High-Solid Loading Enzymatic Hydrolysis of Waste Office Paper for Poly-3-Hydroxybutyrate Production Through Simultaneous Saccharification and Fermentation

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Research Article

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Abstract

Waste paper holds great potential as a substrate for the microbial production of bioplastic (Poly-3-hydroxybutyrate (PHB)). This study aimed to produce PHB by utilizing office paper as a substrate using *Cupriavidus necator* through batch and fed-batch simultaneous saccharification and fermentation (SSF) approach. For the batch experiment, different loadings of shredded office paper (3, 5 and 10%) with two different pretreatments H_2O_2 (OPH) and H_2O_2 and Triton X-100 (OPTH) were carried out. For the fed-batch experiment, paper loading started with 3% and two more additions were made at 36 and 84 h. Both experiments were conducted at 30°C, 200 rpm and pH 7 using 55.5 FPU/g of cellulase and 37.5 CBU/g of β -glucosidase with a fixed amount of nitrogen source. High PHB yield was observed with OPH in all loadings, though the OPHT showed a better hydrolysis. Maximum PHB yield (4.27 g/L) was achieved with 10% OP at six days of fermentation in batch SSF. Whereas, maximum PHB yield (4.19 g/L) was obtained within a shorter time (66 h) in the fed-batch OPH paper. The extracted PHB showed well-matched characteristic features to the standard PHB. Finally, this study proves the feasibility of employing SSF process for PHB production using waste paper as an alternative approach to overcome the shortcoming of the separate hydrolysis and fermentation (SHF) process.

1. Introduction

Waste paper can be utilized as a cheap alternative substrate of noble sugars for poly-3-hydroxybutyrate (PHB) production [1–4]. The major components of waste paper are cellulose, hemicellulose, and lignin. It also has filling materials such as calcium carbonate, clay, and other chemical additives, for instance, rosin, alum, and starch were added in various proportions depending upon the paper type. Besides, residuals of talc or sodium silicate used during paper processing also remain in the paper materials [5–8, 2]. The presence of these chemicals reduces the efficiency of the enzymatic hydrolysis of the cellulose present in the waste paper. Therefore, waste paper pretreatment is a critical step to remove these chemicals and expose more cellulose in the paper to the enzymatic hydrolysis.

Recently, waste paper has been widely exploited, and studies have been conducted to increase the conversion percentage of cellulose to fermentable sugars by applying different pretreatments, in addition to increased solid loading [9–11, 1, 2, 6, 7]. Previously, we have reported that the ground waste office paper treated with H_2O_2 at 121 °C for 30 min. with 3% paper loading was the most effective pretreatment to get high sugar from waste office paper [12, 3]. Further optimization of H_2O_2 -pretreated paper loading using central composite design reached the maximum saccharification at 5% paper loading [13]. However, further higher paper loading was not possible due to high water absorption by ground paper. Hence, in this study, the shredded paper was used instead of the ground waste office paper to increase the substrate loading. In addition, the combined pretreatment using H_2O_2 and the combined pretreatment using H_2O_2 and Triton X-100 were evaluated for its effect on the saccharification process. Triton X-100 was reported as an enhancer for the enzymatic hydrolysis when associated with other pretreatments. Triton X-100, along with ionic liquid pretreatment, leads to a significant increase of rice straw

saccharification than the untreated and ionic liquid treated biomass [14]. Generally, the application of surfactant is one of the promising approaches to improve the enzymatic hydrolysis and reduce enzyme loading [15].

In our previous studies, the waste paper was tried as a substrate for PHB production through separate hydrolysis and fermentation (SHF) mode. However, the maximum saccharification occurred while using 3% paper loading and decreased in higher paper loading due to the end-product inhibition [12, 13]. Therefore, the simultaneous saccharification and fermentation (SSF) mode, integrating the enzymatic hydrolysis of the lignocellulosic waste and PHB production [16, 17], was tried to overcome the drawback in SHF. Although the SHF was conducted at the optimal conditions for both saccharification and fermentation processes, applying SSF will reduce the production cost by simplifying the production process, avoiding end-product inhibition of the hydrolysis process, and reducing the enzyme loading [18–21, 17]. In addition, the sugar concentration available for PHB accumulation can be increased by increasing the paper loading in SSF mode [22, 23]. Indeed, there are meager studies on PHB production through SSF using lignocellulosic wastes as substrates. Dahman and Ugwu [24] observed a better yield of PHB using a wheat straw through SSF mode than SHF with both wheat straw and pure glucose. García-Torreiro [25] reported > 50% higher yield in SSF configuration than SHF, in conjunction with 47% shortage of processing time.

Hence, this study aimed to examine the feasibility of PHB production by *Cupriavidus necator* H16 through SSF using H₂O₂ treated shredded waste office paper with and without Triton X-100 pretreatment at various solid loadings.

2. Materials And Methods

2.1. Bacterial culture and maintenance

C. necator H16 NCIMB 11599 was purchased from the National Collection of Industrial Food and Marine Bacteria (NCIMB), Aberdeen, Scotland. The bacterial culture was maintained on YPM agar which contained (g/L): yeast extract, 10; peptone, 10; meat extract, 5; (NH₄)₂SO₄, 2; agar, 15 g/L and stored at 4 °C, and then transferred to a fresh broth culture to be incubated at 30 °C for 24 h.

2.2. Pretreatment of waste office paper

Raw materials were collected from college of Science, Sultan Qaboos University, Oman. Waste paper were shredded into small pieces (2×5 mm) in bulk using a paper shredder (Atlas, China) to utilize the same source of raw material for the whole study. Then, the shredded materials (5%) were pretreated using 0.5% H₂O₂ at 121°C and 15 psi for 30 min. The solid material was filtered through a cloth sheath and washed till the neutral pH of the filtrate is achieved and dried overnight in a hot oven at 60°C. Half amount of the H₂O₂ pretreated-shredded paper was subjected to a second pretreatment using Triton X-100 by adding 1 ml/ 10 g paper and incubated at 40 °C at 250 rpm for 2 h. After that, the solid residues were separated by filtration through cloth sheath and washed thoroughly by distilled water until the drained water reached

neutral pH. The recovered substantial fraction was dried at 60 °C for 24 h and then stored in air tight bags at room temperature for further work.

2.3. PHB production through SSF

SSF experiment was conducted in 500 mL shaking flasks each containing 200 mL of the mineral salt medium (MSM) which contained (g/L): KH_2PO_4 , 2.4; Na_2HPO_4 , 2.5; MgSO_4 , 0.5; Ferric ammonium citrate, 0.05; CaCl_2 , 0.02; $(\text{NH}_4)_2\text{SO}_4$, 2. For the batch SSF experiment, the shredded paper substrate was added to the fermentation medium as a carbon source with different percentages (3, 5, 10% w/v), then sterilized at 121 °C for 15 min. The $(\text{NH}_4)_2\text{SO}_4$ was maintained at 2 g/L for all paper loadings. After sterilization, the medium was loaded with 55.5 FPU/g and 37.5 CBU/g of cellulase (C2730) and β -glucosidase (49291) (Sigma), respectively. To this, 3% (v/v) inoculum was added aseptically and incubated at 35 °C with 200 rpm in a rotary shaker (Thermo scientific MAXQ 420 HP). In the fed-batch experiment, 3% shredded paper was added at the beginning, and then 3% was added after 36 and 84 h of incubation. During fermentation, 3 mL samples were collected in pre-weighed tubes and centrifuged at 4000 rpm. The supernatant was separated to analyze the reducing sugar content, and the pellet was dried overnight at 60 °C. The weight of the pellet represents the microbial biomass and the unhydrolyzed paper, as the bacteria adhered to the residual paper cannot be separated. All experimental data represent the average value of three replicate of the fermentation experiments.

2.4. Analytical methods

2.4.1. Reducing sugar determination

Sugar concentrations were analyzed using HPLC (Shimadzu; LC10AD) equipped with an ion-exchange column (Aminex HPX-87H; 300 mm × 7.8 mm; Bio-Rad, USA), a pump series 200 (Shimadzu), autosampler series 200 (Shimadzu), and a refractive index detector (Shimadzu; RI 20). Samples of 1 mL were diluted 10-fold with Milli-Q water, and 20 μL from each diluted sample was injected into the column at 65 °C using 5 mM sulfuric acid as a mobile phase with a flow rate of 1 mL/min. Sugar concentrations were quantified by external calibration of the pure sugar standards.

2.4.2. PHB extraction and quantification

PHB was quantified by gas chromatography (GC) using the methanolysis method [3]. Briefly, 2 mL of 3% acidified methanol (2.8 M H_2SO_4 in methanol) containing 1% octanoic acid as an internal standard, and 2 mL of chloroform was added to a known amount of dried biomass in a Teflon capped tube and heated for 4 h at 100 °C using heat block (DRB 200, HACH). After cooling, 1 mL of distilled water was added to the mixture, vortexed vigorously for 3 min and left at room temperature to form two layers. The organic layer of the dissolved methylated ester was filtered, and 1 μL with 1:20 split ratio was injected to the GC-FID (Agilent Technologies 5890 series II) equipped with Agilent J & W HP-5 capillary column (30 m × 0.32 mm × 0.25 mm), flame ionization detector (FID), and a 7683B injector. The temperatures of the injector and FID was kept constant at 250 and 270 °C, respectively, while the oven temperature was programmed

to increase from 100 °C (1 min) to 250 °C (4 min) with a heating rate 15 °C/ min. PHB obtained from Sigma was used as a standard calibration and was subjected to the same procedure as the samples.

2.4.3. Scanning electron microscopy (SEM)

The solid residue remained after the experiment was filtered using Whatman no.1 filter paper and dried overnight at 60 °C. A tiny piece of each sample was fixed on the sample holder using carbon conducting tape, coated thoroughly with carbon, and then observed under SEM (Jeol JSM-6360A).

2.4.4. Fourier-transform infrared spectroscopy (FTIR)

The dried materials of the SSF experiment were analyzed using FTIR spectrophotometer (BOMEN, Canada), and the spectrum was recorded between 600 and 4000 cm^{-1} . Different functional groups were analyzed by comparing various pretreatments and paper loadings with standard cellulose and lignin spectrum.

2.4.5. Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) was conducted to determine the decomposition temperature (T_d) of the extracted and standard PHB. A TGA7 thermo-analyzer (Perkin-Elmer, USA) was used with a temperature range of 30 to 900°C at a heating rate of 20°C/min in a nitrogen environment. The weight (%) curve against temperature was obtained by observing the weight loss at specific ranges of temperature.

2.4.6. Nuclear magnetic resonance (NMR)

Chemical structure of the extracted PHB was achieved using NMR. ^1H NMR and ^{13}C NMR spectra were recorded at room temperature in chloroform using a 500 MHz spectrophotometer (Bruker, Switzerland).

2.4.7. X-ray diffraction (XRD)

Crystallinity nature of both the extracted and the standard PHB was using XRD spectroscopy (The PANalytical X, Pert Pro) with Cu-K α ($\lambda = 1.5405 \text{ \AA}$) in the range of $2\theta = 1-70^\circ$.

3. Results And Discussion

Integrating the processes of enzymatic hydrolysis and PHB production has a great potential to avoid product inhibition, and simplify the conversion of cellulosic biomass into PHB. This study focused on PHB production using waste paper through SSF by *C. necator*, where both enzymatic hydrolysis and sugar fermentation coincide. *C. necator* accumulates high quantity of PHB inside the cell as a reserve material of carbon and energy. It synthesizes the PHB by catalyzing the condensation of two molecules of acetyl CoA from the tricarboxylic acid cycle, into acetoacetyl CoA with the help of the enzyme β -ketothiolase encoded by the gene *phbA*. Then the reduction of acetoacetyl CoA into 3-hydroxybutyryl-CoA is mediated by acetoacetyl CoA dehydrogenase enzyme encoded by *phbB* gene. Finally, the PHB synthases (encoded by *phbC*) facilitated the polymerization of 3-hydroxybutyryl-CoA monomers into PHB homopolymer [26].

In the SHF study, the hydrolysis was conducted at 50 °C, and pH 4.8, while the microbial fermentation was carried out at 30 °C and pH 7.2 [3, 13]. However, in the case of the SSF lower temperature and higher pH may affect the efficiency of the enzyme. Therefore, we tried to increase the temperature to a level that will not inhibit the growth of *C. necator*. Hence, in this study, the conditions for microbial growth were fixed as 35 °C and pH 7.0, which may ultimately affect the saccharification process of waste paper. Therefore, the strategy adopted to increase the sugar release was increasing the enzyme concentration by 1.5-fold in the SSF process and an extra-pretreatment using Triton X-100.

3.1. Pretreatment of waste paper

The effect of pretreatment of H₂O₂ without (OPH) and with triton X-100 (OPHT) on the shredded office paper was assessed by FTIR in the range of 750–4000 cm⁻¹ (Fig. 1). Generally, the range of 1000–1770 is known as lignin fingerprint [27]. The bands within the range of 1400–1630 cm⁻¹ were attributed to the aromatic skeleton stretching vibration at 1414, 1456, 1516, and 1605 cm⁻¹ [28]; the bands around 875 cm⁻¹ represents C-H out from the plane bending of syringl content in lignin [29, 30]. The bands around 1500 and 1587 cm⁻¹ refer to the aromatic ring vibration and C = O stretching in lignin polymer [31]. Peak around 1030 cm⁻¹ corresponds to the aromatic C–H bond found in lignin and the stretching of the non-conjugated C = O bond found in both lignin and hemicellulose [32]. The bands at 2875–2940 and 3325 cm⁻¹ are responsible for C–H stretch of methyl, methylene, or methine group and O–H stretching of lignin and cellulose polymer, respectively [33]. Therefore, the decrease or absence in these peaks in the pretreated paper is attributed to delignification. Pretreatment caused a dramatic decline in the intensity at the lignin fingerprint region, especially in OPHT compared to untreated paper, specifically the band at 1587 cm⁻¹. Different studies revealed that the office paper has 65 to 87 % cellulose. 5 to 13% hemicellulose and 1 to 2% to lignin [34–36]. In general, the pretreatment increased the sugar content and decreased the lignin content.

3.2. PHB production through batch and fed-batch SSF mode

Initially, OPH and OPHT treated paper were assessed at different substrate loadings (3, 5, and 10% paper) through batch SSF. The dry weight represents both residual paper and the microbial biomass (Figs. 2 and 3). They have an inverse relationship where the paper was decreasing, and the biomass was increasing during the course of SSF. In addition to the free cells in the liquid medium, a good amount of bacterial cells attached to the shredded paper makes the separation difficult. OPHT showed a higher reduction rate of the dry weight than OPH, particularly between 24 and 72 h, which was visually liquefied faster. At the last 24 h of the fermentation, the paper conversion rate decreased significantly to around 4% in both 5% OPH and 5% OPHT, and even less than 1% in 3% OPHT. Almost 7.03 and 5.77 g/L of dry weight remained in both 3% OPH and 3% OPHT (Fig. 2A and B) while 11.63 and 12.93 g/L in 5% OPH and OPHT (Fig. 2A and B), respectively. Paper conversion was evidenced by the residual glucose content that has reached the maximum at 48 h of incubation (Fig. 2A-D). However, more residual glucose was found in both OPHT loading paper, reaching a maximum of 3.07 and 6.19 g/L from 3 and 5%, respectively. At the end of the

fermentation, glucose concentration in both treatments at 3% loading was almost utilized by *C. necator*, while 0.71 and 3.37 g/L remained in 5% OPH and OPHT, respectively. PHB content was higher in OPH than the OPHT medium in both paper loadings. After 24 h, 5% OPH reached the maximum PHB (0.46 g/L) (Fig. 2C) while 3% OPH reached the maximum PHB (0.31 g/L) at 48 h (Fig. 2A) due to the C/N ratio.

Similarly, higher paper loading (10%) using both OPH and OPHT was tested on their effect on PHB production, and the fermentation period was extended for seven days (168 h). At the beginning of the experiment, OPHT took 48 h to liquefy, and hence the first samples were taken; however, OPH was faster in liquefaction. The results were consistent with the previous solid loading experiments regarding residual glucose, dry weight, and PHB (Fig. 3). The general overview of the OPH experiment showed that the residual glucose and dry weight were almost constant between 48 to 144 h with an increment of PHB yield. This is due to the simultaneous increment of enzymatic hydrolysis and, consequently, bacterial biomass, which is acknowledged by PHB accumulation. The maximum PHB yield 4.27 g/L was achieved using OPH at 6 days of incubation. Nevertheless, there was a sudden decrease in the PHB yield at the end of the incubation. Unlike OPH, the OPHT experiment showed a delay in the hydrolysis at the beginning because OPHT was processed twice, which resulted in a semi-ground structure. This increased the paper surface area, and consequently, higher absorption of water. Despite the higher glucose in the medium, the PHB yield was almost nil till 144 h of incubation. After that, the PHB production slightly increased in coinciding with glucose utilization.

The higher sugar concentration in OPHT treated paper proves the efficiency of additional triton X-100 treatment in improving the enzymatic hydrolysis of paper fibers [37, 38]. Application of surfactant in pretreatment and enzymatic digestion favored enzymatic hydrolysis of various biomass through different ways; 1) surfactant reduces the irretrievable adsorption of the enzyme keeping it free with superior activity; 2) surfactant preserves the enzyme from thermal denaturation by reducing the surface tension; 3) surfactant intensifies the electrostatic interaction between the enzyme and the biomass; 4) surfactant increases the lignin and hemicellulose removal, the primary hurdle for effective saccharification, through emulsions process since both contain hydrophobic part and 5) surfactant causes an alteration in biomass structure and increases surface area makes it suitable for enzymatic hydrolysis [39–42, 15]. Despite the very close paper conversion rate in terms of dry weight (paper and bacterial culture) of both treatments at 10% solid loading, the PHB yield was low in the OPHT. This is due to the presence of residual amount of Triton X-100 remained after pretreatment, which affected the microbial growth. Qu *et al.* [38] found that Triton X-100 affected the transportation and utilization of reducing sugar, leading to a detrimental effect on the metabolism and growth of *Clostridium thermocellum*. Moreover, Triton X-100 boosts the enzymatic hydrolysis, led to the release of high sugars compared to OPH treatment affected the growth of the bacteria by a very high C/N ratio in the production medium. Despite nitrogen limitation is a favorable condition for higher PHB content, a higher C/N ratio than the optimum condition minimized *C. necator* growth and hence, the PHB yield [3]. In the case of 10% OPH, the higher PHB yield was achieved due to the higher released-sugar, which increased the C/N ratio compared to the previous paper loadings. In contrast, the later reduction in the PHB after 144 h was due to the polymer degradation by

bacteria for its growth or due to the concomitant occurrence of both saccharification and fermentation, causing a fluctuation in the C/N ratio during SSF mode of fermentation.

A fed-batch SSF was also tried to increase the yield of PHB production by the gradual increase of the paper load in the medium of both OPH and OPHT, starting with 3% (Fig. 4). The results showed that the residual glucose increased after the addition of the paper (36 and 84 h) and decreased later, apparently due to the bacterial growth. In both cases, the production of PHB was deficient at the first stage of the growth, like the batch SSF (3% paper of both OPH and OPHT) experiment. The first addition of paper (3%) in fed-batch experiment led to an increase in the PHB reaching the maximum (4.19 g/L) at 66 h of incubation in the OPH, but not in OPHT. Hereafter, PHB production decreased in the OPH experiment despite the second batch addition of the paper. The results showed that the second supplement of the substrate increases the residual sugar concentration but not PHB production. However, the PHB production started to increase at the end of the fermentation (after 115 h). OPHT showed almost half the PHB yield (2.05 g/L) of the OPH, only after 114 h of incubation. This indicates that double pretreatment, specifically Triton X-100, did not improve the PHB production; instead, it affects the growth of *C. necator* by interfering with sugar transport.

Dahman and Ugwn [24] used both SHF and SSF processes for PHB production using *C. necator* by utilizing wheat straw as a feedstock. The results showed that SSF is more influential than SHF and even pure sugar in terms of PHB yield and microbial growth. More recently, cereal mash was used as a substrate for PHB production through SSF using *Halomonas boliviensis*, resulted in a 60% higher PHB polymer than SHF mode with a 47% reduction in the overall processing time [25]. Although the PHB yield of our fed-batch mode (4.19 g/L) was almost closer to the 10% batch mode (4.27 g/L) experiment, its production was achieved within a shorter time almost half period (66 h) in fed-batch. However, unlike previous studies, the production of PHB from waste paper through SSF was still lower than that produced through SHF. The PHB yield of this study is higher than that obtained by *B. sacchari* (3.6 g/L) [13], but slightly lower than using *C. necator* (5.3 g/L) in shake flask experiments using SHF [3]. Table 1 represented the PHB yield of *C. necator* and *Burkholderia* sp. by utilizing various lignocellulosic biomass. The fluffy nature of waste paper materials interfere during the analysis of PHB in the samples and affected the final yield. Thus, SSF application simplified the processes while achieving the comparable PHB production. However, this result was achieved using 10% paper loading, unlike the SHF, which achieved using 3% paper loading only. Indeed, the coordination of waste paper enzymatic hydrolysis and sugars assimilation is essential for further improving the efficiency of the SSF approach. Further optimization studies on SSF conditions such as temperature, substrate loading, enzyme loading, and agitation will increase PHB production. Also, the fed-batch SSF process can be improved further by optimizing the quantity and time of paper loading.

Table 1

Cell dry weight (CDW) and PHB yield of *C. necator* and *Burkholderia* sp. by utilizing various lignocellulosic biomass (LCB) through SHF and SSF mode

PHB producer	LCB	Operation mode	CDW (g/l)	PHB (%)	PHB (g/l)	Yield (P/S)	Ref.
<i>C. necator</i>	Sugarcane Bagasse	SHF, Batch	6.0	65	3.9	ND	[53]
<i>C. necator</i>	Water hyacinth	SHF, Batch	12.0	58	7.0	0.24	[54]
<i>C. necator</i>	Sunflower	SHF, Batch	10.9	73	7.8	0.40	[55]
<i>C. necator</i>	Wheat bran	SHF, Batch	24.5	63	14.8	0.32	[56]
<i>C. necator</i>	Sargassum	SHF, Batch	5.3	74	3.9	0.47	[49]
<i>B. sacchari</i>	Waste OP	SHF, Flask, Batch	3.6	44	1.6	0.15	[13]
<i>C. necator</i>	Waste OP	SHF, Flask, Batch	7.7	57	4.5	0.21	[3]
<i>C. necator</i>	wheat straw	SHF, Flask, Batch	12.2	58	7.1	0.13	[24]
<i>C. necator</i>	wheat straw	SSF, Flask, Batch	15.3	65	10.0	0.16	[24]
<i>C. necator</i>	Waste OP	SSF, Flask, Batch	ND	ND	4.27	ND	This study
		SSF, Flask, Fed-batch	ND	ND	4.19	ND	This study
*ND: Not Defined, *SHF: separate hydrolysis and fermentation, *SSF: simultaneous saccharification and fermentation							

The structural changes of the paper after the fermentation was observed through SEM. Figure 5A-F showed representative figures of 3 and 5% OPH and OPHT filtered residues, containing paper residues and bacterial culture, at different magnifications. Figure 5B and C illustrated the abundant growth of *C. necator* attached to the paper fibers. Paper fibers were particularly hydrolyzed into fermentable sugars and utilized by the microbes (Fig. 5D). At the end of the fermentation, the toner particles were visually noticed after the hydrolysis (Fig. 5E). Interestingly, it was observed that the toner crystal did not affect the growth of *C. necator*. Instead, the bacterial cells used the toner crystal as a surface for their growth (Fig. 5F).

FTIR spectra of both OPH and OPHT before and after SSF were carried out in the range of 750–4000 cm^{-1} (Fig. 6) to assess the nature of residues remained at the end of the experiment. Overall, peaks intensity decreased after hydrolysis compared to unhydrolyzed materials, specifically at 1000–1770 cm^{-1}

¹. Besides, the peak at 875 cm^{-1} , which represents C-H of syringl content in lignin is almost disappeared. Lignin and cellulose peaks are significantly declined. Cellulose was utilized by bacteria in the form of glucose, while lignin derivatives were definitely released in the medium, especially in OPH treatment. Although PHB is higher in the OPH experiments, lignin derivatives could have a side effect compared to the PHB yield of SHF [3].

3.3. PHB characterization

Thermal properties of PHB differs among PHB producing microbes [43]. The thermal strength of the PHB produced by *C. necator* (PHB/CN) was evaluated using thermogravimetric analysis (TGA) having the standard/pure PHB (PHB/STD) procured from Sigma (363502) as a reference (Fig. 7A). The TGA is used to analyse the changes in the mass of a sample with increased temperature in a time period. The dehydration of solvents in the PHB resulted in a weight loss from $130\text{--}230^\circ\text{C}$. Further decrease in PHB weight occurred $\sim 300^\circ\text{C}$ led to the crotonic acid formation [44, 45]. From $300\text{--}800^\circ\text{C}$ the PHB/CN produced using waste paper substrate exhibited a high stability and residual weight. The delay in complete degradation compared to the PHB/STD showed the possible presence of some impurities. The differential thermogravimetric (DTG) curves deduced from TGA confirms the thermal stability of the produced PHB. The degradation temperature of the PHB/CN and the PHB/STD were in the same range, 293.39 and 301.50 respectively (Fig. 7B).

The molecular structure of the PHB was studied using ^1H and ^{13}C NMR spectra (Fig. 8). The presence of methyl group was represented by peaks near 1.3 ppm in ^1H NMR. The methylene group was presented by two peaks at 2.4 and 2.6 ppm and the methine group by 5.2 ppm. The ^{13}C NMR spectrum showed the presence of CH_3 , CH_2 , CH and $\text{C}=\text{O}$ groups at 19, 40, 67 and 169 ppm respectively. The above studies demonstrated that the PHB produced by SSF was a homoplomer and comparable to the previous studies [45, 46].

The FTIR spectrum results (Fig. 9) revealed the presence of C–O stretching ester band at 1055.8 cm^{-1} and C–O–C symmetric stretching amorphous band at 1183.2 cm^{-1} [47]. The bands at 1277.8 cm^{-1} , 2930.5 cm^{-1} and 1379.7 cm^{-1} are assigned to methylene, methane and methyl groups respectively. The strong band at 1721 cm^{-1} denotes the crystalline carbonyl group in the ester group of PHB. The FTIR spectral study supported the NMR results and corroborates the earlier reports [48, 49, 47, 45].

The XRD diffractograms of the extracted PHB (PHB/CN) and standard (PHB/STD) are presented in Fig. 10. The diffractogram of PHB is predominated by five major peaks at the corresponding $2\theta = (020)$ at 13.5° , (110) at 16.9° , (101) at 21.4° , (111) at 22.5° , and (121) at 25.5° . The crystallinity of the PHB revealed by the strong peak located at $2\theta = 13.5^\circ$ at 020 plane. The PHB produced through SSF, PHB/CN, is less crystalline or slightly amorphous when compared to standard PHB. The less intensified peak at 2θ of 020 in the extracted PHB evidenced the low crystallinity [50–52]. The other peaks observed at various

2θ of both PHB/CN and PHB/STD are reflecting the same pattern and similar to the PHB produced from sugarcane bagasse (Salgaonkar and Bragança, 2017) and rice straw [50].

4. Conclusion

OPH showed better PHB yield compared to OPTH in all loadings. Increasing the paper loading resulting in higher PHB due to higher C/N ratio, 4.27 g/L was attained at 10% OPH at six days of fermentation. A comparable yield was achieved through fed-batch SSF within a shorter time. The produced PHB showed a good thermal stability and the other characteristic features are similar to the commercial PHB from Sigma. However, the PHB production from waste paper through SSF was lower than the PHB production through SHF. Hence, further novel approaches to increase the PHB yield through SSF.

Declarations

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Conflicts of interest

The authors declare that they have no conflicts of interest

Availability of data and material (data transparency)

Not applicable

Code availability

Not applicable

Authors' contributions

Huda Al Battashi: designed the experiment, carried out the experimental work and prepared the original draft of the MS. Nallusamy Sivakumar: conceptualization of the work, analyzed the data, supervised the research and finalized the MS.

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References

1. Ma Y, Hummel M, Määttänen M, et al (2016) Upcycling of waste paper and cardboard to textiles. *Green Chem* 18:858–866. <https://doi.org/10.1039/C5GC01679G>
2. Rodriguez C, Alaswad A, El-Hassan Z, Olabi AG (2017) Mechanical pretreatment of waste paper for biogas production. *Waste Manag* 68:157–164. <https://doi.org/10.1016/j.wasman.2017.06.040>
3. Annamalai N, Al-Battashi H, Al-Bahry S, Sivakumar N (2018) Biorefinery production of poly-3-hydroxybutyrate using waste office paper hydrolysate as feedstock for microbial fermentation. *J Biotechnol* 265:25–30. <https://doi.org/10.1016/j.jbiotec.2017.11.002>
4. Ma Y, Hummel M, Kontro I, Sixta H (2018) High performance man-made cellulosic fibres from recycled newsprint. *Green Chem.* <https://doi.org/10.1039/c7gc02896b>
5. Sangkharak K (2011) Optimization of enzymatic hydrolysis for ethanol production by simultaneous saccharification and fermentation of wastepaper. *Waste Manag Res* 29:1134–1144. <https://doi.org/10.1177/0734242X10387656>
6. Adu C, Jolly M, Thakur VK (2018) Exploring new horizons for paper recycling: A review of biomaterials and biorefinery feedstocks derived from wastepaper. *Curr Opin Green Sustain Chem* 13:21–26. <https://doi.org/10.1016/j.cogsc.2018.03.003>
7. Al Azkawi AS, Sivakumar N, Al Bahry S (2018) Bioprocessing of cardboard waste for cellulase production. *Biomass Convers Biorefinery* 8:1–10. <https://doi.org/10.1007/s13399-018-0309-7>
8. Al Battashi H, Al-Kindi S, Gupta VK, Sivakumar N (2020) Polyhydroxyalkanoate (PHA) Production Using Volatile Fatty Acids Derived from the Anaerobic Digestion of Waste Paper. *J Polym Environ.* <https://doi.org/10.1007/s10924-020-01870-0>
9. Wang L, Templer R, Murphy RJ (2012) High-solids loading enzymatic hydrolysis of waste papers for biofuel production. *Appl Energy* 99:23–31. <https://doi.org/10.1016/j.apenergy.2012.03.045>
10. Mota HG da, Gouveia ER (2016) Improvement in Enzymatic Hydrolysis of Waste Office Paper with Chemical Pretreatment and Enzyme Loading Reduced. *Waste and Biomass Valorization* 7:507–512. <https://doi.org/10.1007/s12649-015-9475-z>
11. Bilal M, Asgher M, Iqbal HM, Ramzan M (2017) Enhanced bio-ethanol production from old newspapers waste through alkali and enzymatic delignification. *Waste and biomass valorization* 8:2271–2281
12. Annamalai N, Al Battashi H, Anu SN, et al (2018) Enhanced Bioethanol Production from Waste Paper Through Separate Hydrolysis and Fermentation. *Waste and Biomass Valorization* 1–11
13. Al-Battashi H, Annamalai N, Al-Kindi S, et al (2019) Production of bioplastic (poly-3-hydroxybutyrate) using waste paper as a feedstock: Optimization of enzymatic hydrolysis and fermentation employing *Burkholderia sacchari*. *J Clean Prod* 214:236–247. <https://doi.org/10.1016/j.jclepro.2018.12.239>
14. Chang K-L, Chen X-M, Wang X-Q, et al (2017) Impact of surfactant type for ionic liquid pretreatment on enhancing delignification of rice straw. *Bioresour Technol* 227:388–392. <https://doi.org/10.1016/j.biortech.2016.11.085>

15. Qing Q, Yang B, Wyman CE (2010) Impact of surfactants on pretreatment of corn stover. *Bioresour Technol* 101:5941–5951
16. Kádár Z, Szengyel Z, Réczey K (2004) Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. *Ind Crops Prod* 20:103–110. <https://doi.org/10.1016/j.indcrop.2003.12.015>
17. Marques S, Alves L, Roseiro JC, Gírio FM (2008) Conversion of recycled paper sludge to ethanol by SHF and SSF using *Pichia stipitis*. *Biomass and Bioenergy* 32:400–406. <https://doi.org/10.1016/j.biombioe.2007.10.011>
18. Alkasrawi M, Eriksson T, Börjesson J, et al (2003) The effect of Tween-20 on simultaneous saccharification and fermentation of softwood to ethanol. *Enzyme Microb Technol* 33:71–78. [https://doi.org/10.1016/S0141-0229\(03\)00087-5](https://doi.org/10.1016/S0141-0229(03)00087-5)
19. de Jesus Assis D, Gomes GVP, da Cunha Pascoal DR, et al (2016) Simultaneous Biosynthesis of Polyhydroxyalkanoates and Extracellular Polymeric Substance (EPS) from Crude Glycerol from Biodiesel Production by Different Bacterial Strains. *Appl Biochem Biotechnol* 180:1110–1127. <https://doi.org/10.1007/s12010-016-2155-z>
20. Al-Battashi H, Annamalai N, Sivakumar N, et al (2019) Lignocellulosic biomass (LCB): a potential alternative biorefinery feedstock for polyhydroxyalkanoates production. *Rev Environ Sci Bio/Technology* 18:183–205. <https://doi.org/10.1007/s11157-018-09488-4>
21. Al-Battashi H, Sivakumar N (2020) Chapter 17 - Bioenergy technology for integrated production of polyhydroxybutyrate/bioplastic. In: Gupta VK, Treichel H, Kuhad RC, Rodriguez-Cout S (eds) *Recent Developments in Bioenergy Research*. Elsevier, pp 325–347
22. Pillai AB, Kumar AJ, Thulasi K, Kumarapillai H (2017) Evaluation of short-chain-length polyhydroxyalkanoate accumulation in *Bacillus aryabhattai*. *brazilian J Microbiol* 48:451–460
23. Zhou W, Gong Z, Zhang L, et al (2017) Feasibility of lipid production from waste paper by the oleaginous yeast *Cryptococcus curvatus*. *BioResources*. <https://doi.org/10.15376/biores.12.3.5249-5263>
24. Dahman Y, Ugwu CU (2014) Production of green biodegradable plastics of poly(3-hydroxybutyrate) from renewable resources of agricultural residues. *Bioprocess Biosyst Eng* 37:1561–1568. <https://doi.org/10.1007/s00449-014-1128-2>
25. García-Torreiro M, López-Abelairas M, Lu-Chau TA, Lema JM (2016) Production of poly(3-hydroxybutyrate) by simultaneous saccharification and fermentation of cereal mash using *Halomonas boliviensis*. *Biochem Eng J* 114:140–146. <https://doi.org/10.1016/j.bej.2016.07.002>
26. Masood F (2017) Polyhydroxyalkanoates in the Food Packaging Industry. In: *Nanotechnology Applications in Food: Flavor, Stability, Nutrition and Safety*
27. Nair AS, Al-Battashi H, Al-Akzawi A, et al (2018) Waste office paper: A potential feedstock for cellulase production by a novel strain *Bacillus velezensis* ASN1. *Waste Manag*. <https://doi.org/10.1016/j.wasman.2018.08.014>

28. Allison GG, Thain SC, Morris P, et al (2009) Quantification of hydroxycinnamic acids and lignin in perennial forage and energy grasses by Fourier-transform infrared spectroscopy and partial least squares regression. *Bioresour Technol* 100:1252–1261.
<https://doi.org/10.1016/j.biortech.2008.07.043>
29. Jahan MS, Mun SP (2007) Characteristics of Dioxane Lignins Isolated at Different Ages of Nalita Wood (*Trema orientalis*). *J Wood Chem Technol* 27:83–98.
<https://doi.org/10.1080/02773810701486865>
30. Zhang M, Qi W, Liu R, et al (2010) Fractionating lignocellulose by formic acid: Characterization of major components. *Biomass and Bioenergy* 34:525–532.
<https://doi.org/10.1016/j.biombioe.2009.12.018>
31. Sills DL, Gossett JM (2012) Using FTIR to predict saccharification from enzymatic hydrolysis of alkali-pretreated biomasses. *Biotechnol Bioeng* 109:353–362. <https://doi.org/10.1002/bit.23314>
32. Sharma S, Sharma V, Kuila A (2016) Cellulase production using natural medium and its application on enzymatic hydrolysis of thermo chemically pretreated biomass. *3 Biotech* 6:139.
<https://doi.org/10.1007/s13205-016-0465-z>
33. Li X, Yu H, Sun D, et al (2015) Comparative Study of Enzymatic Hydrolysis Properties of Pulp Fractions from Waste Paper. *BioResources* 10:3818–3830.
<https://doi.org/10.15376/biores.10.3.3818-3830>
34. Barlaz MA (2006) Forest products decomposition in municipal solid waste landfills. In: *Waste Management*
35. Chu KH, Feng X (2013) Enzymatic conversion of newspaper and office paper to fermentable sugars. *Process Saf Environ Prot.* <https://doi.org/10.1016/j.psep.2011.12.003>
36. Guerfali M, Saidi A, Gargouri A, Belghith H (2015) Enhanced enzymatic hydrolysis of waste paper for ethanol production using separate saccharification and fermentation. *Appl Biochem Biotechnol.* <https://doi.org/10.1007/s12010-014-1243-1>
37. Qu X-S, Zhu M-J (2016) Investigation on the Enhancing Biological Saccharification of Cellulose by *Clostridium thermocellum* with Triton X-100 Addition. *J Biobased Mater Bioenergy* 10:362–369.
<https://doi.org/10.1166/jbmb.2016.1614>
38. Qu X-S, Hu B-B, Zhu M-J (2017) Enhanced saccharification of cellulose and sugarcane bagasse by *Clostridium thermocellum* cultures with Triton X-100 and β -glucosidase/Cellic®CTec2 supplementation. *RSC Adv* 7:21360–21365. <https://doi.org/10.1039/C7RA02477K>
39. Chen L, Fu S (2013) Enhanced cellulase hydrolysis of eucalyptus waste fibers from pulp mill by Tween80-assisted ferric chloride pretreatment. *J Agric Food Chem* 61:3293–3300.
<https://doi.org/10.1021/jf400062e>
40. Jin W, Chen L, Hu M, et al (2016) Tween-80 is effective for enhancing steam-exploded biomass enzymatic saccharification and ethanol production by specifically lessening cellulase absorption with lignin in common reed. *Appl Energy* 175:82–90

41. Putro JN, Soetaredjo FE, Lin S-Y, et al (2016) Pretreatment and conversion of lignocellulose biomass into valuable chemicals. RSC Adv 6:46834–46852. <https://doi.org/10.1039/C6RA09851G>
42. Kleingesinds EK, José ÁHM, Brumano LP, et al (2018) Intensification of bioethanol production by using Tween 80 to enhance dilute acid pretreatment and enzymatic saccharification of corncob. Ind Crops Prod 124:166–176. <https://doi.org/10.1016/j.indcrop.2018.07.037>
43. Heng K-S, Hatti-Kaul R, Adam F, et al (2017) Conversion of rice husks to polyhydroxyalkanoates (PHA) via a three-step process: optimized alkaline pretreatment, enzymatic hydrolysis, and biosynthesis by *Burkholderia cepacia* USM (JCM 15050). J Chem Technol Biotechnol 92:100–108. <https://doi.org/10.1002/jctb.4993>
44. Hablot E, Bordes P, Pollet E, Avérous L (2008) Thermal and thermo-mechanical degradation of poly(3-hydroxybutyrate)-based multiphase systems. Polym Degrad Stab 93:413–421. <https://doi.org/10.1016/j.polymdegradstab.2007.11.018>
45. Pradhan S, Borah AJ, Poddar MK, et al (2017) Microbial production, ultrasound-assisted extraction and characterization of biopolymer polyhydroxybutyrate (PHB) from terrestrial (*P. hysterophorus*) and aquatic (*E. crassipes*) invasive weeds. Bioresour Technol 242:304–310. <https://doi.org/10.1016/j.biortech.2017.03.117>
46. Liu X-J, Zhang J, Hong P-H, Li Z-J (2016) Microbial production and characterization of poly-3-hydroxybutyrate by *Neptunomonas antarctica*. PeerJ 4:e2291. <https://doi.org/10.7717/peerj.2291>
47. Bayari S, Severcan F (2005) FTIR study of biodegradable biopolymers: P(3HB), P(3HB-co-4HB) and P(3HB-co-3HV). J Mol Struct 744–747:529–534. <https://doi.org/10.1016/j.molstruc.2004.12.029>
48. Zhou Y, Huang Z, Diao X, et al (2015) Characterization of the effect of REC on the compatibility of PHBH and PLA. Polym Test 42:17–25. <https://doi.org/10.1016/j.polymertesting.2014.12.014>
49. Azizi N, Najafpour G, Younesi H (2017) Acid pretreatment and enzymatic saccharification of brown seaweed for polyhydroxybutyrate (PHB) production using *Cupriavidus necator*. Int J Biol Macromol 101:1029–1040. <https://doi.org/10.1016/j.ijbiomac.2017.03.184>
50. Saratale GD, Oh M-K (2015) Characterization of poly-3-hydroxybutyrate (PHB) produced from *Ralstonia eutropha* using an alkali-pretreated biomass feedstock. Int J Biol Macromol 80:627–635. <https://doi.org/10.1016/j.ijbiomac.2015.07.034>
51. Salgaonkar BB, Bragança JM (2017) Utilization of Sugarcane Bagasse by *Halogeometricum borinquense* Strain E3 for Biosynthesis of Poly(3-hydroxybutyrate-co-3-hydroxyvalerate). Bioengineering 4:50. <https://doi.org/10.3390/bioengineering4020050>
52. Saratale RG, Saratale GD, Cho SK, et al (2019) Pretreatment of kenaf (*Hibiscus cannabinus* L.) biomass feedstock for polyhydroxybutyrate (PHB) production and characterization. Bioresour Technol 282:75–80. <https://doi.org/10.1016/j.biortech.2019.02.083>
53. Yu J, Stahl H (2008) Microbial utilization and biopolyester synthesis of bagasse hydrolysates. Bioresour Technol. <https://doi.org/10.1016/j.biortech.2008.03.071>
54. Radhika D, Murugesan AG (2012) Bioproduction, statistical optimization and characterization of microbial plastic (poly 3-hydroxy butyrate) employing various hydrolysates of water hyacinth

(*Eichhornia crassipes*) as sole carbon source. Bioresour Technol.

<https://doi.org/10.1016/j.biortech.2012.06.107>

55. Kim HS, Oh YH, Jang YA, et al (2016) Recombinant *Ralstonia eutropha* engineered to utilize xylose and its use for the production of poly(3-hydroxybutyrate) from sunflower stalk hydrolysate solution. Microb Cell Fact. <https://doi.org/10.1186/s12934-016-0495-6>

56. Annamalai N, Sivakumar N (2016) Production of polyhydroxybutyrate from wheat bran hydrolysate using *Ralstonia eutropha* through microbial fermentation. J Biotechnol 237:.. <https://doi.org/10.1016/j.jbiotec.2016.09.001>

Figures

Figure 1

FTIR analysis of untreated (OP), pretreated office paper (OPH and OPHT), pure cellulose, and lignin

Figure 2

PHB production utilizing two differently pretreated OP as a substrate through SSF using *C. necator* at different paper loadings: 3% OPH (A), 3% OPHT (B), 5% OPH (C), and 5% OPHT (D)

Figure 3

PHB production utilizing waste OP as a substrate through SSF using *C. necator* at 10% paper loading of both OPH (A) and OPHT (B)

Figure 4

PHB production utilizing waste OP as a substrate through fed-batch SSF using *C. necator* with OPH (A) and OPHT (B)

Figure 5

SEM of the residues remained after SSF shows *C. necator* cells and paper fiber at different magnifications (A, B, C, and D). The suspended tonner remained in the medium tonner (E) was used as a surface for microbial growth (F)

Figure 6

FTIR analysis of the paper before and after SSF for both OPH and OPHT

Figure 7

TGA (A) and DTG% (B) thermograms of the extracted PHB produced by *C. necator* (PHB/CN) and standard (PHB/STD). The temperature was increased from 30 to 990 °C at a heating rate of 20 °C /min.

Figure 8

NMR spectra of PHB produced by *C. necator* using waste office paper (A) ¹H NMR spectrum (B) ¹³C NMR spectrum

Figure 9

FTIR spectra of the standard PHB (Sigma) (PHB/STD) and PHB produced by *C. necator* (PHB/CN) through SSF process using waste office paper

Figure 10

XRD diffractograms of the exacted PHB from the *C. necator* fermentation of waste office paper through SSF process and standard PHB (Sigma)