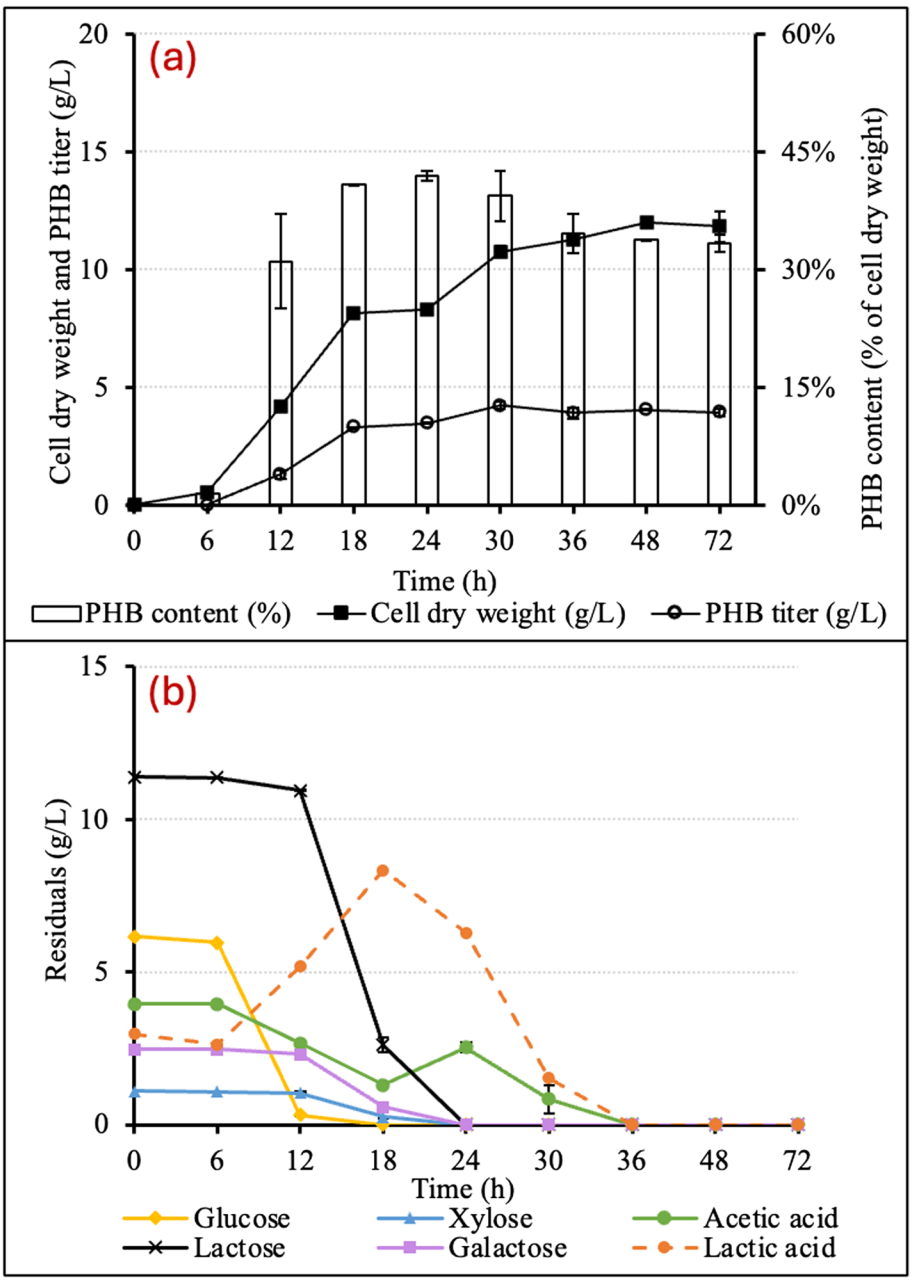


Fig. 4 Time evolution of (**a**) cell dry weight, PHB inclusion, and PHB titer (**b**) Substrate consumption and intermediate concentration during PHB production on 20 g/L of mixture (FR:AW 1:2)

Subsequently, lactic acid and acetic acid were gradually consumed, leading to a slight increase in CDW to a maximum of 12.0 g/L. PHB content decreased slightly to 44.6% and stabilized resulting in the highest PHB production of 5.2 g/L at 30 h. Notably, during the period when glucose was the primary carbon source and PHB was accumulating rapidly, an increase in lactate concentration was not observed as with other substrate mixtures during AWWF fermentation. This condition was observed to favor PHB accumulation over cell growth. In comparison to previous studies on PHB production using recombinant *E. coli*, our results demonstrate a competitive performance when utilizing AWWF. A study using brewers' spent grain as a substrate reported a titer of 3.5 g/L PHB, lower than the results with AWWF [21].

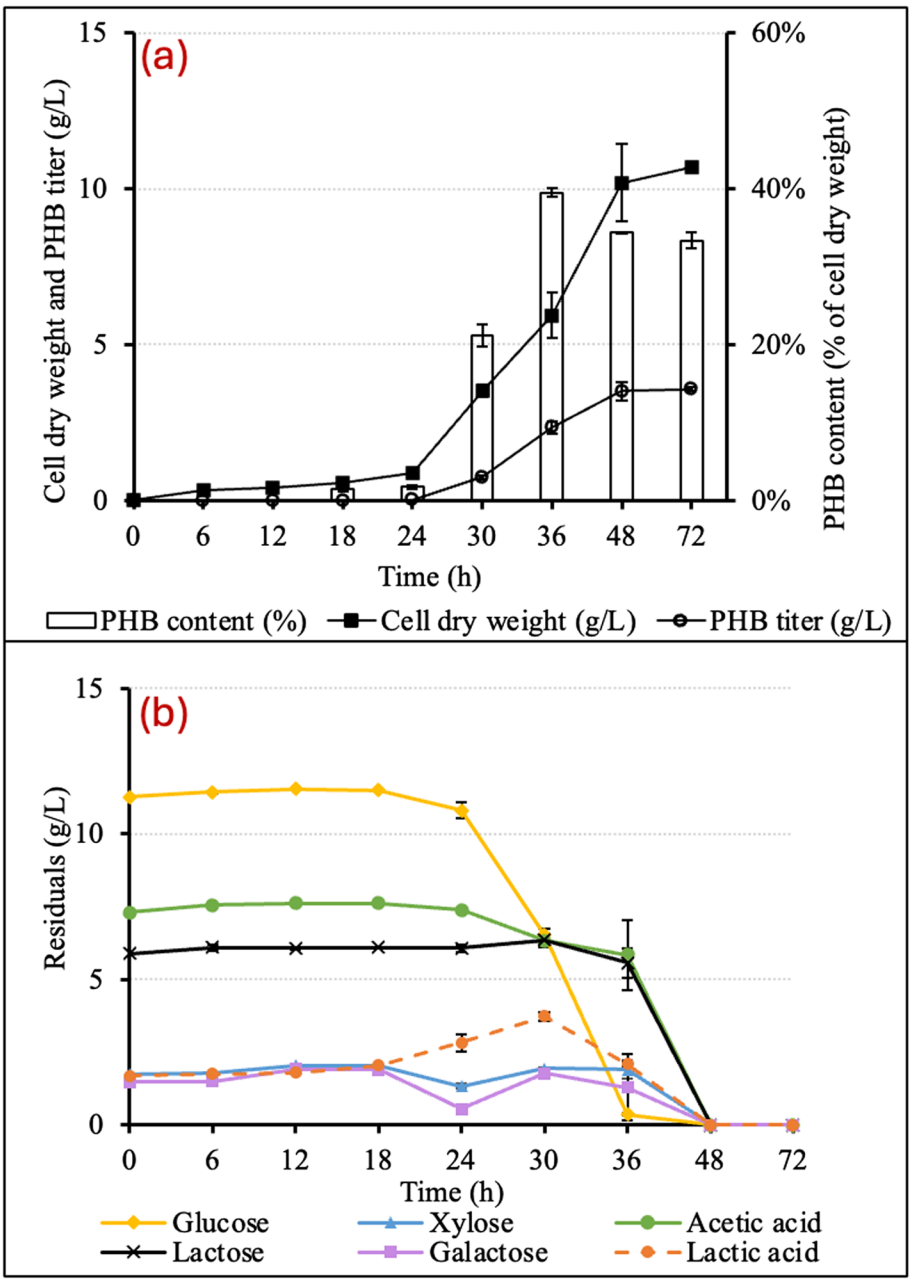


Fig. 5 Time evolution of (**a**) cell dry weight, PHB inclusion, and PHB titer (**b**) Substrate consumption and intermediate concentration during PHB production on 20 g/L of mixture (FR:AW 2:1)

Comparison of different mixed approaches

In comparing the PHB titer during the 72 h fermentation in kinetic studies (Figs. 7 and 8), it was observed that the presence of AW facilitated faster cell growth and attainment of maximum PHB titer, which is crucial for industrial applications as it shortens the overall production time. This reduction in cultivation time can lead to increased efficiency and cost-effectiveness in large-scale PHB production processes. In one of the conditions (2:1 WF: AW), however, results were contrary. The ANOVA results demonstrate that the mixing methods have varying levels of effectiveness on the PHB production. Significant pairwise differences suggest that certain mixing methods (such as AWWF and 1FR1AW) perform better than others (ANOVA table provided in supplementary materials). When this mixture (FR: AW 2:1) was used as substrate, the PHB titer was significantly lower than that observed with

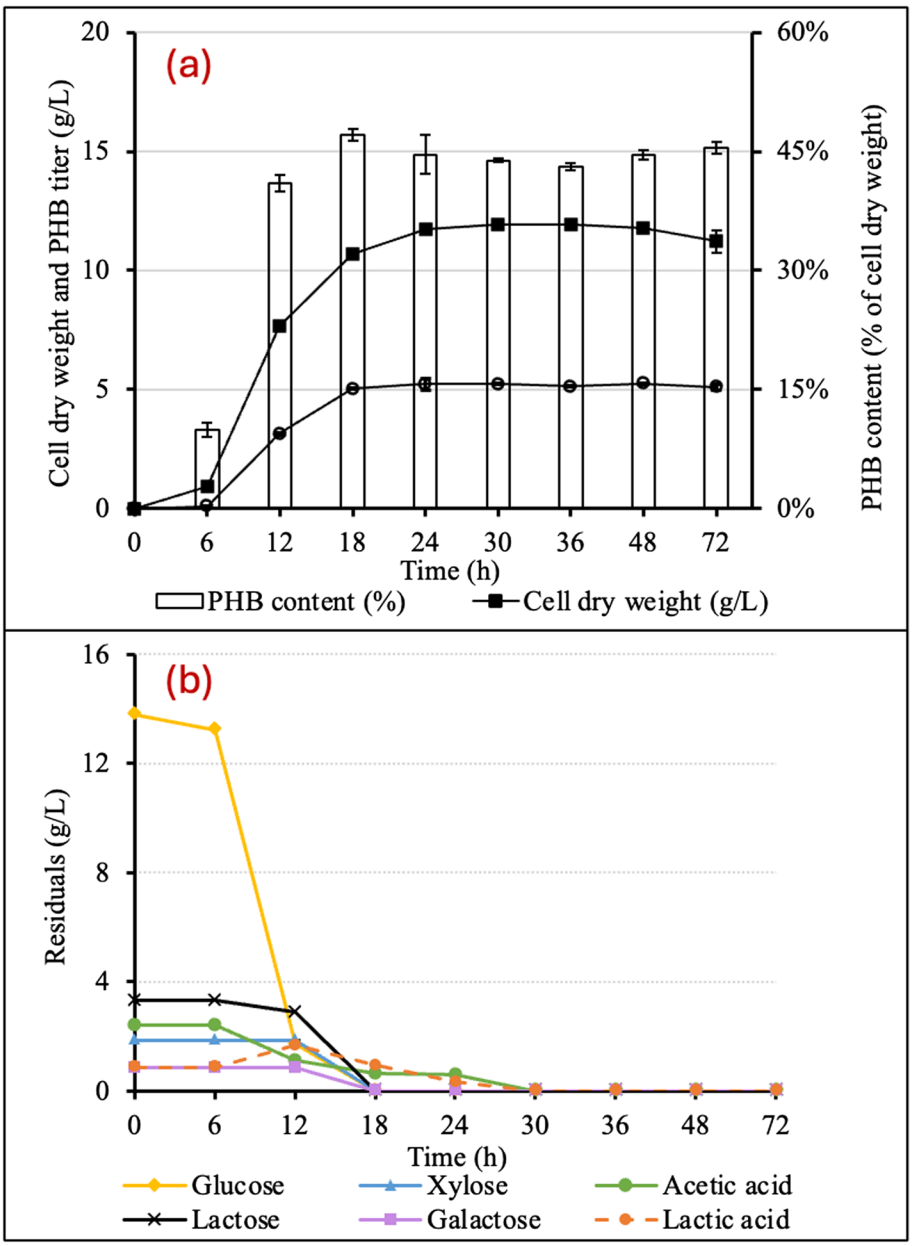


Fig. 6 Time evolution of (**a**) cell dry weight, PHB inclusion, and PHB titer (**b**) Substrate consumption and intermediate concentration during PHB production on 20 g/L of mixture (AWWF)

other ratios, and the lag phase was longer. This may be attributed to the higher glucose content in the medium which inhibits lactose utilization. It is well established that the presence of glucose in the extracellular medium hinders the induction of the lactose operon in *E. coli* cultures [31]. In other conditions, the presence of minerals, proteins, and vitamins in acid whey supported microbial growth and enhanced metabolic activity, thereby increasing PHB productivity. The acetic acid in the FR hydrolysate was diluted to varying concentrations depending on the different ratios used, which exerted differing degrees of inhibition on cell growth and PHB accumulation, resulting in a delay in PHB synthesis [25].

Conclusion

This study aimed to utilize non-recyclable fiber rejects and acid whey as co-substrates for PHB production. The effect of various ratios of AW and FR hydrolysate (1:1, 1:2, 2:1 v/v) and effect of different mixed

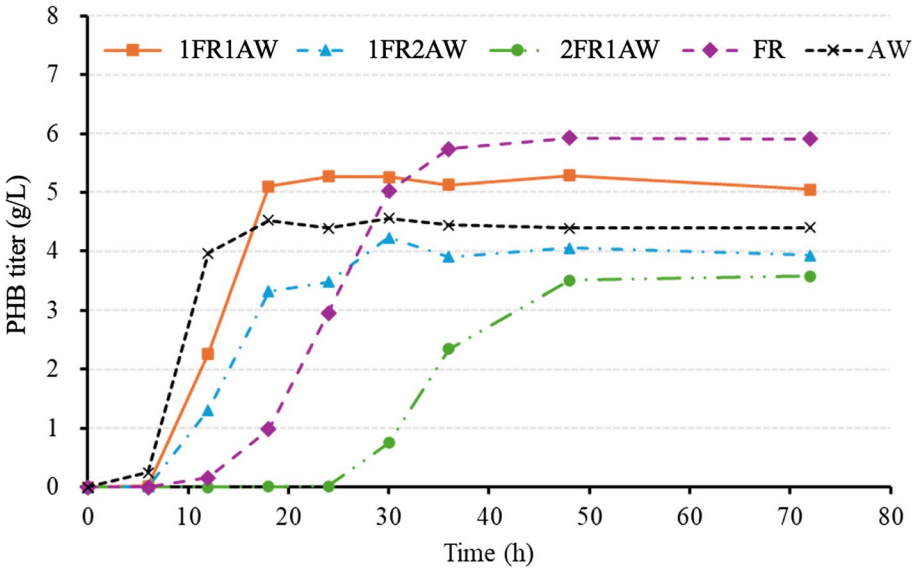


Fig. 7 Time evolution of PHB titer during PHB production on different ratio of blends

approaches of two wastes have been investigated on cell growth and PHB inclusion in recombinant *E. coli* during fermentation. The results showed that adding AW during the enzymatic hydrolysis of FR obtained a carbon-rich hydrolysate, leading to the highest PHB titer of 5.2 g/L with 45.4% PHB inclusion. This study provides a promising approach for the production of PHB utilizing AW and FR as co-substrates. Future research should focus on exploring additional mixing strategies and scaling up the process in bioreactor systems to assess the commercial viability of PHB production. Moreover, the use of acid whey offers environmental benefits such as significant water and acetate savings. Acid whey serves as a viable alternative to water in the process, and its inherent lactic acid content obviates the need for additional acetic acid to adjust pH levels. These factors collectively underscore the efficiency and sustainability of utilizing acid whey in PHB production processes.

Materials and methods

Materials

Fiber reject samples were collected from a local paper mill (WestRock Paperboard Mill, Syracuse, NY, USA). The biomass was dried at 50 °C for 48 h to reduce moisture below 10%, and then milled using a Wiley Mill to achieve a particle size of less than 2 mm. The samples were subsequently stored in a refrigerator at 4 °C for further experiments. The chemical composition of fiber rejects was determined using the methods reported earlier [8]. Acid whey was obtained from the Chobani production facility in Norwich, NY, and stored in a -20 °C freezer until use. The profile of acid whey is characterized by applying the procedure reported [18].

Two-step hydrothermal pretreatment

Fiber rejects were pretreated using a two-step hydrothermal pretreatment (hot water pretreatment followed by disk refining). The hot water pretreatment was performed in a 300-mL stainless-steel Parr reactor vessel (Parr Instrument Company, Moline, IL, USA) at 150 °C and 15% solid loading for 10 min. The pretreated slurry was subsequently milled for 3 cycles using a lab-scale disk mill (Quaker City Mill model 4E, Philadelphia, PA, USA). Detailed methodology of the pretreatment process can be found in prior literature [8].

Enzymatic hydrolysis

Following the hot water pretreatment and disk milling, enzymatic hydrolysis of the pretreated biomass was performed using the procedure reported earlier [8]. Briefly, hydrolysis was conducted at 10% solids, pH 5.0, and 50 °C for 72 h. In the case when hydrolysate was generated from fiber rejects only, the pH was adjusted to 5.0 using glacial acetic acid, and the solid loading was brought to 10% using DI water. In another approach, acid whey was added to the pretreated fiber rejects and the pH was further adjusted to 5.0 using acetic acid. In both cases, commercial cellulase and hemicellulase cocktails Cellic®Ctec2 and Cellic®Htec2 (Novozymes North America, Inc., Franklinton, NC, USA) were added to each flask at dosages of 0.17 mL (15 FPU) /g biomass and 0.04 mL/g biomass, respectively. At the end of the hydrolysis, the slurry

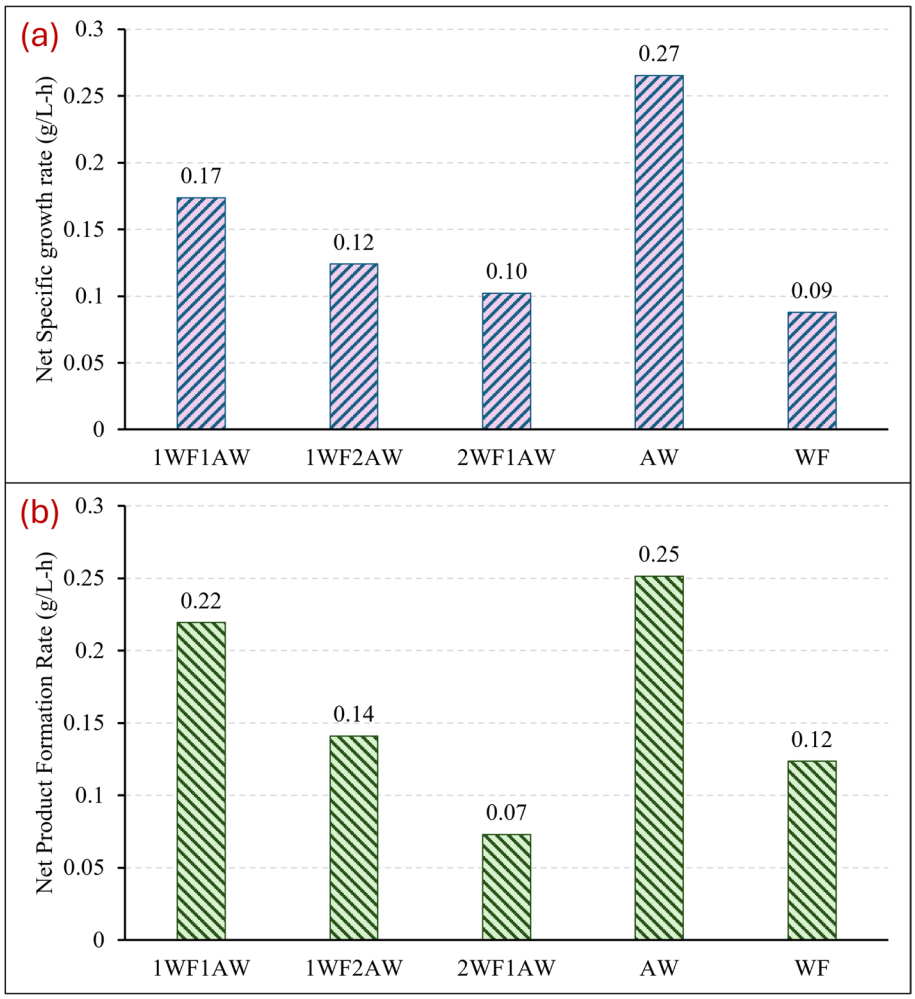


Fig. 8 Kinetic parameters (**a**) specific growth rate, and (**b**) PHB production rate calculated for fermentation with different ratio of blends

was filtered with a Whatman No. 4 cellulose filter paper to remove the solids, and the filtrate was sterile-filtered and stored at 4 °C until used for fermentation.

PHB Fermentation

Fermentation of hydrolysate, acid whey, and mixtures of acid whey and hydrolysate was conducted using recombinant *E. coli* LSBJ, using the protocol described in the previous report [8]. Details about the strain's origin and PHB synthesis pathway have been provided in the published study [18, 21]. Fermentation was carried out at a 40 mL scale in 500 mL baffled shake flasks containing Luria–Bertani (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L sodium chloride) (Sigma-Aldrich, St. Louis, MO, USA) adjusted to pH 7, supplemented with 50 mg/L Kanamycin, variable amounts of carbon source sourced from hydrolysate and/or acid whey, and 1% (v/v) seed culture. The carbon source was either from pure hydrolysate, pure acid whey or mixtures of both in ratios of 1:1, 2:1, and 1:2 (volume basis), maintaining a fixed total sugar concentration of 20 g/L. The flasks were incubated in a rotary shaker at 200 rpm and 30 °C. The strategy of sacrificing flasks was employed because of the high amount of sample needed for all analyses at every time point. Sampling was performed at 6 h, 12 h, 18 h, 24 h, 30 h, 36 h, 48 h, and 72 h. For each sample, pH levels were measured using a pH meter, while cell density at 600 nm was determined using a spectrophotometer. Samples collected were centrifuged at 10,000 rpm (5415 D, Brinkmann Eppendorf, Hamburg, Germany) for 5 min, and the supernatant was passed through 0.2 µm syringe filters (nylon Acrodisc WAT200834, Pall Life Sciences, Port Washington, NY) into 150 µL HPLC vials. The vials were immediately stored at − 20 °C until analysis. The remainder was harvested by centrifugation at 4,000 rpm for 20 min at 4 °C. After decanting the supernatant, the cell pellet was washed with 35% ethanol, followed by nanopure water, and subsequently lyophilized

for 48 h. The cell dry weight was determined by dividing the total mass of the cells by the volume of the culture. All experiments were carried out in duplicates.

PHB extraction and quantification

A detailed description of PHB extraction and quantification is provided in the previous report [8, 18]. Briefly, the PHB extraction from lyophilized cells was conducted using the dispersion of chloroform in acidic methanol. After filtration, the organic layer was analyzed using gas chromatography with a Rtx®-5 column and flame ionization detector (Shimadzu, Kyoto, Japan). The samples were injected at 280 °C, with an oven temperature program: holding at 100 °C for 7 min, ramping 8 °C/min to 280 °C, holding for 2 min, then ramping 20 °C/min to 310 °C with a final 2-min hold. Methyl octanoate was used as the internal standard.

HPLC analysis

The filtrate of the samples was subsequently analyzed using HPLC (Thermo Fisher Scientific, MA, USA) equipped with a refractive index detector (RID-10A) and multiple standards. The amounts of sugars were quantified using an Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA). The mobile phase was nanopure water at 85 °C at a flow rate of 0.6 mL/min. The amounts of organic acids were quantified using the Aminex HPX-87H (Bio-Rad, Hercules, CA, USA) at 60 °C with 0.005 M sulfuric acid at 0.6 mL/min.

Statistical analysis

The data were analyzed with a one-way analysis of variance (ANOVA) and followed by Tukey's HSD test using the software SPSS version 29, with a 95% confidence level set to assess differences between the treatments.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44316-024-00013-y.

Supplementary Material 1.

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Authors' contributions

L.J.: Conceptualization, methodology, formal analysis, investigation, writing—original draft. G.K.: Methodology, formal analysis, investigation. A.J.: Methodology, writing—review & editing. E.L.-W.M.: Writing—review & editing, and funding acquisition. B.V.R.: Writing—review & editing, and funding acquisition, D.K.: Conceptualization, resources, supervision, validation, writing, review and editing, and funding acquisition. All authors read and approved the final manuscript.

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Availability of data and materials

Most of the data generated or analyzed during this study are included in this published article. Any additional data will be made available on request.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

This study does not contain any studies involving human or animal participants.

Consent for publication

This study does not contain data from any individual person.

Competing interests

The authors declare no competing interests.

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